The Effect of Endocrine Disrupting Chemicals on Female Rat Reproductive System

メタデータ	言語: eng 出版者: 公開日: 2017-08-10 キーワード (Ja): キーワード (En): 作成者: HAOLIN ZHANG メールアドレス: 所属:
URL	http://hdl.handle.net/20.500.12099/56190

The Effect of Endocrine Disrupting Chemicals on Female Rat Reproductive System

（雌ラットの生殖機能に対する内分泌かく乱物質の作用に関する研究）

2016

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Table of Contents

Table of Contents i

List of abbreviations vi

Chapter 1 Introduction 1

1.1 Endocrine disrupting chemicals 1

1.1.1 What is endocrine disrupting chemicals? 1

1.1.2 The occurrence of EDCs exposure 1

1.1.3 General adverse effect of EDCs on human's or animals' health 2

1.1.4 17α-ethynyl estradiol (EE) 2

1.1.5 4-nitrophenol (PNP) 3

1.2 Hypothalamus-pituitary-ovary axis 3

1.3 The ovary development 4

1.4 The objective of the present study 5

Chapter 2 The effects of neonatal exposure to EE on female reproduction during adult period 6

2.1 Introduction 6

2.2 Materials and Methods 7

2.2.1 Animals 7

2.2.2 Experimental design 7

2.2.3 Hormone assays 8

2.2.4 Quantitative real-time PCR 9

2.2.5 Western blot analysis 9

2.2.6 Histological analysis 10

2.2.7 Immunohistochemistry 10

2.2.8 Statistical analysis 10

2.3 Results 11

2.3.1 Effect of neonatal EE exposure on reproductive parameters 11

2.3.2 Effect of neonatal EE exposure on hormonal changes at PND90 11

2.3.3 Effect of neonatal EE exposure on ovarian follicular composition at PND90 12

2.3.4 Effect of neonatal EE exposure on ovarian gene expression at PND90 12

2.3.5 Expression and localization of LHCGR protein in neonatal EE exposed ovary at PND90 13

2.4 Discussion 13

Chapter 3 The effects of neonatal exposure to EE on hormone profile during the pre-pubertal period in the female rats 24

3.1 Introduction 24

3.2 Materials and methods 25

3.2.1 Animals 25

3.2.2 Experiment design 25

3.2.3 Histology 26

3.2.4 Immunohistochemistry 26

3.2.5 QRT-PCR analysis 26

3.2.6 Hormone Assay 27

3.2.7 Statistical analysis 27

3.3 Results 27

3.3.1 Body weight and ovary weight 28

3.3.2 Cell proliferation and histological examination in the ovaries at PND21 28

3.3.3 Hormones changes in the control and EE treatment rats 28

3.3.4 The expressions of steroidogenic enzymes and inhibin/activin subunits in the developmental ovaries. 29
3.3.5 The immunolocalizaition of P450arom in the ovaries at PND14 29
3.3.6 The expression of kisspeptin in the hypothalamus in the developmental rats........29
3.4 Discussion 29
Chapter 4 Neonatal exposure to EE disrupts follicle formation by inhibiting the pro-apoptotic factor *Harakiri* (*Hrk*) 39
4.1 Introduction 39
4.2 Materials and methods 40
4.2.1 Animals 40
4.2.2 Experiment design 41
4.2.3 Microarray analysis 41
4.2.4 Real-time PCR analysis 42
4.2.4 Histology 42
4.2.5 Immunohistochemistry 43
4.2.6 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining 43
4.2.7 Immunofluorescence 43
4.2.8 Plasmid construction and virus infection 44
4.2.9 Statistical analysis 44
4.3 Results 44
4.3.1 Profile of gene expression in neonatal EE-exposed ovary.....................................45
4.3.2 Down-regulation of *Hrk* expression in neonatal EE-exposed ovary 45
4.3.3 Co-localization of Hrk and cleaved caspase 3 in neonatal ovary 45

4.3.4 Decrease of apoptotic oocytes and number of primordial follicles in neonatal EE-exposed ovaries 46

4.3.5 Effect of *Hrk* knockdown in neonatal ovary 46

4.3.6 Estrogen responsive element (ERE) localized in the promoter of *Hrk* gene and the expressions of estrogen receptors in the developmental ovaries 47

4.3.7 Downregulation of *Hrk* expression by estrogenic compounds 47

4.4 Discussion 47

Chapter 5 The effects of neonatal exposure to PNP on the female reproduction 62

5.1 Introduction 62

5.2 Materials and methods 63

5.2.1 Animals 63

5.2.2 Experiment design 63

5.2.3 Histology 64

5.2.4 Radio-immunoassay 64

5.2.5 QRT-PCR analysis 65

5.2.6 Statistical analysis 65

5.3 Results 65

5.3.1 High dose of PNP induced neonatal rats' death 66

5.3.2 Body weight and organs weight in PNP treated animals 66

5.3.3 Vaginal opening, estrous cycles and ovary histology in PNP treated animals 66

5.3.4 Gene expression of gonadotropin receptors and steroidogenic enzymes and 17β-estradiol concentration in PNP treated animals 66

5.3.5 Gene expression of inhibin/activin subunits and Inhibin concentration in PNP treated animals 67

5.3.6 Circulating LH and FSH concentration in PNP treated rats 67

5.3.7 The effect of PNP on the expressions of gonadotropin receptors, steroidogenic enzymes and inhibin/activin subunits *in vitro* 67

5.3.8 The effect of PNP on the expressions of estrogen receptors *in vivo* and *in vivo* 67

5.4 Discussion 68

Chapter 6 Summary 79

Acknowledgements 82

References 83

List of abbreviations

3βHSD: 3β-hydroxysteroid dehydrogenase

ARC: arcuate nucleus

AVPV: anteroventral periventricular nucleus

BBB: blood-brain barrier

D: diestrous

DAPI: 4',6-diamidino-2-phenylindole

DDT: dichloro-diphenyl-trichloroethane

DDX4: DEAD-Box Helicase 4

DES: diethylstilbestrol

DMEM: Dulbecco's Modified Eagle Medium

DMSO: dimethyl sulfoxide

E: estrous

E_2: 17β-estradiol

EB: estradiol monobenzoate

EDCs: endocrine disrupting chemicals

EE: 17α-ethynyl estradiol

ERα: estrogen receptor α

ERβ: estrogen receptor β

ERE: estrogen responsive element

FSH: follicle stimulating hormone

FSHR: follicle stimulating hormone receptor

GAPDH: glyceraldehyde phosphate dehydrogenase

GnRH: gonadotropin releasing hormone

GO: Gene ontology

HPG axis: hypothalamus-pituitary-gonad axis

Hrk: Harakiri

INH: inhibin

INH α: inhibin α subunit

INHβA: inhibin/activin βA subunit

INHβB: inhibin/activin βB subunit

ITS: insulin-transferrin-selenium

LD_{50}: lethal dose 50%

LH: luteinizing hormone

LHCGR: luteinizing hormone/choriogonadotropin receptor

P450scc: cytochrome P450 cholesterol side-chain cleavage

P450c17: cytochrome P450 17α-hydroxylase/17,20 lyase

P450arom: cytochrome P450 aromatase

PAHs: polycyclic aromatic hydrocarbons

PCNA: proliferating cell nuclei antigen

PE: proestrous

PGCs: primordial germ cells

PND: postnatal day

PNP: 4-nitrophenol

POA: preoptical area

RIA: radio immunoassay

sc: subcutaneous injection

shRNA: short hairpin RNA

SRY: sex determining region Y gene

StAR: steroidogenic acute regulatory

TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling

WWF: World Wildlife Federation

Chapter 1 Introduction

With the development of human society, human-made chemicals are becoming part of everyday life in this living world. However, some of those chemicals turn to the pollutants after introducing into the environment with or without intention. And those chemical pollutants have developed into a more and more serious problem attracting public concerns. Over the past decades, the increasing human reproductive dysfunction and other endocrine-related health problems are accompanied with the increasing chemical pollutant in the environment. The phenomenon related with disrupted endocrine system is also observed in the wildlife. And in truth, the correlation between chemical pollutants and disease outcomes in the laboratory animals is identified in a lot of research work. The present study focuses on the chemical pollutants, especially the issue of endocrine disrupting chemicals (EDCs) acting on the reproductive system.

1.1 Endocrine disrupting chemicals

1.1.1 What is endocrine disrupting chemicals?

Although there is no precise definition for EDCs until now, generally EDCs represent a wide range of chemicals that have the potential to interfere with the body's endocrine system (28). Most of EDCs are humman-made chemicals. Since the pesticide dichloro-diphenyl-trichloroethane (DDT) was found in penguins of the south pole during 1970s, the public concerns on the EDCs have greatly increased (43). Until 2013, close to 800 chemicals are known or suspected to be EDCs dues to the ability to interfere with hormones receptors, hormones synthesis or other endocrine functions (141). These suspected EDCs include not only some persistent organic pollution chemicals and pesticides, but also many common chemicals, such as 17α-ethynyl estradiol (EE) and bisphenol A, used in the daily life. To be aware, a vast majority of chemicals used in the commercial products are still waiting for test.

1.1.2 The occurrence of EDCs exposure

EDCs are both human-made and natural chemicals, which were found among many classes of chemicals, including persistent organic pollutions, current-use pesticides, phytoestrogens, metals, active ingredients in pharmaceuticals, and additives or contaminants in food, personal care products, cosmetics, plastics, textiles and construction materials (56; 141). Once released into the environment, the more persistent chemicals can be carried by air and water currents to remote locations, and many can be biomagnified through food webs to high levels in humans and other top predators. Other chemicals have shorter lifespans in the environment but are regularly released in effluents, in agricultural runoff or from urban environments, resulting in the high environmental levels near the sources (141).

Human exposure to EDCs occurs via ingestion of food, dust and water, by inhalation of gases and particles in the air and through dermal uptake (141). Transfer of EDCs from the pregnant female to the developing fetus through the placenta and to offspring in mothers' milk also occurs in both wildlife and human (17; 36; 144). Children can have higher exposures to EDCs because of their hand-to-mouth activities (87).

1.1.3 General adverse effect of EDCs on human's or animals' health

As mentioned in the definition, EDCs induce the adverse effect via interfering with hormone action on the health of human, wildlife or other animals. Since the physiological system is linked with all the endocrine organs and different types of hormones, EDCs could possibly disrupt the whole endocrine system and induce the huge health problem ranging from the organ development defect to the energy metabolism disorder, and from the dysfunction of reproductive organs to the cardiovascular disease (56; 64; 115).

1.1.4 17α-ethynyl estradiol (EE)

One endocrine disrupting chemical used in the present study is 17α-ethynyl estradiol. The abbreviated name for 17α-ethynyl estradiol is EE. EE is an orally bioactive estrogen used in many formulations of combined oral contraceptive pills and is one of the most commonly used

medications for this purpose (34). The dose for hormonal therapy treatment using EE is ranging from 15 to 50 μg per tablet (34; 92). In addition, EE now is also used for the medicine treatment in the livestock (71; 72). EE is released into the environment as a xenoestrogen from the urine and feces of human or livestock who take it as a medication (72; 101; 138). EE has become an environmental pollution problem due to the high resistance to the process of degradation (22). Due to the above reason, EE could be easy to spread widely via the raining or water transport and accumulate and concentrate in the biota system via the food chain (31; 84).

1.1.5 4-nitrophenol (PNP)

The other endocrine disrupting chemical applied in the present study is 4-nitrophenol. The abbreviated name for 4-nitrophenol is PNP. We can find PNP existing in our daily life in three different ways. PNP is one degradation of insecticide parathion, which you are probably using in your backyard garden (41; 139). PNP is also isolated from the diesel exhaust particles and there is about 169 mg PNP in the 1 kg diesel exhaust particles (96). And more, PNP is presented in the drug or medicine as the contamination during the medicine production (2; 33). Those lines of evidence show that people could easily be exposed to PNP in the daily life via breathing or eating, and this makes it meaningful to investigate carefully the effect of PNP on the animal physiology in the laboratory work.

1.2 Hypothalamus-pituitary-ovary axis

As known, the hypothalamic–pituitary–gonadal axis (HPG axis) plays a center role on the regulation of the reproductive system (21; 54). As shown in the name, HPG axis consists of the hypothalamus, pituitary gland, and gonadal glands. The hypothalamus is located in the brain and produces kisspeptin and GnRH. Kisspeptin neurons are localized in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC) of the hypothalamus and project to the GnRH neurons, which localize in the preoptical area (POA) of the hypothalamus. Kisspeptin produced from the kisspeptin neurons are transported to gonadotropin releasing

hormone (GnRH) producing neurons and binds with kisspeptin receptor-GPR54, which induces the production of GnRH from the hypothalamus. GnRH travels down the anterior portion of the pituitary via the hypophyseal portal system and binds to receptors on the secretory cells of the adenohypophysis (18). In response to GnRH stimulation, these cells in the pituitary produce gonadotropins including luteinizing hormones (LH) and follicle stimulating hormone (FSH), which travel into the blood stream (136). These two hormones play an important role in the gonads. In females, FSH and LH act on the ovary and stimulate the expressions of steroidogenic enzymes in granulosa cells and theca cells to produce estrogen. During the menstrual cycle in human or estrous cycle in the mammals, the low dose of estrogen exerts a negative feedback effect to inhibits the production of GnRH and gonadotropins; before the ovulation, the high dose of estrogen exerts a positive feedback effect to stimulate the production of GnRH and gonadotropins. One mechanism involved in the positive and negative feedback loop is that estrogen can stimulate the expression of kisspeptin in the AVPV of hypothalamus and inhibit the expression of kisspeptin in the ARC of hypothalamus (106). Moreover, inhibin and activin produced by the ovary also modulate the activity of HPG axis (79). Inhibin goes to the pituitary by the circulation and inhibits the production of gonadotropins, and activin works oppositely (79).

1.3 The ovary development

The origin of ovary during embryo period is from the gonadal ridge. During embryo 6.5 day (E6.5), the primordial germ cells (PGCs) proliferate and migrate into the gonadal ridge in the rodents. Without the sex determining region Y gene (SRY), the PGCs will differentiate into the ovary (7). Before birth, the oocytes are pooled in the germ cell cyst. Then, the germ cell cyst starts to breakdown and the oocyte is surrounded by the somatic cells to form the primordial follicle during the first three days after birth. Within one week after birth, along with the flat granulosa cell becoming the cubical granulosa cell, the primordial follicles grow

into the primary follicles. The granulosa cells continue to proliferate and with the layers of granulosa cells in the primary follicles increasing, the primary follicle becomes the secondary follicle (118). Under the stimulation of gonadotropins, the secondary follicles grow into antral follicles, and eventually, the first ovulation occurs with the stimulation of LH during the puberty. The rest of the ovulated follicle forms corpus luteum (32). During the adult period, the follicle recruitment occurs with the responsive of hormone rehearsal. And the selected follicle would growth and ovulate; other follicles will undergo the fate of atresia.

1.4 The objective of the present study

The aim of the present study is to investigate the mechanism underlying the effect of endocrine disruptor chemicals including EE and PNP on ovary function in the mammalian animal model (female Wistar-Imamichi rats), especially during the early stage of the life.

Chapter 2 The effects of neonatal exposure to EE on female reproduction during adult period

2.1 Introduction

It has been approximately 20 years since the first World Wildlife Federation (WWF) Wingspread Conference focused on endocrine-disrupting chemicals (EDCs) (42). EDCs represent a broad class of synthetic and natural chemicals, most of which have estrogenic activity (42). 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 (98).

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 (9; 86). 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 (104; 118). Estrogens are involved in these processes and predicate the limited number of oocytes formed during the reproductive lifespan of the female (20; 61). Exposure to estrogenic compounds may delay reproductive competence in adult female rodents by affecting the brain and/or the ovarian cascade.

The synthetic estrogen, 17α-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 (94; 121) because it is excreted in urine and feces and not completely removed during wastewater treatment, thus eventually being dis- charged into aquatic environments (22;

103). Because the contraceptive pills contain 20–50 μg EE, corresponding to 0.2–1.0 μg/kg/day in humans, the doses selected in many studies using laboratory animals, 1.0–50 μg/kg/day, were approximately 10–100 times higher than exposure of women. it was observed that single injected EE to neonatal animals also induced delayed effects similar to the long- term perinatal exposure, such as the early onset of persistent estrous (116; 126). It is reported that injected EE was excreted from animal bodies within 24 h (126) 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. To gain better insight into the mechanism of the delayed effects on the reproductive system by single neonatal exposure of EE, the reproductive functions in the adult female rodents were investigated in the present study after the single exposure to EE during neonatal period.

2.2 Materials and Methods

2.2.1 Animals

Adults Wistar-Imamichi rats were maintained in an animal room under standard housing conditions of controlled lighting (lights on 0500-1900h), temperature (25±2°C) and humidity (50±10%). They were feed a 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 (23-1). All the procedures were permitted by the ethical committee of animal experimentation of Tokyo University of Agriculture and Technology.

2.2.2 Experimental design

Figure 2-1 summarizes the experiment conducted. Newborn female pups were assigned to one of the following neonatal treatment: 1) controls given 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 given on within 24 hours 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 PND 90 or PND180. Once vaginal opening had occurred, daily vaginal smear was collected from each rat and the cytology was recorded until PND180 (each treatment *n*=8). Another set of pups around PND90 were euthanized at 1100h on second diestrous day (D), 1100h on proestrous day (PE11), 1700h on proestrous day (PE17) and 1100h estrous day (E), and blood and ovaries were collected. The collected blood samples were immediately centrifuged (3000rpm for 15min at 4°C), and serum was harvested and stored at -20°C until use for hormonal assays. Four sets of ovaries were snap frozen and stored at -80°C for RNA and protein extraction. For histological analysis, another four sets of ovaries were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned.

To evaluate the acute effects of EE, ovaries at PND1 were collected and stored at -80°C for RNA extraction (each treatment *n*=5). In addition, ovaries at PND0 were collected and cultured with or without 1 ng/ml EE in Dulbecco's Modified Eagle Medium (DMEM) containing insulin-transferrin-selenium (ITS) supplement and antibiotic-antimycotic (Invitrogen). After 24h culture, ovaries were snap frozen and stored at -80°C for RNA extraction (each treatment *n*=5).

2.2.3 Hormone assays

Serum concentrations of luteinizing hormone (LH) and follicle stimulating hormone (FSH) were measured using an NIDDK rat radio immunoassay (RIA) kits. 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 intra- and interassay coefficients of variations were 5.4 and 11.2% for LH, 7.2 and 15.7% for FSH, respectively.

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 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) kits (Perkin-Elmer, Waltham, MA) in accordance with the manifacturer's instructions. All samples to be compared were analyzed in the same assay.

2.2.4 Quantitative real-time PCR

Total RNA from each sample was extracted using ISOGEN (Nippon Gene, Tokyo, Japan). Complementary DNA (cDNA) was synthesized using PrimeScript reverse transcriptase (TaKaRa bio, Shiga, Japan) according to the manufacturer's protocol. Oligonucleotide primers were designed using a web-based Primer3 software and are listed in Table 2-1. PCR reactions were run using SYBR Premix Ex Taq II (TaKaRa Bio), and the expression of each target mRNA relative to glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA was determined using the $2^{-\Delta\Delta CT}$ method.

2.2.5 Western blot analysis

Whole-cell lysate was prepared in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 mM $NaVO_4$, and 50 mM NaF) supplemented with Protease Inhibitor Cocktail (Roche Applied Science, Penzberg, Germany). The 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% skimmed milk at room temperature for 1 h and then incubated with anti-luteinizing hormone/ choriogonadotropin receptor (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 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).

2.2.6 Histological analysis

Ovaries were fixed in 4% paraformaldehyde, embedded in paraffin, serially sectioned at 10 μm and stained with hematoxilin and eosin. Ovarian follicles were categorized as previously described (Braw, 1980, Flaws et al 2001). Follicles were classified as primordial if they contained an intact oocyte surrounded by a single layer of flat granulosa cells. Other follicles were classified as small-size, medium-size and large-size by their follicular diameter, <50 μm, 50-170 μm and 170 μm<, respectively. The number of follicles and corpora lutea were counted in the mid-portion of each ovary (20 serial sections per ovary, 3 ovaries per group).

2.2.7 Immunohistochemistry

Serial sections of ovary were incubated with 10% normal goat serum to reduce background staining caused by the second antibody. Then the sections were incubated with primary antibody, anti-LHCGR (Proteintech) or anti-cleaved caspase3 (Cell signaling, Beverly, MA) for 12 h at room temperature. Then the sections were incubated with a secondary antibody, anti-rabbit lgG conjugated with biotin and peroxidase with avidin, using a rabbit VECTASTAIN ABC kit (Vector lab., Burlingame, CA) and subsequently visualized with 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) instead of the primary antibodies.

2.2.8 Statistical analysis

The data are presented as mean ± SEM of 3 independent experiments, each performed in triplicate. The level of significance was analyzed using one-way analysis of variance, followed

by multiple range tests (GraphPad Prism5). Differences were considered statistically significant when $p < 0.05$.

2.3 Results

2.3.1 Effect of neonatal EE exposure on reproductive parameters

Body weight and reproductive organ's weight, ovary and uterus, are shown in Table 2-1. The rats in all treatment groups grew normally, and there were no significant differences in the body weight, ovary weight and uterus weight between control, low and high dose treated groups (Table 2-2).

The days of vaginal opening, an indicative of puberty, are shown in Figure 2-2A. The slight earlier puberty onset was observed in EE treated animals, but there were not significant differences among the groups. The results of estrous cycles are shown in Figure 2-2B. 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. 2-2B). 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. 2-2B).

2.3.2 Effect of neonatal EE exposure on hormonal changes at PND90

Changes in serum levels of LH, FSH, inhibin, 17β-estradiol, testosterone, and progesterone at PND90 are shown in Fig. 2-3. 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 during PE17, and a low level of inhibin during PE11 and PE17 (Fig. 2-3 A–C). Animals from the high-dose group also showed the suppression of

increment in the levels of testosterone during PE11 (Fig. 2-3 E), and the suppression of increment in progesterone level from PE11 to PE17 (Fig. 2-3 F). Comparisons of hormone levels in the control and low-dose groups revealed that 17β-estradiol was the only hormone for which levels were affected, as the level of 17β-estradiol was higher in the low-dose group than in the control group at PE17 (Fig. 2-3 D).

2.3.3 Effect of neonatal EE exposure on ovarian follicular composition at PND90

To find any potential alterations between control and low dose group at PND90 when there was no significant difference on estrous cyclicity and hormonal changes, ovarian morphology was examined. Control and low dose ovaries contained follicles at all stages of the development and some corpora lutea (Fig. 2-4A). The number of follicles per section did not change between control and low dose treated groups (Fig. 2-4B). Examination of follicular composition demonstrated that there were no significant changes in the percentage of small, medium-sized and large follicles in the ovaries (Fig. 2-4C). The percentage of primordial follicles decreased in low dose EE-treated ovaries while corpus luteum number increased (Fig. 2-4C).

2.3.4 Effect of neonatal EE exposure on ovarian gene expression at PND90

To explore the change of ovarian gene expression in EE exposed ovaries at PND90, the levels of LHCGR, FSHR, cytochrome p450scc, inhibin α subunit, inhibin/activin βA subunit and inhibin/activin βB subunit mRNA were quantified by real-time PCR. It was observed that LHCGR mRNA was higher at PE11 and FSHR mRNA was higher at PE17 in in high dose group than in control group (Fig. 2-5). The expressions of cytochrome p450scc and inhibin α subunit were higher from PE11 to E in the high dose group (Fig. 2-5). The expression of inhibin/activin βA subunit was higher at PE11 and E and the expression of inhibin/activin βB subunit was high at PE11 and PE17 in high dose group than in control group (Fig. 2-5). Inhibin α subunit mRNA was higher at PE17 and inhibin/activin βB subunit mRNA was also higher at PE11 and PE17 in low dose group.

2.3.5 Expression and localization of LHCGR protein in neonatal EE exposed ovary at PND90

As we suspected that LHCGR might contribute to the change of follicular composition, the decrease of primordial follicles and/or the increase of corpus luteum number, LHCGR protein expression and localization in ovary was investigated by western blotting and immunohistochemistry analysis. Consistent with mRNA change, LHCGR protein was increased in low dose EE-treated ovaries (Fig. 2-6A). Immunohistochemical analysis shows that positive signals of LHCGR protein were mainly observed in luteal cells and interstitial theca cells in both groups (Fig. 2-6B). In low dose EE-treated ovaries, however, luteal-like cells in the interstitial area were increased and these cells exhibited cleaved-caspase-3 proteins, one of apoptosis marker (Fig. 2-6B).

2.4 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 (29; 88). 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 (91). Because the binding affinity of the estrogen receptor (ER) of fish for EE is approximately five times higher than the binding affinity for fish estrogen (130), 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. During normal follicular development, LHCGR is expressed in thecal cells, interstitial cells, differentiated granulosa cells in preovulatory follicles, and corpus luteal cells (6). The major function of LHCGR is to induce ovulation of preovulatory follicles and formation of the corpus luteum (74). The increase in LHCGR expression after exposure of ovaries to EE was mostly observed in the interstitial cells, which also expressed cleaved caspase-3. The gonadotropin LH enhances caspase-3 activity and apoptosis in thecal-interstitial cells (145; 146). 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 (25; 80). It has been reported that neonatal and perinatal treatment of EE is associated with the formation of follicular cysts and abnormal estrous cycling (83). 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 (oral administration vs subcutaneous administration) and/or different times of treatment (embryonic vs. postnatal).

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 (38). 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 (95), which also impacts ovarian development (78). 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 (126, 134). Furthermore, these effects of EDCs exposure are complicated by the fact that under natural, rather than experimental, situations, exposure involves a complex mixture of chemicals (36). It remains challenging to provide a mechanistic explanation for these effects.

Table 2-1. Nucleotide sequences of the primers used for real-time PCR

Gene	Forward	Reverse
LHCGR	5′-GCATTCAATGGGACGACTCT-3′	5′-GTAGGAAGACAGGGCGATGA-3′
FSH-R	5′-TTTACTTGCCTGGAAGCGACTAA-3′	5′-CCCAGGCTCCTCCACACA-3′
p450scc	5′-GGAGGAGATCGTGGACCCTGA-3′	5′-TGGAGGCATGTTGAGCATGG-3′
INHα	5′-GCTCTACCAGGGAGCATGAG-3′	5′-CACCTTCCTCCTAGCTGACG-3′
INHβA	5′-TTTCTGTTGGCAAGTTGCTG-3′	5′-CGGGTCTCTTCTTCAAGTGC-3′
INHβB	5′-GCCACGTATCCCTGACTTGT-3′	5′-CTGCTCCATGGTCTCTGTGA-3′
GAPDH	5′-GGCACAGTCAAGGCTGAGAATG-3′	5′-ATGGTGGTGAAGACGCCAGTA-3′

Table 2-2. Body weights, ovary and uterus weights at 1100 h on the day of proestrous (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

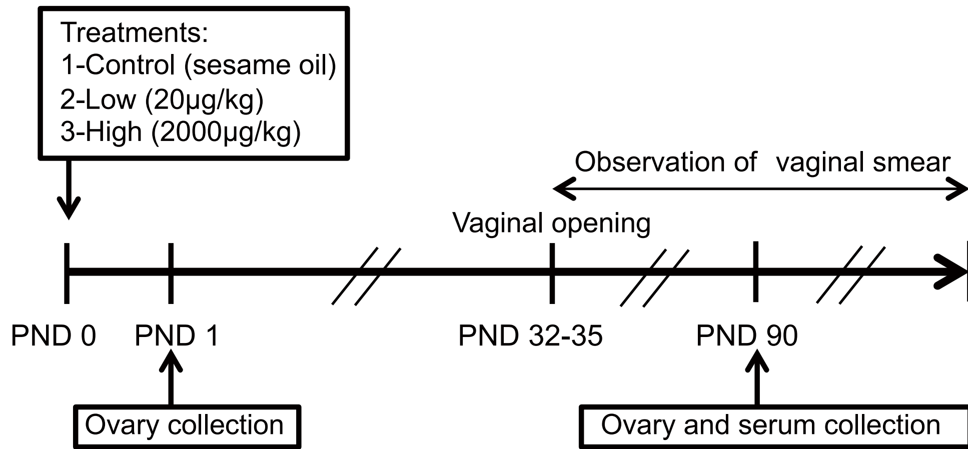


Fig. 2-1. Schematic representation of experimental protocol. PND, postnatal day.

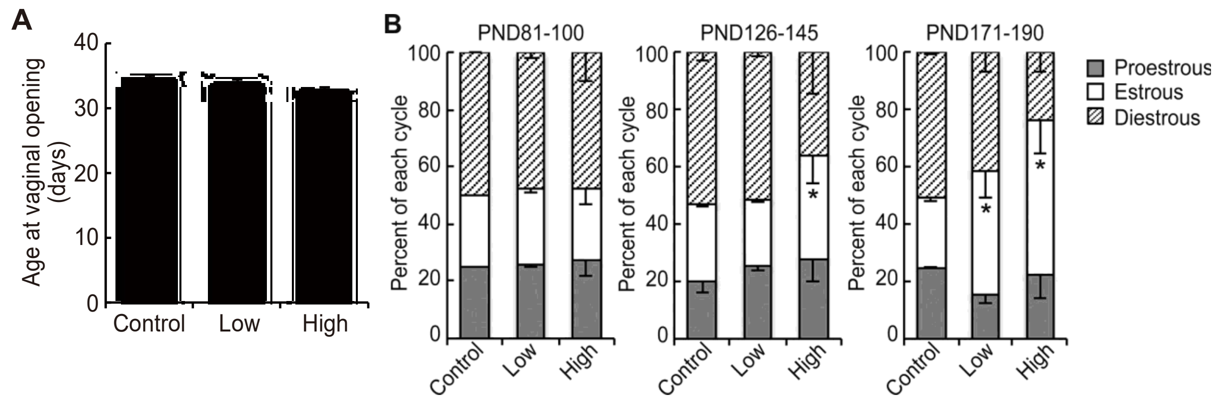


Fig. 2-2. Effect of neonatal EE exposure on reproductive parameters, days of vaginal opening (A) and percent of time spent in each cycle during PND81–100, PND126–145 and PND171–190 (B). Rats were neonatally treated with sesame oil and with two concentrations of EE (low, 20 μg/kg; high, 2000 μg/kg). Estrous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

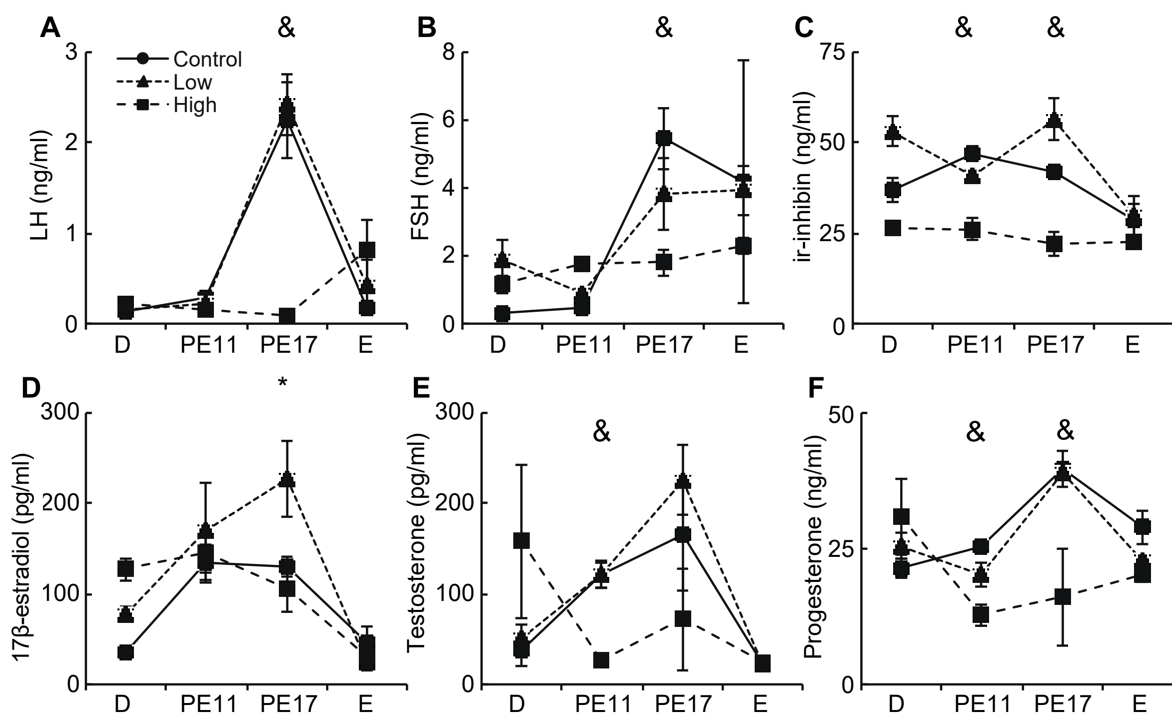


Fig. 2-3. Changes in serum levels of LH (A), FSH (B), ir-inhibin (C), 17β-estradiol (D), testosterone (E), and progesterone (F) in neonatal EE-treated rats. Blood was collected at PND90 from animals treated with sesame oil and with two concentrations of EE (low, 20 μg/kg; high, 2000 μg/kg). Hormone level was measured by RIA at 1100h at diestrous day (D), 1100h proestrous day (PE11), 1700h on proestrous day (PE17) and 1100h estrous day (E). And data are presented as the mean ± SEM. * and & represent the significant difference between control and low-dose or high-dose EE-treated rats, respectively.

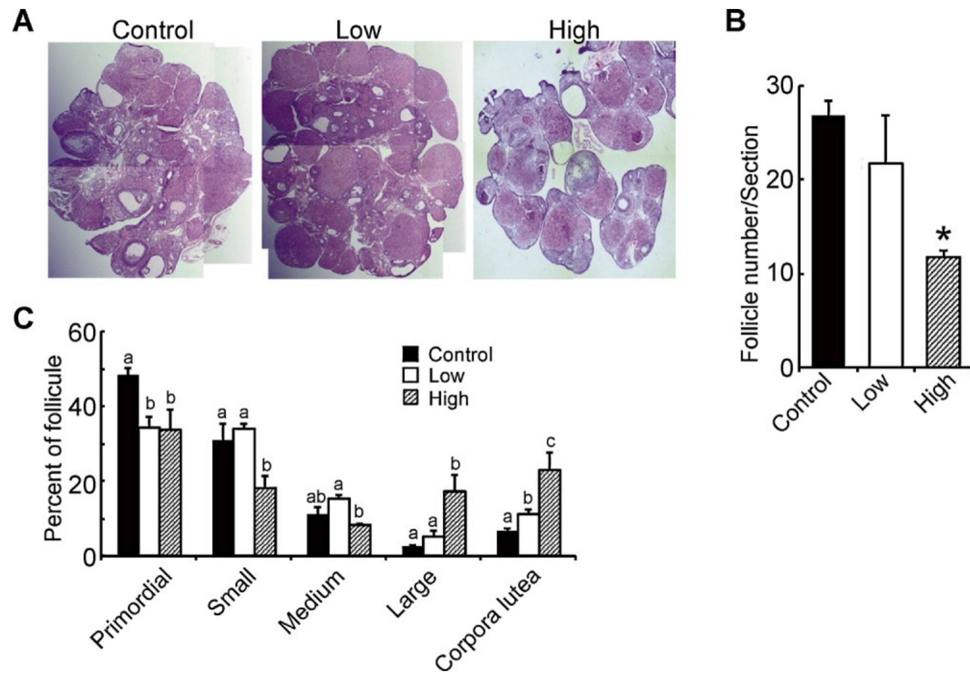


Fig. 2-4. Effect of neonatal EE exposure on ovarian follicular composition. Ovaries were collected from control, low- and high-dose-treated animals at PND90. Histology (A), total follicle count (B), and composition analysis (C) were performed. Histogram represents the mean ± SEM. Means with different superscripts are significantly difference ($p < 0.05$).

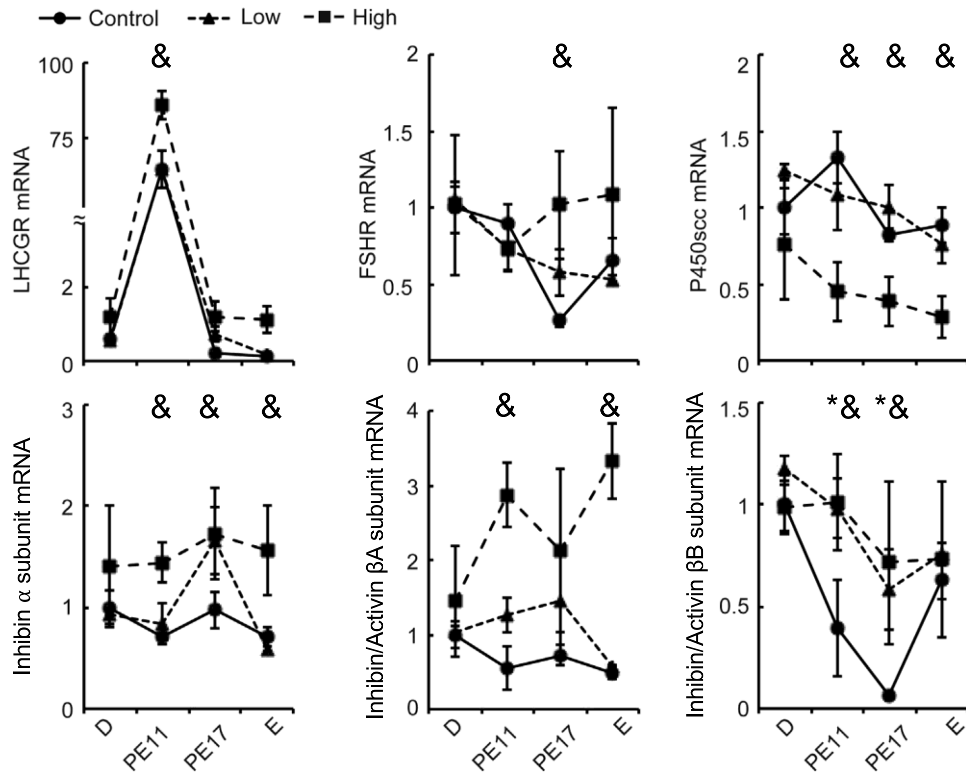


Fig. 2-5. Changes in LHCGR, FSHR, P450scc, inhibin α subunit, inhibin/activin βA subunit and inhibin/activin βB subunit mRNA expression in neonatal EE-treated ovaries. (A) Ovaries were collected at 1100 h on the second diestrous day (D), 1100 h on the proestrous day (PE11), 1700 h on the proestrous day (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. * and & represent the significant difference between control and low-dose or high-dose EE-treated rats, respectively.

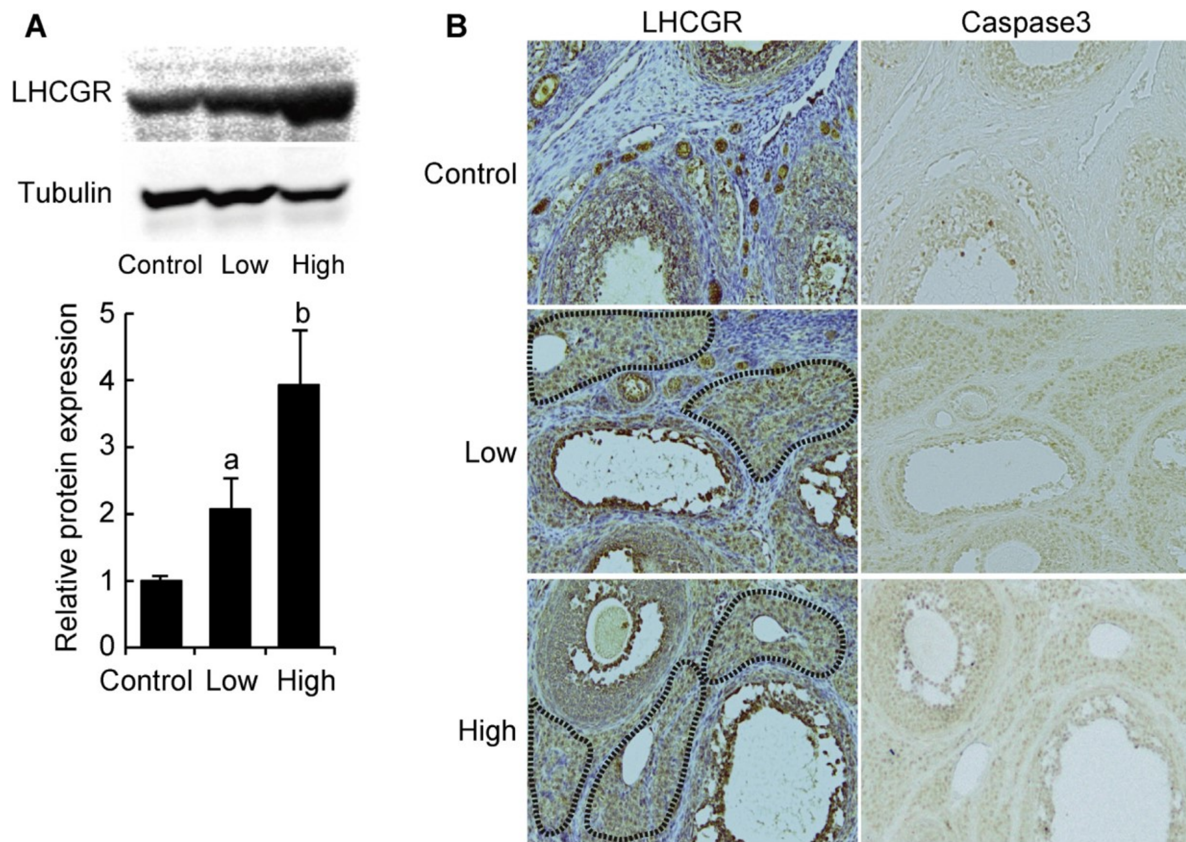


Fig. 2-6. Protein expression of LHCGR in neonatal EE-treated ovaries. Ovaries were collected at PE17 from PND90 animals treated with sesame oil, low-dose EE (20 μg/kg) and high-dose EE (2000 μg/kg). (A) LHCGR protein level was determined by western blot analysis. The letters indicate the significant difference ($p < 0.05$). (B) LHCGR and cleaved-caspase-3 protein localizations were demonstrated by immunohistochemistry. Circles represent the interstitial area expressing LHCGR and cleaved-caspase-3.

Chapter 3 The effects of neonatal exposure to EE on hormone profile during the pre-pubertal period in the female rats

3.1 Introduction

Endocrine disruptor chemicals (EDCs) are referred to as the human-made or natural chemicals that may interfere with the body's endocrine system (28). EDCs have been proved to be associated with the reproductive dysfunction, abnormal fetus development, breast cancer and other physiological defect or disease (16; 100; 122; 133). In particular, exposure to EDCs during the fetal and neonatal periods may be very risky and the toxic effects during these critical periods may be irreversible and last for the whole life (28). Many human-made EDCs have been found in the environment, such as 17α-ethynyl estradiol (EE) in water, bisphenol A in food product and 4-nitrophenol (PNP) from diesel exhaust (72; 96; 107). Due to the increasing public concerns on the effect of EDCs on the human and animal health in recent years, the basic data collection about the adverse effects of EDCs on human and animal physiology becomes more and more important.

EE is a kind of synthetic estrogen, which has been used as oral contraception drug for women. And now it becomes a well-known EDC in the environment since it gets into the aquatic environment via wastewater discharges (22; 103). Many reports from experimental animals showed neonatal exposure of EE in rats would induce reproductive dysfunction during the adulthood (81; 83; 112; 116; 126). Our previous study showed earlier disrupted estrous cycle, shorter reproductive lifespan and disrupted expressions of Kiss1 in the hypothalamus occurred in adult rats after neonatal exposure to EE (97; 134). However, the molecular or physiological changes in the pre-pubertal rats after neonatal exposure to EE are still unclear.

Hypothalamus-pituitary-ovary axis is the classical reproductive axis (21). As known, kisspeptin can stimulate the production of gonadotropin releasing hormone (GnRH) in the hypothalamus and GnRH can stimulate the secretions of gonadotropins including follicle-

stimulating hormone (FSH) and luteinizing hormone (LH) from pituitary, which can promote the steroidogenesis through stimulating the expression of steroidogenic enzymes including steroidogenic acute regulatory (StAR), cytochrome P450 cholesterol side-chain cleavage (P450scc), cytochrome P450 17α-hydroxylase/17,20 lyase (P450c17), 3β-hydroxysteroid dehydrogenase (3β-HSD) and cytochrome P450 aromatase (P450arom) the in the ovary (5). In addition, the ovary could produce activins or inhibins to promote or inhibit gonadotropins' production (21). It remains unclear how the neonatal exposure to EE influence the hypothalamus-pituitary-ovary axis, especially concerning on the hormone profiles. The aim of the present study is to investigate the steroidogenic enzymes expression in the developmental ovaries, the peptide and steroid hormone profile difference in the pre-pubertal rats between control and EE treatment group.

3.2 Materials and methods

3.2.1 Animals

Wistar-Imamichi male and female rats were purchased from SLC (Shizuoka, Japan) and maintained at 23 ± 2 ˚C under a 14-hour lighting schedule (lights on 05:00 to 19:00 h). Food and tap water were given *ad libitum*. After mating and delivery, the female pups were used for the experiment. All procedures were carried out in accordance with the animal welfare regulations of Tokyo University of Agriculture and Technology (23-1). All the procedures were permitted by the ethical committee of animal experimentation of Tokyo University of Agriculture and Technology.

3.2.2 Experiment design

Newborn female pups were assigned to one of the following neonatal treatment: controls given sesame oil vehicle alone (control group) or EE at 200 μg/kg. Treatments were given on within 24 hours of delivery, postnatal day 0 (PND0), by subcutaneous (sc) injection in the nape of the

neck. All compounds were dissolved in sesame oil. The ovaries in control and treatment group were collected at PND1, PND3, PND7, PND14 and PND21.

3.2.3 Histology

Ovaries were fixed with 4% paraformaldehyde, embedded in paraffin, serially sectioned at 6 μm and stained with hematoxylin and eosin. The diameter of all antral follicles in ovaries of PND21 were measured with cellSens software (Olympus, PA, USA) every five sections. The antral follicles with a clear oocyte nucleus were examined in a total of 80 sections per ovary (3 ovaries per group).

3.2.4 Immunohistochemistry

Serial sections of the ovary were incubated with 10% normal goat serum to reduce background staining caused by the second antibody. Then the sections were incubated with primary antibody (1:1000 dilution), rabbit against human placental cytochrome P450 aromatase (P450arom) or mouse against proliferating cell nuclei antigen (PCNA) antibody (Thermo Fisher scientific, IL, USA, #13-3900) for overnight at 4°C. Then the sections were incubated with the biotinylated anti-rabbit or anti-mouse IgG conjugated with peroxidase and avidin, using a rabbit or mouse VECTASTAIN ABC kit (Vector lab., CA, USA) and subsequently visualized with 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, CA, USA) or normal mice IgG (EMD Millipore, MA, #12-371) instead of the primary antibodies. Images were captured using a microscope, BX-50 (Keyence, Osaka, Japan).

3.2.5 QRT-PCR analysis

Total RNA was extracted from ovaries or brains using TRIzol Reagent (Invitrogen Co., CA, USA) according to the protocol. And cDNA was synthesized using PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan). The oligonucleotide primers for qRT-

PCR analysis were designed using the Primer 3 program (Table 3-1). The PCR reactions were carried in a 10 μl volume using ExTaqR Hot Start Version containing SYBR-Green I (Takara Bio) and performed with chrome4 Real-Time PCR System (Bio-Rad, CA, USA) using the following conditions: 95℃ for 30 sec, followed PCR reaction by 40 cycles of 95°C for 5 sec, 60°C for 30 sec, and dissociation protocol. The expression level of each target mRNA relative to GAPDH mRNA was determined using the $2^{-\Delta\Delta Ct}$ method.

3.2.6 Hormone Assay

Serum concentrations of LH and FSH were measured using an NIDDK rat RIA kits. 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 intra- and interassay coefficients of variations were 5.4 and 11.2% for LH, 7.2 and 15.7% for FSH, respectively.

Serum concentrations of 17β-estradiol were measured using ^{125}I-labeled radioligands ([^{125}I] MP01738226), MP Biomedicals, CA, USA). Antiserum to 17β-estradiol (GDN244) were kindly provided by Dr. G.D. Niswender (Fort Collins, CO, USA). The intra- and interassay coefficients of variations were 3.4% and 5.2% for E_2, respectively.

Serum concentrations of immunoreactive (ir-) inhibin were measured using rabbit antiserum against bovine inhibin (TNDH-1) and 125I-labeled 32-kDa bovine inhibin. The intra- and interassay coefficients of variation were 7.1 and 14.7%, respectively.

All samples to be compared were analyzed in the same assay.

3.2.7 Statistical analysis

Statistical comparisons were made with the Student's *t*-test or One-way ANOVA followed by Turkey's multiple range test using Prism 5 (Graphpad Software Inc., CA, USA). A value of $p<0.05$ was considered indication of statistical significance.

3.3 Results

3.3.1 Body weight and ovary weight

The body weight and ovaries weight were checked in the control and EE treatment rats at the developmental stage. There is no significant difference in the body weight between the control group and EE treatment group from PND1 to PND21 (Fig. 3-1A). However, there were significant decreases in the ovary weight of EE treated rats at PND14 and PND21 (Fig. 3-1B).

3.3.2 Cell proliferation and histological examination in the ovaries at PND21

Due to the decreased ovarian weight in the EE treatment group, the cell proliferation and the ovarian histology were checked at PND21. The PCNA staining in control (Fig. 3-2A-E) and EE treated ovaries (Fig. 3-2F-J) were showed in the figure 3-2. PCNA were stained in theca cells, granulosa cells and oocytes in the different types of follicles. However, there is no too much difference between control and EE treated ovaries, which indicates that the cell proliferation is not different between control and EE treated ovaries.

The ovary histology was also checked in the control and EE treated animals (Fig. 3-2K and L, respectively). The number of antral follicles with the diameter more than 300μm in control ovary was more than that in EE treated ovary, however, the number of antral follicles with the diameter between 100-150 μm in control ovary was fewer than that in EE treated ovary (Fig. 3-2M).

3.3.3 Hormones changes in the control and EE treatment rats

The different types of hormones including gonadotropins, 17β-estradiol and inhibin were investigated in the control and EE treatment rats from PND3 to PND21. Though there was no difference in the circulating FSH levels between the control and EE treatment groups (Fig. 3-3A), the levels of circulating LH is significantly higher in the EE treated rats when compared with that in the control rats (Fig. 3-3B). There was a peak in 17β-estradiol concentration in both the control and EE treated rats at PND14; however, the level of 17β-estradiol is significantly higher in the EE treated rats than the control rats at PND14 (Fig. 3-3C). The

circulating level of inhibin was constantly low from PND3 to PND14 and increased to a relatively high level at PND21 (Fig. 3-3D). However, there was no significant difference in the inhibin concentration between the control and EE treatment groups.

3.3.4 The expressions of steroidogenic enzymes and inhibin/activin subunits in the developmental ovaries.

The genes expressions profile including steroidogenic enzymes and inhibin/activin subunits in the developmental ovaries were investigated by real-time PCR. There is no difference in the expression of P450c17 in the developmental ovaries of control and EE treatment groups from PND1 to PND21 (Fig. 3-4A). The expression of P450arom in the EE treatment ovaries increased significantly compared with that in the control ovaries at PND14 (Fig. 3-4B). The expression of inhibin α was not changed by EE exposure, but the expression of inhibin/activin subunits βA and βB were decreased by EE exposure at PND21 (Fig. 3-4C-E, respectively).

3.3.5 The immunolocalizaition of P450arom in the ovaries at PND14

P450arom were localized in the theca cells and granulosa cells of different types of follicles including primary follicles, secondary follicles and antral follicles at PND14 in the control (Fig. 3-5A) and EE (Fig. 3-5B) treated ovaries. There was slightly decrement in the immunoactivity of P450arom in the control ovaries than EE treated ovaries (Fig. 3-5).

3.3.6 The expression of kisspeptin in the hypothalamus in the developmental rats

Although the expressions of kisspeptin in anteroventral periventricular nucleus (AVPV) of the hypothalamus were no difference between control and EE treatment rats (Fig. 3-6A), the expression of kisspeptin in the arcuate nucleus (ARC) of the hypothalamus decreased in the EE treatment group compared with that in the control group at PND14 (Fig. 3-6B).

3.4 Discussion

The adverse effect after exposure to EDCs has been studies for many years. The causal relationship between neonatal exposure to EDCs and health defect during adulthood has been

reported in many published papers (81; 83; 112; 116; 126). In our previous studies, neonatal exposure to EE induced the disrupted estrous cycles in the adult period (97; 134). To clarify the mechanism for this delayed effect, the influence of EE on the ovarian development in the pre-pubertal period was checked in the present study. The results of this study showed that the ovary weight decreased at PND14 and PND21. However, the cell proliferation in the control and EE treated ovaries is not different. Moreover, the size of antral follicle decreased in the EE treated ovaries. Those results suggest that the decreased ovary weight may be due to the impaired follicle development in EE treatment group. Estrogen plays an important role in the ovary function and follicle development (1; 14). The high dose of 17β-estradiol could delay or inhibit the oocyte meiotic maturation and induce abnormal follicle development (129). The increased circulating 17β-estradiol accompanied with the increased expression of P450arom was found in the EE treated rats at PND14, which is the timing for the antral follicle formation. Taken together, the increased 17β-estradiol at PND14 may retard the follicle development and result in the high ratio of small size follicles at PND21.

EE is one small molecule, which can not only act directly on ovary via binding with hormone receptors, but also can pass the blood-brain barrier (BBB) and exert its functions on the developmental brains (114; 123). The evidence from previous reports also showed that endocrine disruptors including bisphenol A directly affect the expression of P450arom in human fetal osteoblastic and granulosa cell-like cell lines (14; 140). However, the present data showed that the difference in the expressions of P450arom between control and EE treatment group were not found at PND1 but at PND21. This data suggests that EE may disrupt the expression of P450arom in the ovary via the indirect pathway. Moreover, the LH level in the EE treated animals is higher than control animals. Taken those data together, it is indicated that neonatal EE exposure may affect the hypothalamus-pituitary function and therefore induce high circulating estrogen and ovary dysfunctions.

Kisspeptin has been proved to be the controller for the hypothalamus-pituitary-gonad axis (99; 110). The previous reports showed EDCs would induce the epigenetic effect on the gene expression in the exposed individuals (50; 82; 119; 120), and estrogenic compounds exert the ability to regulate the expression of kisspeptin via acetylation and deacetylation (132). The present data showed single neonatal exposure to EE changed the kisspeptin expression after two weeks, which suggests EE may regulate kisspeptin expression in the developmental hypothalamus via epigenetic pathway. As we known, the kisspeptin neuron subpopulations localized in AVPV regulates or in ARC are the LH surge regulator or pulse regulator, respectively (108). The decreased kisspeptin expression in ARC at PND14 may reduce the LH pulse frequency and influence the follicle development. Although we could not explain the increased circulating LH concentration with the decreased expression of kisspeptin in the ARC of hypothalamus after EE exposure at PND14, we could not exclude that EE may directly influence the LH expression or secretion in the pituitary.

Inhibins or activins are one of the important factors involved in the ovary functions and development (66; 109; 142). In the present study, although neonatal exposure to EE would not affect the inhibin, the altered expressions of inhibin/activin subunit βA and βB were observed at PND21 in the EE treated animals. It indicates neonatal exposure to EE may affect the activin production in from the ovary. And the decreased activin production may also related to the retarded follicle development at PND21. However, the further confirmation need to be done.

In conclusion, the present study showed neonatal exposure to EE could altered the kisspeptin expression in the hypothalamus and the circulating concentration of LH, which disrupted the steroidogenesis in the developmental ovary and circulating 17β-estradiol. And this early disruption in the hypothalamus and the impaired follicle development by neonatal exposure to EE may relate to the delayed effect in the adult period.

Table 3-1. Nucleotide sequences of the primers used for real-time PCR

Gene	Forward	Reverse
LHR	5′-GCATTCAATGGGACGACTCT-3′	5′-GTAGGAAGACAGGGCGATGA-3′
FSHR	5′-TTTACTTGCCTGGAAGCGACTAA-3′	5′-CCCAGGCTCCTCCACACA-3′
P450c17	5’-CCATCCCGAAGGACACACAT-3’	5’-CTGGCTGGTCCCATTCATTT-3’
P450arom	5’-GAACGGTCCGCCCTTTCT-3’	5’-TGGATTCCACACAGACTTCTACCA-3’
ERα	5’-CATCGATAAGAACCGGAGGA-3’	5’-AAGGTTGGCAGCTCTCATGT-3’
ERβ	5’-TATCTCCTCCCAGCAGCAGT-3’	5’-CTCCAGCAGCAGGTCATACA-3’
INHα	5′-GCTCTACCAGGGAGCATGAG-3′	5′-CACCTTCCTCCTAGCTGACG-3′
INHβA	5′-TTTCTGTTGGCAAGTTGCTG-3′	5′-CGGGTCTCTTCTTCAAGTGC-3′
INHβB	5′-GCCACGTATCCCTGACTTGT-3′	5′-CTGCTCCATGGTCTCTGTGA-3′
Kisspeptin	5’-CTCAGTGTGCTCCAACTACCC-3’	5’-AGGCCAAAGGAGTTCCAGTT-3’
GAPDH	5′-GGCACAGTCAAGGCTGAGAATG-3′	5′-ATGGTGGTGAAGACGCCAGTA-3′

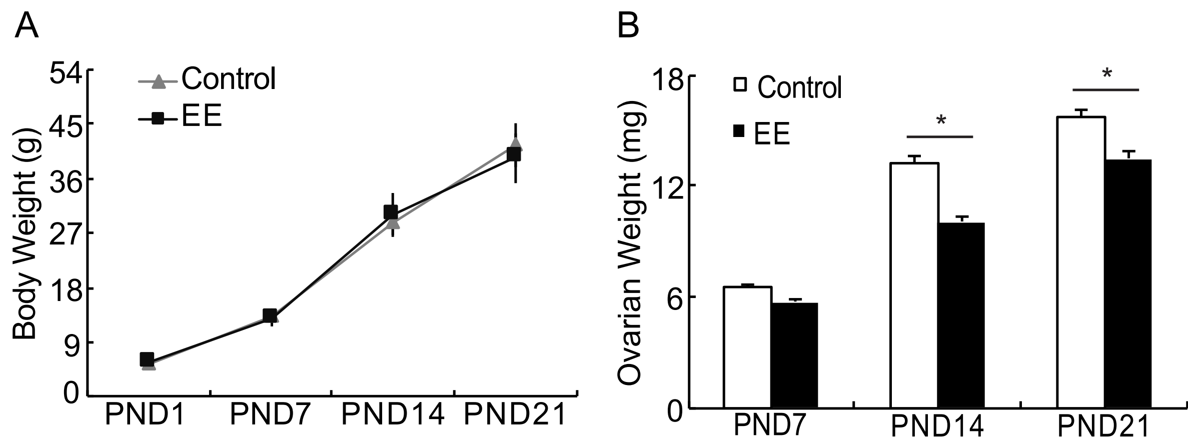


Fig. 3-1. The body weight of control and 200 μg/kg EE treated animals from PND1 to PND21 (A). The ovary weight of control and EE treated animals from PND7 to PND21 (B). Asterisks represent the significant difference ($p<0.05$).

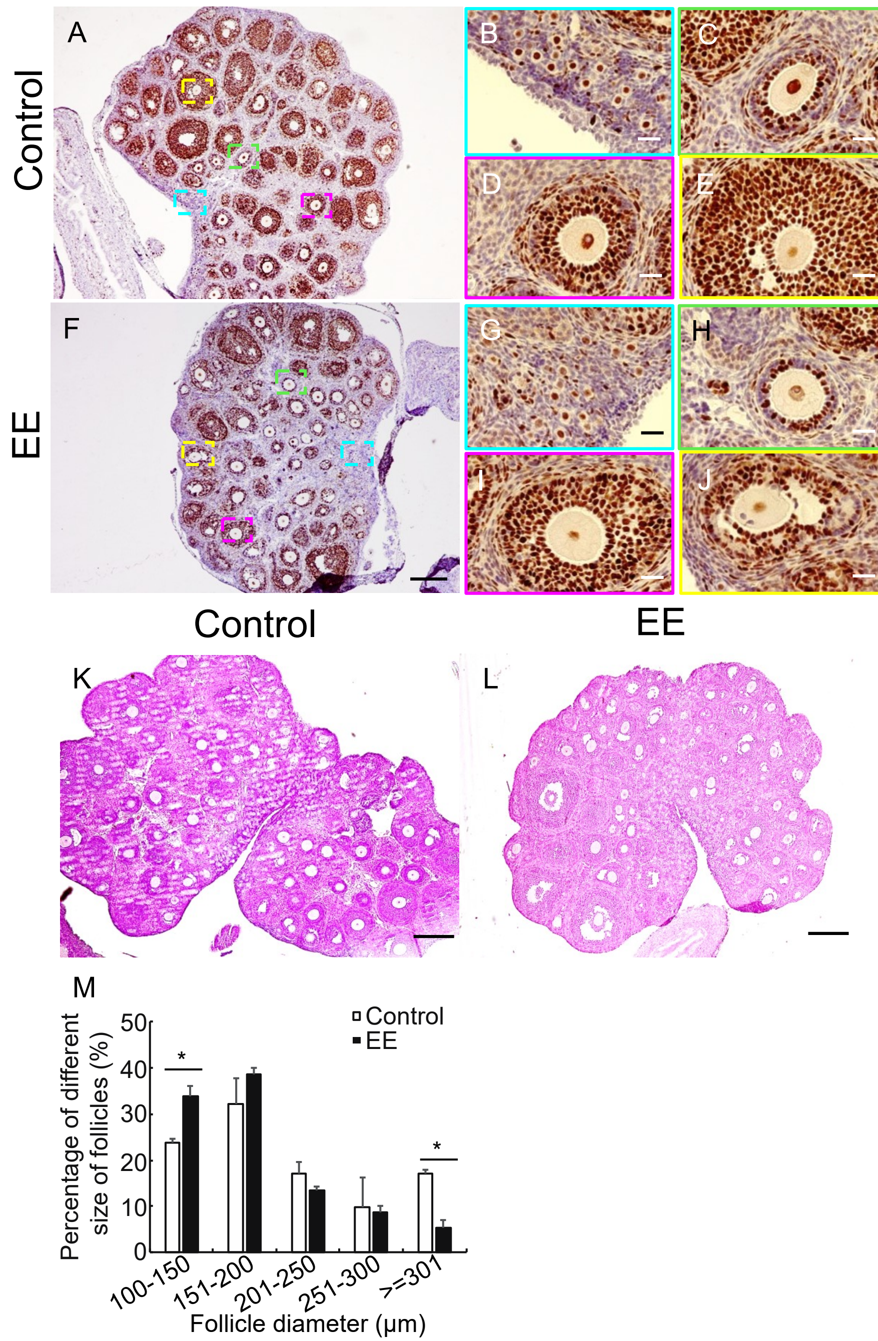


Fig. 3-2. The PCNA staining in the different type of follicles in the ovaries of control (A-E) and 200 μg/kg EE (F-J) treated animals at PND21. The ovary histology of control (K) and EE (L) treated animals at PND21. Bars in the A, F, K, L represent 200 μm; Bars in B-E and G-J represent 20 μm. (M) The percentage of different size of the antral follicles in the ovaries of control and EE treated animals at PND21. Asterisks represent the significant difference (p<0.05).

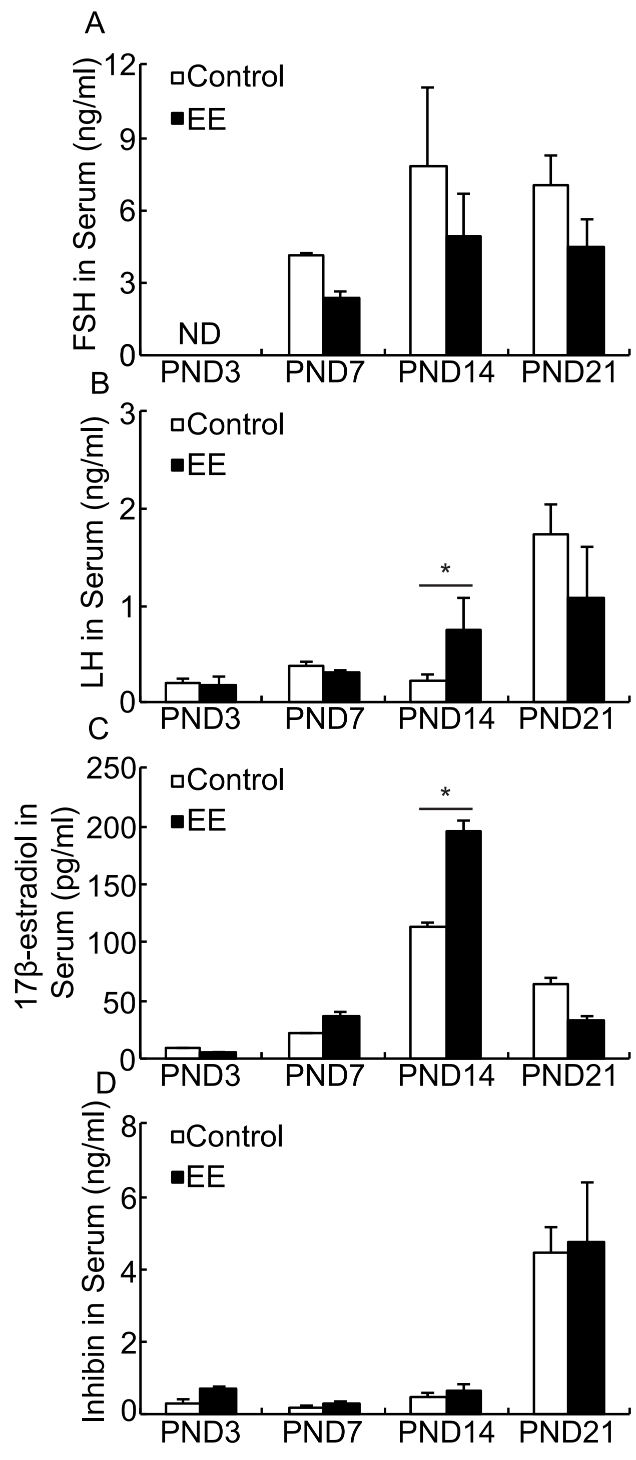


Fig. 3-3 The circulating concentrations of LH (A), FSH (B), 17β-estradiol (C) and inhibin (D) in the control and 200 μg/kg EE treatment animals from PND3 to PND7. Asterisks represent the significant difference (p<0.05).

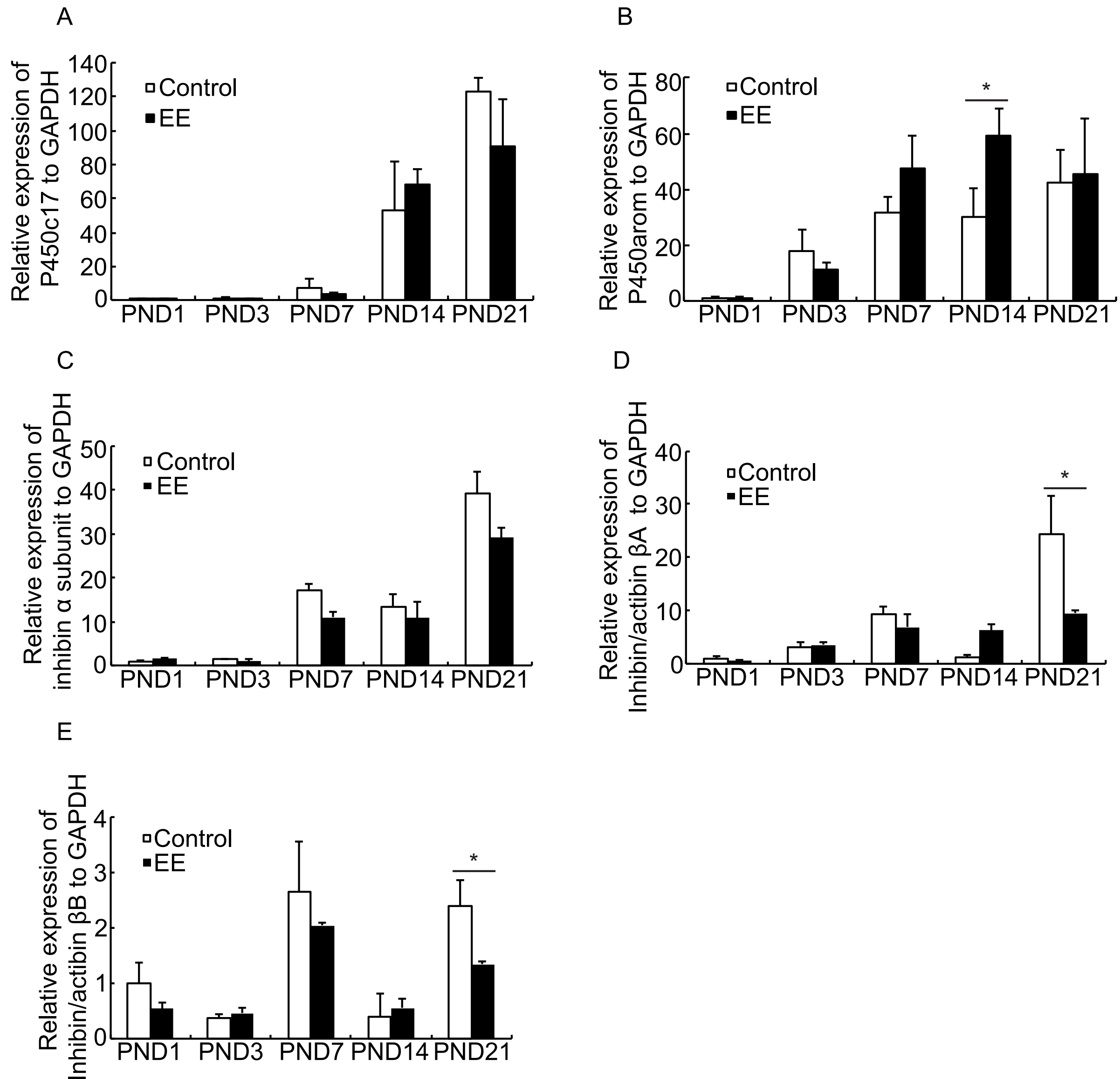


Fig. 3-4. The gene expression of P450c17 (A), P450arom (B), Inhibin subunit α (C) inhibin/activin subunits βA (D) and βB (E) in the developmental ovaries of control and 200 μg/kg EE treated animals. Asterisks represent the significant difference (p<0.05).

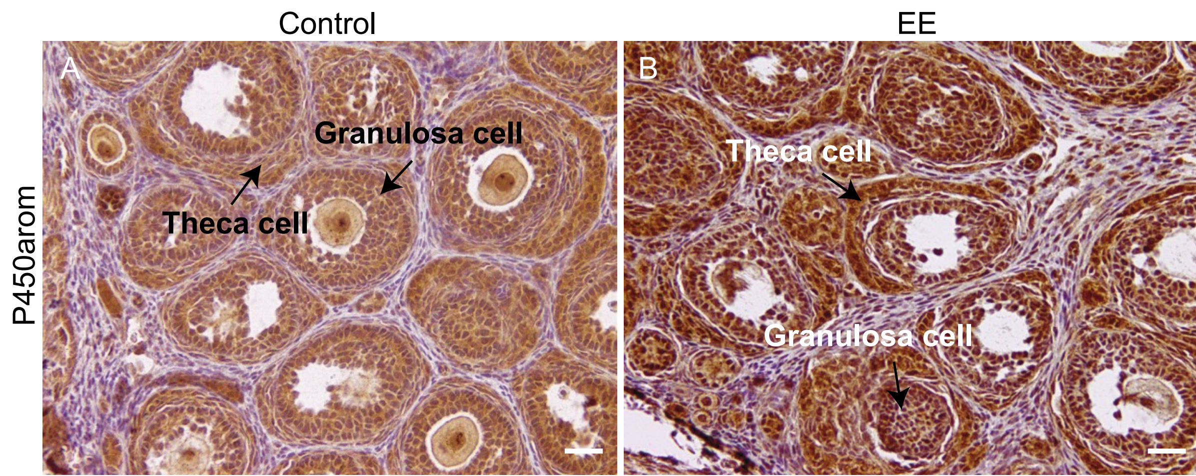


Fig. 3-5. The immunohistochemistry of P450arom in the ovaries of control (A) and EE (B) treated animals at PND21. Bar represents 100 μm.

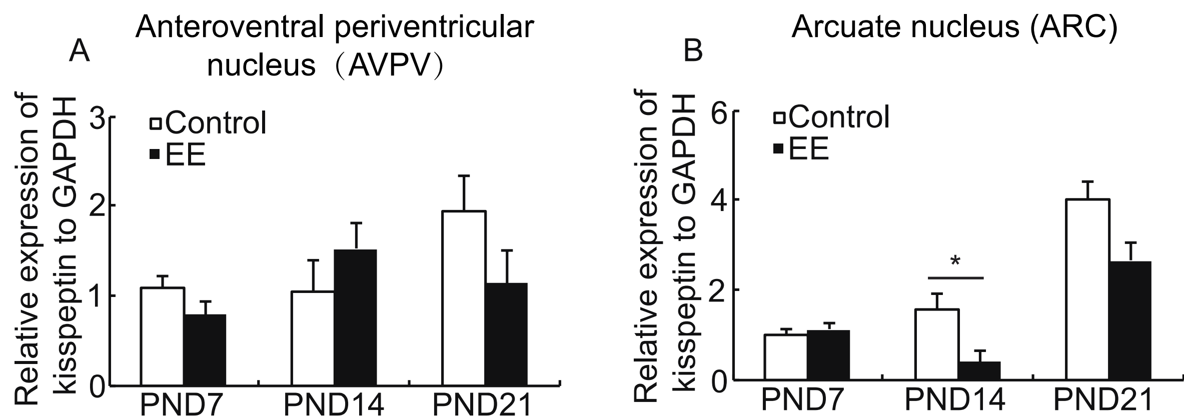


Fig. 3-6. The expressions of kisspeptin in the anteroventral periventricular nucleus (AVPV) (A) and arcuate nucleus (ARC) (B) of hypothalamus in control and EE treated animals from PND1 to PND21. Asterisks represent the significant difference (p<0.05).

Chapter 4 Neonatal exposure to EE disrupts follicle formation by inhibiting the pro-apoptotic factor *Harakiri* (*Hrk*)

4.1 Introduction

A major function of the ovary is to supply oocytes for periodic ovulation in reproductive animals. This process is intricately related to oogenesis and folliculogenesis. Typically, primordial follicle formation occurs during the late fetal and early postnatal period in rodents. The process consists of the breakdown of germ cell cysts, oocyte apoptosis, and the envelopment of surviving oocytes with pre-granulosa cells. As the flattened pre-granulosa cells take on a cuboid shape, the primordial follicles develop into primary follicles. The single layer of granulosa cells proliferates and forms several layers, which serve as an indicator of the development of primary follicles into secondary follicles. As the follicles growth and form an antrum, the mature follicle will ovulate and eventually form the corpus luteum (32; 118). Numerous hormones and growth factors exert positive or negative effects on a tight regulation of these processes, and estrogen is known to be a central regulator.

The high rate of oocyte death has been observed at the perinatal ovaries in many different species (8; 10; 12; 23; 113). During the process of germ cell cyst breakdown, oocyte apoptosis has been shown to be linked to primordial follicle formation in the perinatal ovaries (47; 60; 104). At the perinatal period in rodents, estrogen levels drop as oocytes undergo apoptosis (23; 73). Fetal ovaries undergo a great amount of oocyte loss when they are removed from an environment with high estrogen levels, and oocyte loss can be reduced by the addition of estrogen (20). Oocyte apoptosis in ovaries is substantially reduced by treatment with estradiol monobenzoate at postnatal day 5 (27). There is ample evidence suggesting that estrogenic compounds inhibit germ cell cyst breakdown and primordial follicle formation (19; 20; 51; 61). To a great extent, the number of assembled primordial follicles determines the number of oocytes to be ovulated in the future, which may dictate the reproductive lifespan of the female.

A variety of endocrine-disrupting chemicals (EDCs) that mimic estrogenic compounds can be found in the environment, which have potentially deleterious effects on the development, growth, metabolism, reproduction, and other physiological functions in animals (16; 42; 58; 68; 87; 100; 111). 17α-ethynyl estradiol (EE), a synthetic estrogen, has been widely used as an oral contraceptive for women. It has been found in the wastewater, where it can disrupt reproductive function of wild aquatic animals (11; 22; 70; 103). Moreover, it can be absorbed and accumulated in plants, and can enter our terrestrial food supply via land-based application of water (57). Previous studies demonstrate that neonatal exposure of EE in rats leads to long-term effects in adulthood, including a prematurely disrupted estrous cycle and a shorter reproductive lifespan (81; 83; 97; 112; 116; 126; 134). The mechanisms for these effects are diverse and still unclear, but likely involve changes in expression levels of genes related to apoptosis and/or follicular assembly during early ovarian development.

To gain a better understanding of the mechanism driving the delayed effects of EE on female reproduction, we investigated the direct effect of EE on neonatal ovarian development. First, microarray analysis was performed using control and EE-exposed neonatal ovaries, and *Harakiri (Hrk)*, a proapoptotic factor, was selected as the target gene. Second, to clarify the impact of Hrk on EE-exposed ovaries, follicular composition and apoptosis analysis were conducted. Finally, to evaluate whether the down-regulation of Hrk by EE is due to the estrogenic activity or not, other estrogenic compounds were also examined.

4.2 Materials and methods

4.2.1 Animals

Adult Wistar-Imamichi male and female rats were purchased from Institute for Animal Reproduction (Ibaraki, Japan) and were maintained at 23 ± 2˚C under a 14-hour lighting schedule (lights on 05:00 to 19:00) with free access to food and tap water. The estrus cycles of

female rats were monitored by vaginal cytology and were mated with male rats at proestrus. 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 (23-1). All the procedures were permitted by the ethical committee of animal experimentation of Tokyo University of Agriculture and Technology.

4.2.2 Experiment design

Figure 4-1 summarizes the *in vivo* and *in vitro* experiments conducted.

In vivo experiments: Newborn female pups received a single subcutaneous (sc) injection of sesame oil vehicle (Sigma-Aldrich Co., MO, USA) or EE (200 µg/kg) (Sigma-Aldrich) in the nape of the neck within 24 hours of delivery at postnatal day 0 (PND0). Ovaries in control and EE treatment groups were collected at PND1 (n=8), PND3 (n=5), PND7 (n=5), PND14 (n=5) and PND21 (n=5). One ovary from each rat was snap frozen for RNA extraction and the other was fixed in 4% paraformaldehyde for histological analysis. To explore key genes affected by EE treatment, ovaries at PND1 were used for microarray analysis.

In vitro experiments: Ovaries were collected from intact pups at PND0 and cultured with varying doses of EE (0.1 nM, 1nM, 10 nM, 100 nM), or different estrogenic chemicals [17β-estradiol (E_2) (Sigma-Aldrich), EE or diethylstilbestrol (DES) (Sigma-Aldrich), 10 nM each] in Dulbecco's Modified Eagle Medium (DMEM) high glucose without phenol red (Invitrogen Co., CA, USA) containing insulin-transferrin-selenium (ITS) supplement (Invitrogen) and antibiotic-antimycotic (Invitrogen). The control group was cultured with the same amount of dimethyl sulfoxide (DMSO) (Wako, Tokyo, Japan) and all chemicals were dissolved in DMSO. The final concentration of DMSO is 0.05%. After 24 hours, cultured ovaries were snap frozen for real-time PCR analysis. A sample size of five was selected for each treatment group.

4.2.3 Microarray analysis

Total RNA was isolated from ovaries of control and EE treatment at PND1 (*n*=5 in each group), and pooled RNA was used for microarray analysis. An aliquot of 1 μg of total RNA was used to synthesize double-stranded cDNA, and biotin-labeled cRNA was amplified from cDNA. Finally, cRNA was hybridized to a GeneChip Rat Genome 230 2.0 Array (Affymetrix, CA, USA) for 16 hours at 45°C. After hybridization, chips were washed and dried, and then scanned by the GeneChip Scanner 3000 (Affymetrix).

Gene ontology (GO) term enrichment analysis was performed using the rat genome database website tools http://amigo.geneontology.org/. The cutoff p value is 0.001.

4.2.4 Real-time PCR analysis

Total RNA was extracted from ovaries using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized using PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan). The oligonucleotide primers for qRT-PCR analysis were designed using the Primer 3 program (table 4-1). The PCR reactions were carried in a 10 μl volume using Ex TaqR Hot Start Version containing SYBR-Green I (Takara Bio) and performed with chrome4 real-time PCR System (Bio-Rad, CA, USA) using the following conditions: 95°C for 30 sec; followed by 40 cycles of 95°C for 5 sec, 60°C for 30 sec, and dissociation protocol. The relative expression level of each target mRNA was calculated by multiple internal controls including beta-Actin and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA (137).

4.2.4 Histology

Ovaries were fixed in 4% paraformaldehyde, embedded in paraffin and serially sectioned at 6 μm. To estimate the follicular composition, ovaries from PND7 and PND21 were used and every three sections were stained with hematoxylin and eosin (*n*=3 ovaries per group). Five PND1 ovaries from each group were used for immunostaining, and three were used for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining.

4.2.5 Immunohistochemistry

Sections were deparaffinized in xylene and rehydrated through a graded series of ethanol. To increase epitope exposure, the sections were heated in 0.01 M sodium citrate buffer, pH 6.0, at 121°C for 10 min in an autoclave. After incubating in 0.3% H_2O_2 in methanol for 30 min, the sections were blocked with 10% normal goat serum for 1 hour to reduce background staining caused by the second antibody. Then the sections were incubated with anti-Hrk primary antibody (Thermo Fisher Scientific, IL, USA, Prod #PA1-86773) overnight at 4°C. The sections were incubated with a secondary antibody, anti-rabbit lgG conjugated with biotin and peroxidase with avidin, using a rabbit VECTASTAIN ABC kit (Vector lab, CA, USA), and subsequently visualized with diaminobenzidine (Nichirei Biosciences Inc., Tokyo, Japan) as a chromogen substrate. Finally, the sections were counterstained with hematoxylin solution. The control sections were treated with normal rabbit IgG (Santa Cruz Biotechnology Inc., CA, USA) instead of the primary antibodies. The immunohistochemical result was quantified via counting the oocytes with Hrk-positive staining.

4.2.6 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

TUNEL staining was performed according to the manufacturer's manual using ApopTag peroxidase in situ apoptosis detection kit (Millipore, Darmstadt, Germany). The number of positively stained oocytes was counted in every three sections.

4.2.7 Immunofluorescence

Double staining for Hrk and cleaved caspase 3 was performed using Opal 4-color fluorescent IHC kit (PerkinElmer, MA, USA) according to the modified manual. Deparaffinized and rehydrated sections were blocked with 10% normal goat serum for 1 hour and then incubate with Hrk antibody (1:200, Thermo Fisher Scientific, Prod #PA1-86773) overnight at 4°C. Horseradish peroxidase conjugated secondary antibody was applied for 1 hour and the coloring step was performed according to the manual by opal-520 amplification reagent. The slides were

treated in the AR buffer in a microwave according to the manual, and the same procedure was carried out for the primary antibody cleaved caspase 3 (1:200, Cell Signaling Technology, MA, USA, #9661) or primary antibody DEAD-Box Helicase 4 (DDX4) (1:100, Abcam, MA, USA, ab13840). Opal-670 amplification reagent was used for cleaved caspase 3 or DDX4 coloring. 4',6-diamidino-2-phenylindole (DAPI) was used for counterstaining and the normal rabbit IgG was used instead of primary antibody as a negative control. Images were captured using an immunofluorescence microscope, BX-51 (Keyence, Osaka, Japan).

4.2.8 Plasmid construction and virus infection

A lentiviral shRNA targeting rat Hrk was cloned into pLentiLox 3.7 vector; shHrk: 5'-GATTCAAGAGCCAAGAAAT-3'. The lentiviral constructs were co-transfected with the packaging plasmids into 293T cells using lipofectamine 2000 (Invitrogen). Virus-containing supernatants were collected 48 hours after transfection. The virus-containing supernatants were mixed gently with PEG solution (13 mM PEG 6000, 100 mM NaCl, 10 mM HEPES, PH 7.4) and rotated at 4°C overnight. The supernatant was removed after 1500Xg centrifuge at 4°C for 30 min, and 1 ml opti-MEM medium was added into the virus. The concentrated virus was kept at -80°C until use.

Neonatal ovaries were collected from intact pups at PND0 and infected with virus-containing supernatant in opti-MEM (Invitrogen). After 24 hours, ovaries were subjected to real-time PCR and TUNEL staining (n=5 each group for each experiment).

4.2.9 Statistical analysis

Statistical comparisons were made with the Student's t-test, One-way ANOVA followed by conservative Tukey's test, or Two-way ANOVA followed by Sidak's multiple comparisons test using Prism 5 (Graphpad Software Inc., CA, USA). A value of $p<0.05$ was used as an indication of statistical significance.

4.3 Results

4.3.1 Profile of gene expression in neonatal EE-exposed ovary

Of the 30,000 genes examined between control and EE-exposed ovaries, changes in mRNA levels were detected in 545 genes: 235 of those were increased (over 2 fold), and 310 were decreased (less than 0.5 fold) in EE-exposed ovaries. The five genes with the most increased or decreased expression identified by the microarray examination were validated with real-time PCR analysis (Table 4-2). The gene ontology (GO) term enrichment analysis revealed that the differentially expressed genes were highly associated with apoptosis (Table 4-3). Of note, one particular proapoptotic gene, *Hrk*, was significantly decreased in expression in the EE-exposed ovaries (Table 4-2).

4.3.2 Down-regulation of *Hrk* expression in neonatal EE-exposed ovary

To confirm the involvement of Hrk in early ovarian development, *Hrk* mRNA expression was measured by real-time PCR from ovaries collected from control and EE-exposed pups at PND1, PND3, PND7, PND14 and PND21 (Fig. 4-2A). In control animals, high expression of *Hrk* mRNA was observed at PND1 and decreased from PND3 to PND21. However, *in vivo* exposure to EE at PND0 suppressed the *Hrk* mRNA expression in ovaries at PND1. Next, ovaries were collected from pups at PND0 and cultured with EE for 24 h *in vitro*, and we found that the expression of *Hrk* mRNA was significantly decreased (Fig. 4-2B). Immunohistochemical analysis revealed that the Hrk protein was expressed in oocytes at PND1, and it had a weaker staining in the EE-exposed ovaries compared to control (Fig. 4-2C and D). The Hrk-positive stained oocytes were decreased in the EE-exposed ovaries compared to control (Fig. 4-2E)

4.3.3 Co-localization of Hrk and cleaved caspase 3 in neonatal ovary

Since Hrk is a known proapoptotic factor, we stained for both Hrk and cleaved caspase 3 in control and EE-exposed ovaries (Fig.4-3A). In control ovaries, Hrk and cleaved caspase 3 were highly colocalized in the oocytes, however in EE-exposed ovaries, Hrk had a weaker

expression and a reduced colocalization with cleaved caspase 3. Moreover, the co-localization of Hrk and oocyte marker DDX4 was confirmed in the ovaries of PND1 (Fig. 4-3B), suggesting Hrk was localized in the oocytes of neonatal ovaries.

4.3.4 Decrease of apoptotic oocytes and number of primordial follicles in neonatal EE-exposed ovaries

We performed a TUNEL staining assay to quantify the number of apoptotic oocytes in control and EE-exposed ovaries (Fig. 4-4A and 4-4B). There was a decreased number of oocytes with positive TUNEL staining in EE-exposed ovaries at PND1 (Fig. 4-4C).

The histological results of ovary staining at PND7 are shown in Figure 4-4E. The primary follicle is represented by the oocyte surrounded by cubical granulosa cells, the primordial follicle is represented by the oocyte surrounded by squamous granulosa cells, and the oocyte that is not surrounded by any granulosa cells represents the oocytes in the germ cell cyst. The total number of different types of follicles was counted in the control and EE-exposed ovaries at PND7. There was a decrease in the number of primordial follicles in the EE-exposed ovaries compared with control ovaries (Fig. 4-4D).

Histological analysis in control and EE-exposed ovaries at PND21 was also investigated. We observed the presence of multiple oocyte follicles in exposure to several EDCs (46; 52; 53). Indeed, the multiple oocyte follicles were found and increased in the ovaries of EE-exposed animals (Fig. 4-4F and G).

4.3.5 Effect of *Hrk* knockdown in neonatal ovary

Neonatal ovaries at PND0 were infected with lentiviruses expressing GFP only (control) or GFP plus short hairpin RNA (shRNA) targeting Hrk (shHrk). Similar GFP expression was observed in both ovaries, and the expression of *Hrk* gene decreased more than 40% in shHrk-treated ovaries (Fig. 4-5A-C). There was a decreased number of apoptotic oocytes in *Hrk*

knockdown ovaries compared to control ovaries (Fig. 4-5D-F). We confirmed that the GFP signal was bleached before the process of TUNEL staining (data not shown).

4.3.6 Estrogen responsive element (ERE) localized in the promoter of *Hrk* gene and the expressions of estrogen receptors in the developmental ovaries

The putative estrogen responsive element was found in the promoter of *Hrk* gene by using the online tool dragon ERE finder (http://datam.i2r.a-star.edu.sg/ereV3/) (Fig. 4-6A). The expression of estrogen receptor α (ERα) decreased in the ovaries of EE treated animals, however, there was no difference in the expression of estrogen receptor β (ERβ) between control and EE treated animals (Fig. 4-6B and C, respectively).

4.3.7 Downregulation of *Hrk* expression by estrogenic compounds

To evaluate the effect of estrogenic compounds on the expression of *Hrk* in the ovary, the ovaries were cultured with E_2 and DES, and the expression of *Hrk* was analyzed by real-time PCR. The expressions of *Hrk* in ovaries treated with E_2 or DES were decreased to a similar extent as ovaries treated with EE (Fig. 4-7).

4.4 Discussion

We are frequently exposed to several types of EDCs. The increasing health risk associated with exposure to EDCs has become an increasing cause for concern. EE, which is commonly prescribed for estrogen replacement therapy, has been found to influence the sexual development and behavior in aquatic wildlife through environmental contamination (11). Studies performed in rats also show that EE can induce an abnormal estrous cycle and other reproductive dysfunction (81; 83; 97; 134). Moreover, once leaked into the environment to contaminate aquatic and/or terrestrial food supplies, EE could pose a health risk for humans.

Although some researches concerning the effect of estrogenic compounds on ovarian development have been reported (19; 20; 51; 61), the mechanism remains unclear until now. The link between cell apoptosis and EE was reported in the different tissues (93; 125).

Interestingly, the present microarray results confirmed that EE regulated many genes related to cell apoptosis, suggesting that EE may regulate cell apoptosis in ovaries. The *Bax* deficient animal model and the relationship between Bcl2 family members in the mitochondria membrane and oocytes apoptosis suggest Bcl2 family may be involved in oocyte apoptosis during follicle assemble (47; 105). In this study, we present another Bcl2 family member, *Hrk*, in the key gene for oocyte apoptosis. The colocalization of Hrk and the well-known apoptotic factor cleaved caspase 3 in the oocytes, indicating Hrk may interact with the activated caspase 3. Further, the number of apoptotic oocytes was also decreased in the *Hrk* knockdown ovaries, which suggest Hrk is responsible for oocyte apoptosis. Moreover, the expression of *Hrk* in the ovary was decreased by EE exposure, along with the decreased apoptotic oocytes. Taken together, the results suggest that *Hrk* is a key target gene in the EE-exposed ovaries, and decreased expression of *Hrk* by EE exposure leads to the reduction of oocyte apoptosis in the early ovarian development.

In the present study, we found the putative estrogen responsive element (ERE) located in the promoter region of *Hrk* gene (Fig. 4-6A), which indicates that EE or estrogens could directly regulate *Hrk* gene expression via ERE-dependent pathway. In theory, estrogen receptors may bind with ERE, which recruits co-repressors and suppress *Hrk* gene expression. Alternatively, EE may affect the *Hrk* expression through differentially regulating the expression of estrogen receptors (ERs). In the present study, the expression of estrogen receptor α (ERα) is suppressed significantly at PND1 in the EE-exposed ovaries; however, estrogen receptor β (ERβ) is not affect by EE exposure (Fig. 4-6B and C, respectively). Those data indicate that EE may regulate the balance of ERα and ERβ expression. And the regulation of estrogen receptor by EE is still unclear in the present study, although other report showed the estrogenic chemicals might affect estrogen receptor expression via cooperating with other cellular or paracrine factors (86). Previous reports showed ERβ acted as ERα antagonist and oppose the ERα-mediating gene

expression (44; 85). The type of ER dimer binding with ERE in *Hrk* gene may be altered due to the decreased expression of ERα, which results in the decreased expression of *Hrk*. Therefore, the disrupted ERs-signaling pathway could be another possible reasons for dysregulation of *Hrk* expression in the EE-exposed ovaries. The further confirmation for the mechanism how EE disrupts *Hrk* expression in the neonatal ovary needs to be done.

In the fetal or neonatal ovary, oocyte apoptosis occurs during the germ cell cyst breakdown and primordial follicle assembly, which is an important process for follicle formation. Previous studies show that neonatal exposure to estradiol or phytoestrogen (genistein) leads to the inhibition of oocyte nest breakdown and primordial follicle formation in rodent ovaries (19; 20; 61). Consistent with this, the present study showed that EE exposure to the neonatal ovaries decreased oocyte apoptosis in PND1 ovaries and primordial follicle number in PND7 ovaries. This calls into question the non-apoptotic oocytes in the EE-exposed ovaries. According to previous reports, abnormal follicle formation and multioocyte follicles were found in the ovaries exposed to other estrogenic chemicals (20; 52; 53). Based on this, we proposed that the non-apoptotic oocytes might form abnormal follicles in the EE-exposed ovaries. To test this, the histology of EE-exposed ovaries at PND21 was examined. As expected, there was an increased number of multioocyte follicles in the EE-exposed ovaries at PND21. The multi-oocyte follicles with antrum were present in the PND21 (Fig. 4F), indicating those kinds of follicles have the capacity to grow and develop. Based on the ovarian histology at PND90, there is no multi-oocyte follicle observed in the EE-exposed ovaries (97). Therefore, the fate of those multi-oocyte follicles is still unclear. A question whether they undergo ovulation or apoptosis during development remains open. In our previous study, the irregular estrous cycle and earlier ceased estrous cycles were observed in the EE-exposed animals (97). Though there is no direct evidence in the present study, the multi-oocyte follicles are suspected to be one reason for this phenomenon. Moreover, the in vitro experiments showed that the fertilization

ratio decreased in the oocyte from multi-oocyte follicles, indicating that the quality of oocytes from multi-oocyte follicles is poor (48). Furthermore, the presence of multi-oocyte follicles may induce the reproductive repercussions by shortening the reproductive lifespan of the female (45).

It has been established that the so-called “delayed effect” of fetal or neonatal exposure to estrogenic compounds causes reproductive defects that appear in adulthood (42). In the previous study, neonatal exposure to EE in rats resulted in an abnormal estrous cycle in early adulthood and a decreased number of primordial follicles at PND90 (97). In the present study, we found that neonatal exposure to EE disrupted oocyte apoptosis at PND1 and decreased primordial follicle formation at PND7. Also, the non-apoptotic oocytes formed the multioocyte follicles in PND21 ovaries. Combining these results, I propose that the decreased oocyte apoptosis and disrupted follicle assembly in ovaries by neonatal exposure to EE may cause abnormal ovarian function in adulthood.

In the present study, neonatal exposure to EE inhibited oocyte apoptosis in ovaries. However, this phenomenon was not only observed in EE-treated animals. Neonatal exposure to another synthetic estrogen, diethylstilbestrol (DES), can also reduce oocyte apoptosis in C57BL/6J mice (62). Also, PND5 mouse ovaries had substantially reduced apoptotic oocytes after neonatal treatment with estradiol monobenzoate (EB) (27). These facts suggest that such estrogenic chemicals may use a similar mechanism to inhibit oocyte apoptosis at an early stage of ovarian development. The *in vitro* data shows that E_2 and DES can also suppress the expression of Hrk in the cultured ovaries. Moreover, although there is limited case numbers on human studies, maternal smoking reduced human fetal ovarian cell numbers and Hrk as one of the target genes were dysregulated (37). Maternal exposure to 1 mg/kg polycyclic aromatic hydrocarbons (PAHs) for three weeks (the accumulation of PAHs is equivalent to 25 packages of cigarettes, consistent with potential exposure in a heavy smoker) reduced the ovarian follicle

pool via *Hrk*-mediating pathway in mice (55). Taken together, the results suggest *Hrk* could be a key target of estrogenic chemicals in the ovary, and Hrk could serve as a screening marker for the effect of the estrogenic chemicals on neonatal ovaries.

In conclusion, I have shown that neonatal exposure to EE directly inhibits oocyte apoptosis in the neonatal ovary by suppressing the expression of the Hrk protein, which is localized in oocytes of neonatal rat ovaries. The decreased amount of oocyte apoptosis and primordial follicles may cause an abnormal estrous cycle and other ovarian defects during adulthood.

Table 4-1. Oligonucleotide primers used for quantitative real time PCR.

Gene	Forward Primer	Reverse Primer
Hrk	5’-TGTGAAGCTCTGGCACAATC-3’	5’-GGATGGATGCACACACACT-3’
Calb3	5’-CTCTGGCAGCACTCACTGA-3’	5’-GCTGGGGAACTCTGACTGA-3’
Mmp7	5’-ATAATGCAGAAGCCCAGGT-3’	5’-TCTGCAGTCCCCCAACTAA-3’
Tnfip6	5’-AAGCAGCCAGAAAGATTGG-3’	5’-TTCGGGTTGTAGCAATAGG-3’
Itmap1	5’-TGCTTCCTACGCTTCAACC-3’	5’-TACACTCGGTCTGCAAGTC-3’
Dhdh	5’-GCAGCAGACAGTGCGACTA-3’	5’-TCTTGCTTCGGATGGCTAGT-3’
Sepp1	5’-GGCCGTCTTGTGTATCACC-3’	5’-GTTTGTCATGGTGCTTGTG-3’
Ca3	5’-CCCTCTCTCTGGACCCTAC-3’	5’-TCAGAAAAATGCCAACCAC-3’
Fxyd7	5’-GGATGGATGCACACACACT-3’	5’-GTCTCTGGGTCTGGGGTACA-3’
Vdr	5’-TGAAGGCTGCAAAGGTTTC-3’	5’-TAGCTTGGGCCTCAGACTG-3’
ERα	5’-CATCGATAAGAACCGGAGGA-3’	5’-AAGGTTGGCAGCTCTCATGT-3’
ERβ	5’-TATCTCCTCCCAGCAGCAGT-3’	5’-CTCCAGCAGCAGGTCATACA-3’
β-Actin	5’-AGCCATGTACGTAGCCATCC-3’	5’-CTCTCAGCTGTGGTGGTGAA-3’
GAPDH	5’-GGCACAGTCAAGGCTGAGAATG-3’	5’-ATGGTGGTGAAGACGCCAGTA-3’

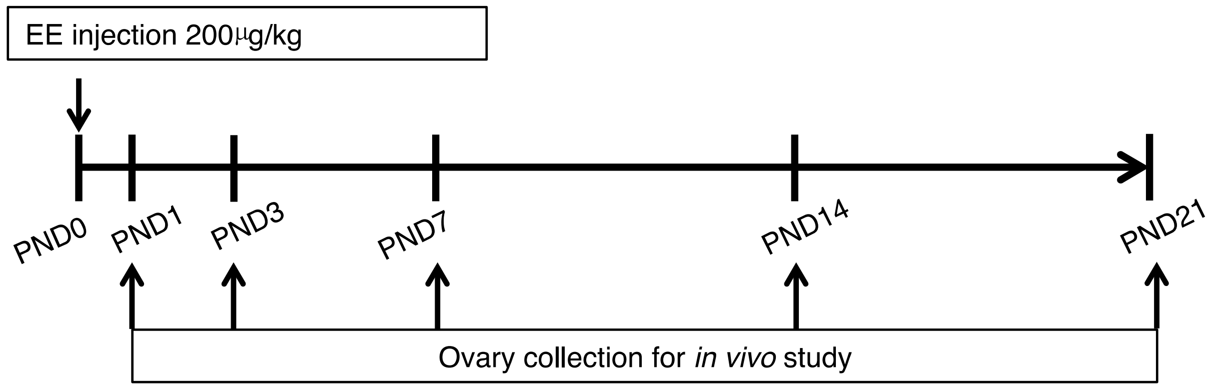
Table 4-2. The top 5 up- and down-regulated genes in the EE treated ovaries in the microarray data validated by real-time PCR.

	Gene Symbol	Full Name	Probe ID	Microarray Data	Real-time PCR data	Gene Expression
Up-regulated genes (EE/Con)	Calb3	Calcium-binding protein, intestinal, vitamin D-dependent (9-kDa CaBP)	1368339_at	27.85506287	7.474619	□
	Mmp7	Matrix metalloproteinase 7 (matrilysin)	1368766_at	10.22323462	9.872922	□
	Tnfip6	TNF-stimulated gene 6 protein	1371194_at	8.531470588	4.304961	□
	Itmap1	Integral membrane-associated protein 1	1368367_at	8.40673944	7.474619	□
	Dhdh	Dihydrodiol dehydrogenase	1370708_a_at	7.181818182	0.035182	
Down-regulated genes (EE/Con)	Sepp1	Selenoprotein P, plasma, 1	1387339_at	0.04494382	1.350794	
	Ca3	Carbonic anhydrase 3	1386977_at	0.057731959	0.3280528	□
	Hrk	BH3 interacting (with BCL2 family) domain, apoptosis agonist, Harakiri7	1368535_at	0.07310705	0.2683151	□
	Fxyd7	FXYD domain-containing ion transport regulator 7	1368696_at	0.083511777	0.1396609	□
	Vdr	Vitamin D (1,25-dihydroxyvitamin D3) receptor	1369454_at	0.090322581	1.454779	

Relative expression is calculated as the ratio of expression levels in EE treatment group/control group. Up-pointing arrows: up-regulated gene validated by both microarray and real-time PCR, down- pointing arrows: down-regulated gene validated by both microarray and real-time PCR.

Table 4-3. The gene ontology (GO) term enrichment analysis for the differentially expressed genes in the ovaries of control and EE treatment rats (cutoff, *p*=0.001).

GO Function	Ratio
Response to nitrogen compound	20.90%
Regulation of apoptotic process	**20.20%**
Cell-cell signaling	17.80%
Regulation of ion transport	15.50%
Response to drug	14.70%
Nitrogen compound transport	14.70%
Cellular response to hormone stimulus	14.70%
Behavior	14.70%
Response to nutrient levels	13.20%
Response to extracellular stimulus	13.20%
Regulation of hormone levels	13.20%
Gland development	13.20%
Mammary gland development	8.50%
Secretion by tissue	7.80%
Regulation of blood pressure	7.80%
Hormone mediated signaling pathway	7.80%
Body fluid secretion	7.80%
Lactation	5.40%



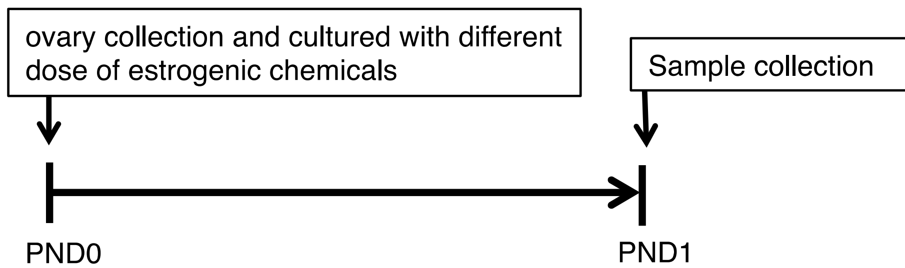


Fig. 4-1. Schematic representation of experimental design for *in vivo* and *in vitro* experiments.

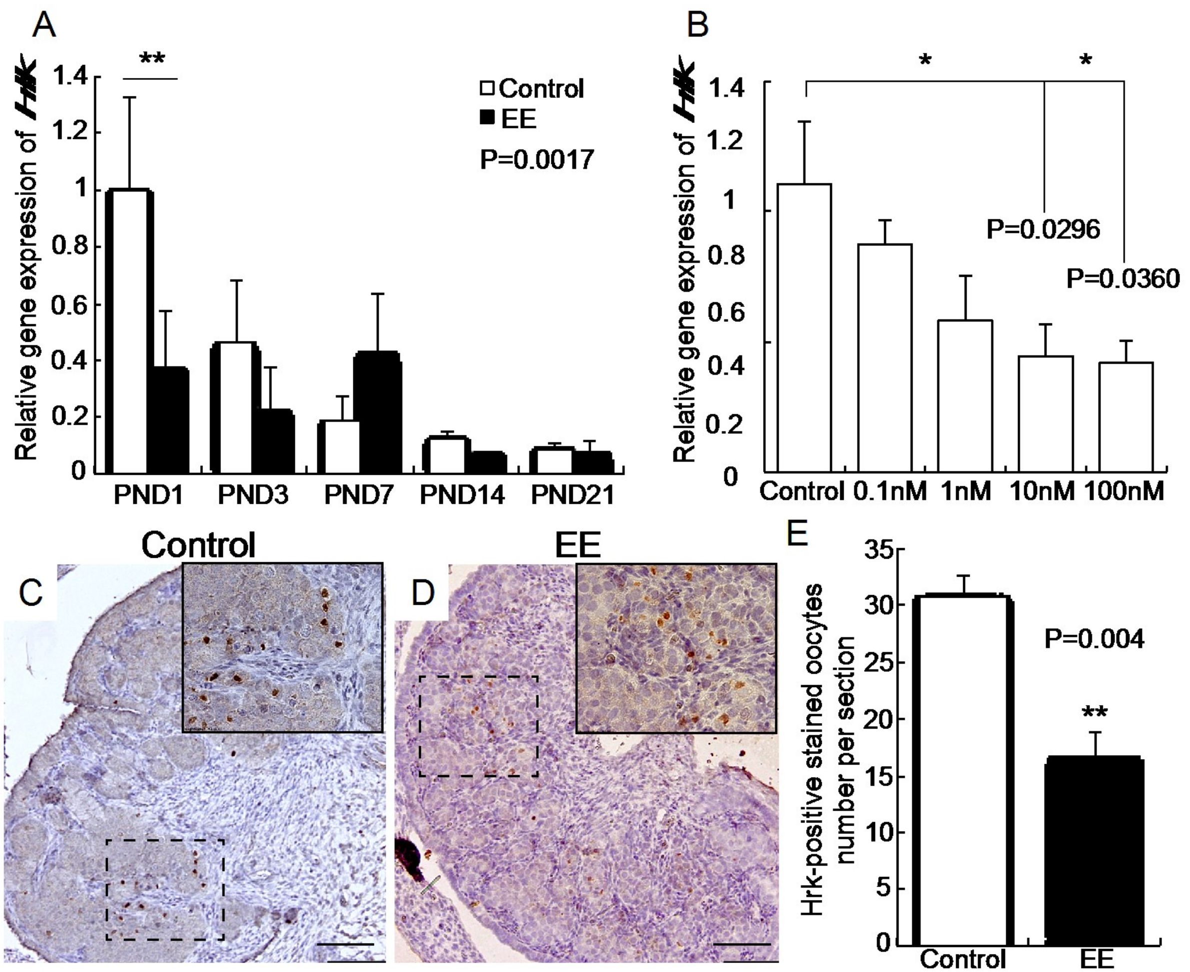


Fig. 4-2. The expressions of *Hrk* in developmental ovaries of control and EE exposed ovaries (A) and ovaries cultured with/without EE (B). Asterisks represent the significant difference ($p<0.05$). The immunolocalization of Hrk in the ovaries of control (C) and EE (D) exposed animals at PND1. Bar, 100μm. (E) The average number of Hrk-positive stained oocytes in the control and EE treated ovaries.

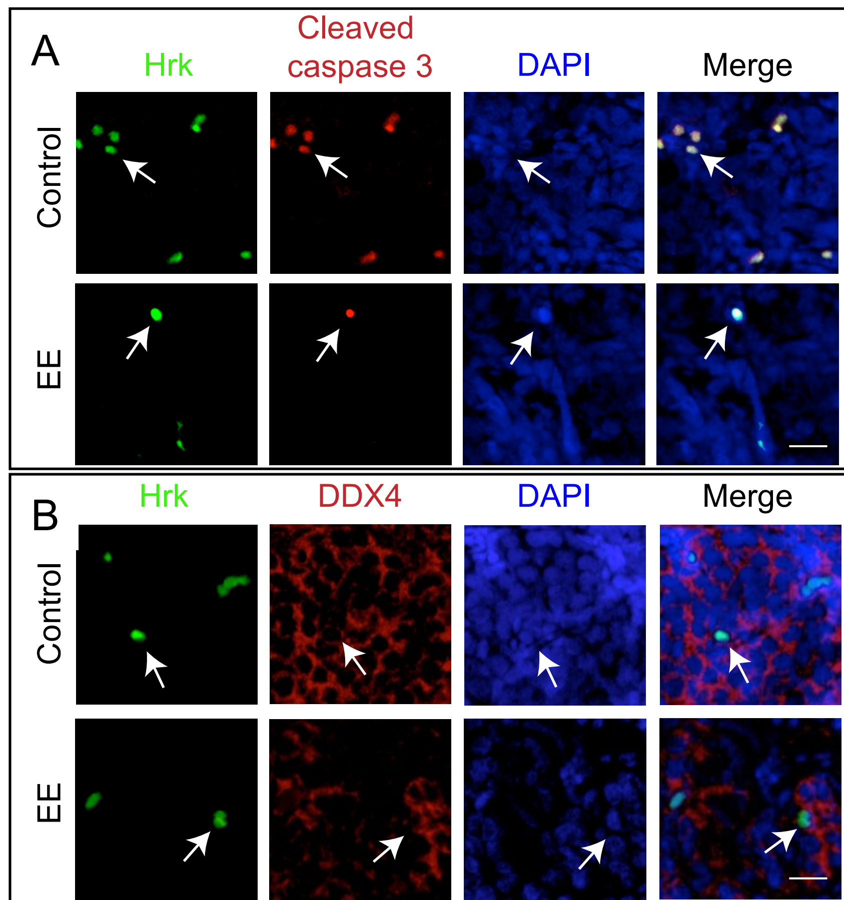


Fig. 4-3. Immunofluorescent images of Hrk (green) and cleaved caspase 3 (red) in control and EE-exposed ovaries of PND1 (panel A). The arrows indicate the colocalization of Hrk and cleaved caspase 3 in the oocytes. The cell nuclei were stained with DAPI (blue). mmunofluorescent images of Hrk (green) and DDX4 (red) in control and EE-exposed ovaries of PND1 (panel B). The arrows indicate the colocalizaiton of Hrk and DDX3 in the oocytes. Bar, 50 μm.

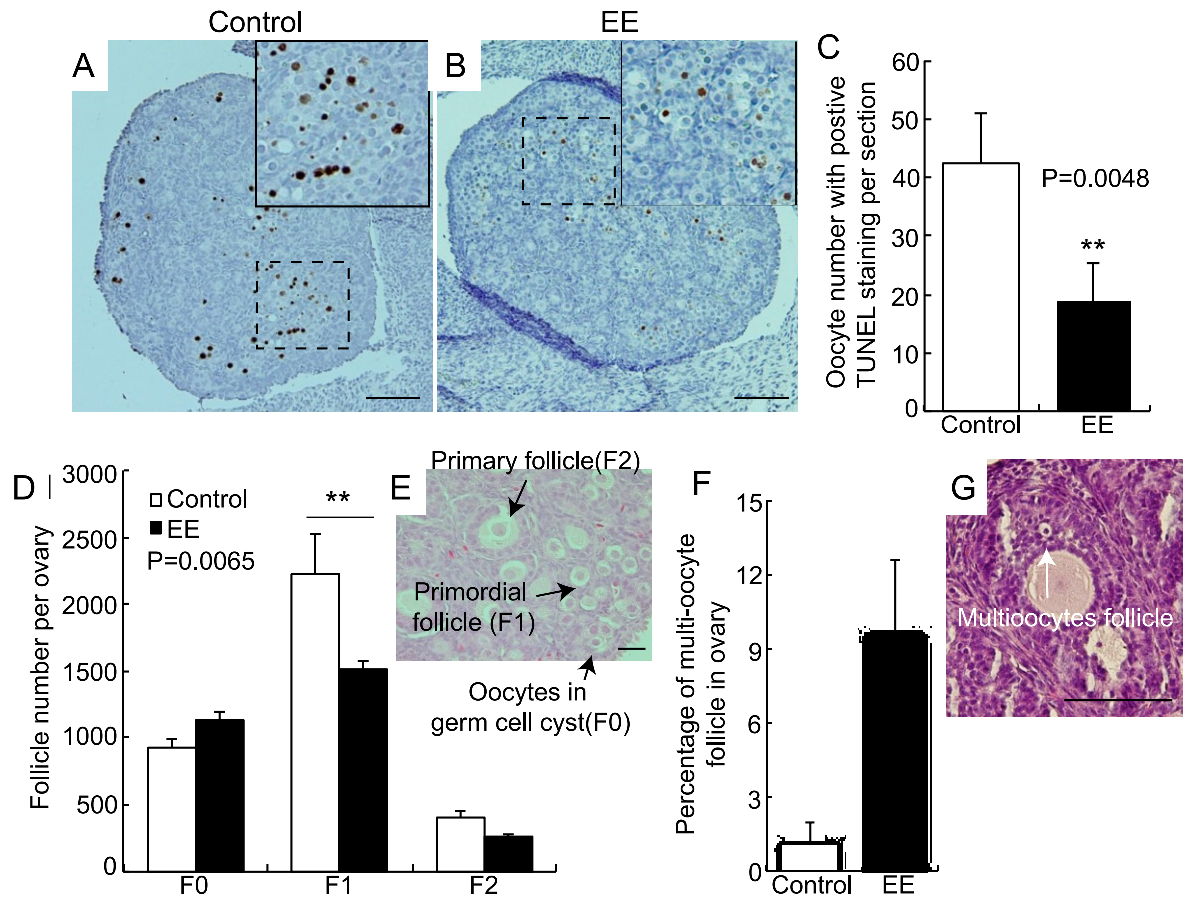


Fig. 4-4. The TUNEL staining in control (A) and EE exposed (B) ovaries of PND1. The average positive staining number of oocytes per paraffin section for TUNEL staining in control and EE exposed ovaries at PND1 (C). The total number of different types of follicles in rat ovaries at PND7 (D). The different types of follicles are shown in histological photograph (E). The percentage of multioocytes follicles increased in the EE treated ovaries at PND21 (F). The histology of multioocytes follicles presented in the EE treated ovaries at PND21 (G). Asterisks represent the significant difference (p<0.05). Bar, 100 µm.

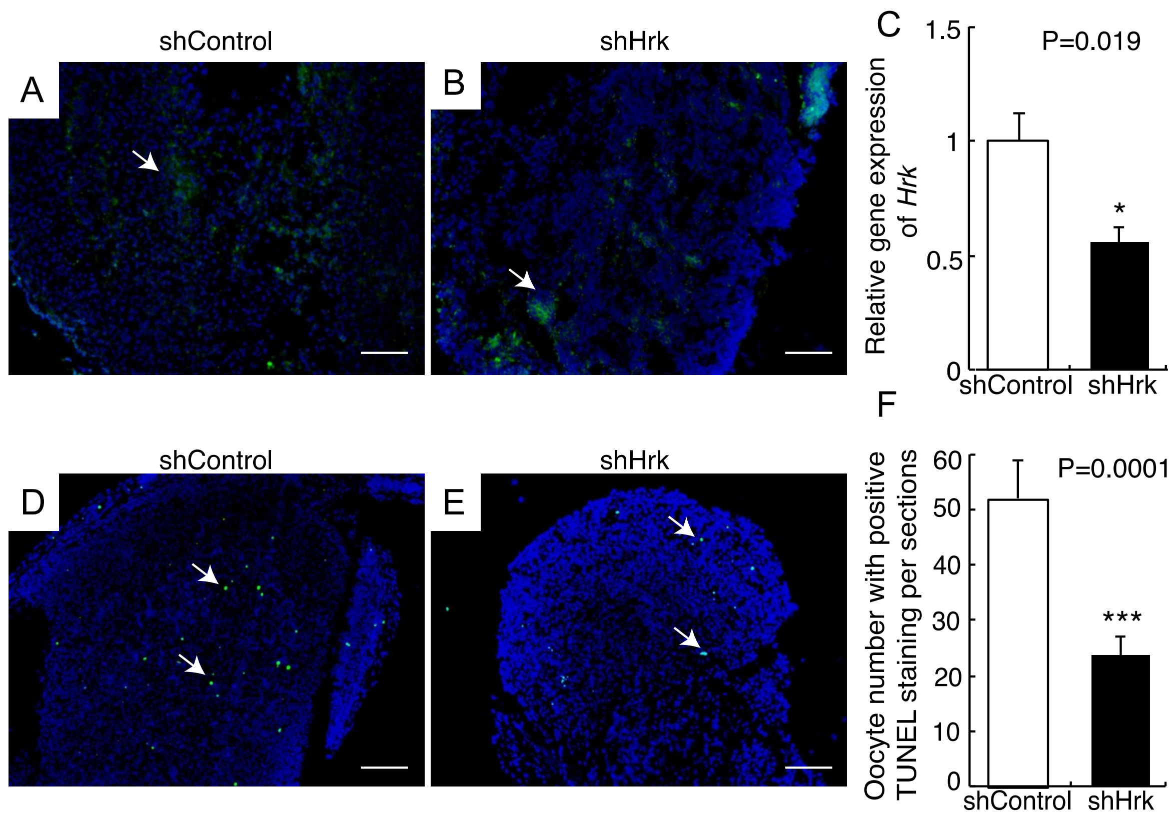


Fig. 4-5. The confirmation for the EGFP containing virus infection in the control (A) and *Hrk* knockdown (B) ovaries. The relative expression of *Hrk* in the control and *Hrk* knockdown ovaries (C). The microscopic photo of TUNEL staining in the control (D) and *Hrk* knockdown (E) ovaries. The average positive staining number of oocytes per section for TUNEL staining in control and *Hrk* knockdown ovaries (F). Asterisks represent the significant difference (p<0.05). Bar, 100 μm.

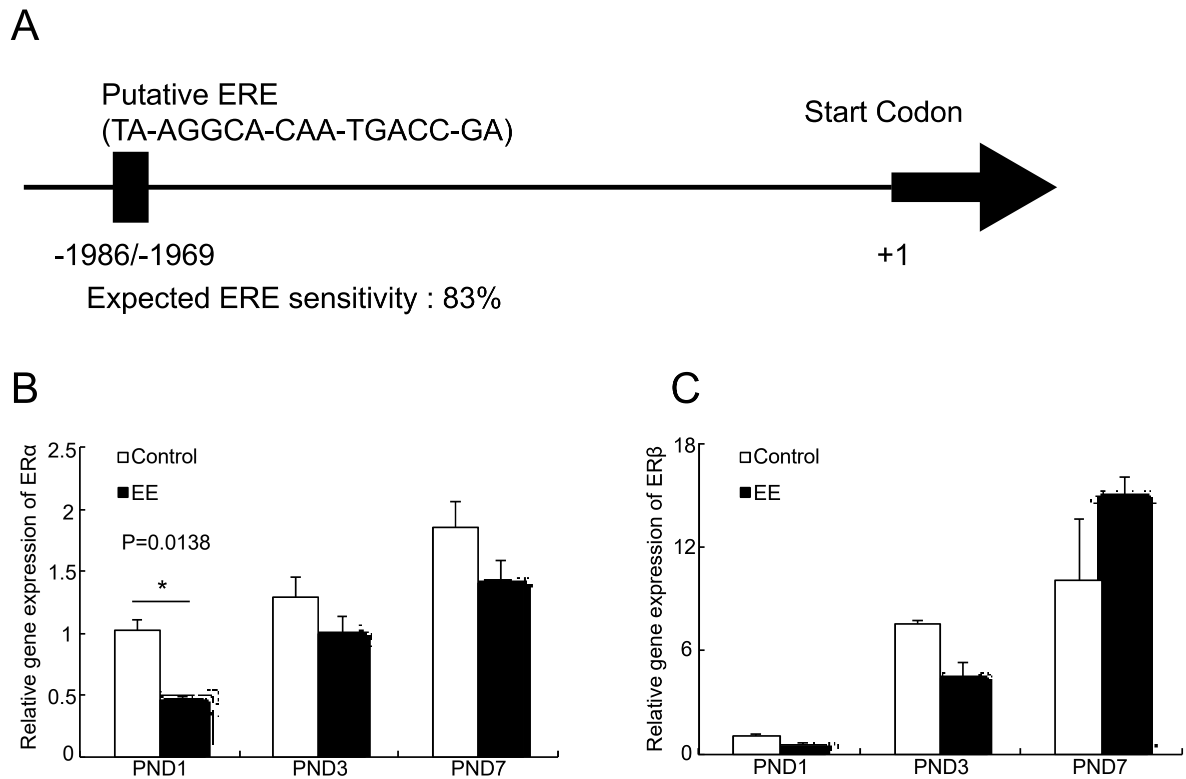


Fig. 4-6. The scheme of putative estrogen responsive element (ERE) localization in the promoter of *Hrk* gene. The relative gene expression of estrogen receptors (ERs) including ERα (B) and ERβ (C) in the developmental ovaries. Asterisk represents the significant difference ($p<0.05$). Bar, 100 μm.

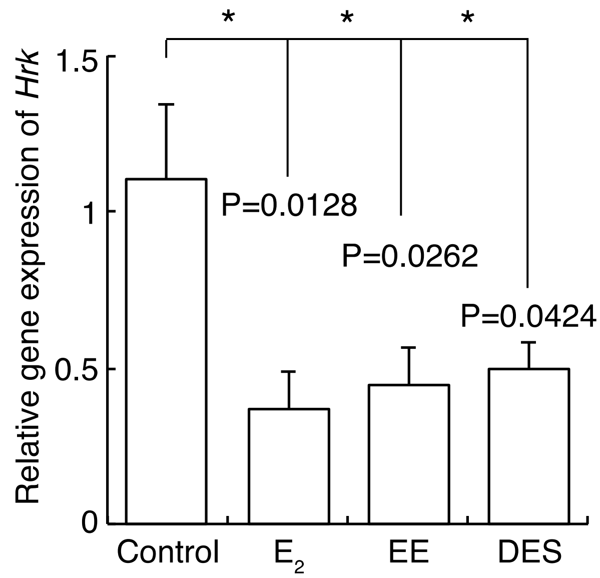


Fig. 4-7. The relative expression of *Hrk* in the ovaries cultured with 10nM different estrogenic chemicals including 17β-estradiol (E2), 17α-ethynyl estradiol (EE) and diethylstilbestrol (DES). Asterisks represent the significant difference (p<0.05).

Chapter 5 The effects of neonatal exposure to PNP on the female reproduction

5.1 Introduction

There is an increasing attention on the environmental disruptor chemicals (EDCs) causing human and wildlife endocrine system dysfunctions from public and scientific communities (4; 65; 68; 117). EDCs are such kind of synthetic and natural chemicals, which can be taken by the body and mimic or interfere with the action of endogenous hormones (28). Exposure to EDCs, especially during fetal or neonatal period, may induce the infertility and other dysfunctions of reproductive organs in adulthoods (35; 83; 97; 147). 4-Nitrophenol (PNP) has been isolated from diesel exhaust particles and is also a degradation product of the insecticide parathion, which suggests PNP might exist in air, water and soil environment (63; 96; 131). PNP highly concentrates in the air pollution due to the diesel exhaust pollution, and the concentration of PNP in the diesel exhaust is 169 mg/kg (96). Based on the human breathing 40000kg air per day, the range from 0.1 mg/kg to 100 mg/kg PNP are applied in the present study.

The adverse effect of PNP on physiological functions including liver, intestine and testis has been studied in the recent years (76; 128; 148). However, there is still little known on the effect of PNP on the ovary function. PNP has been proved to have the estrogenic and anti-androgenic activities via recombinant yeast screening assay and uterotrophic and Hershberger assay in immature rats (75; 127). As known, steroid hormones, such as estrogen, play an important role in the regulation of ovary functions. One of the important function for the ovary is to produce the oocytes after follicle maturation and estrogen is involved in this process. Based on those evidence, lead us to consider whether PNP could influence the follicle development.

In mammals, estrogen is mainly synthesized and secreted by ovaries in females, and influences the ovarian function via binding its receptors including estrogen receptor α and β. The steroidogenesis pathway for estrogen includes the cholesterol transportation into mitochondria

via steroidogenic acute regulatory (StAR) and catalyze reaction via members of cytochrome P450 family and hydroxysteroid dehydrogenase (102; 124). For members of cytochrome P450 family, it includes cytochrome P450 cholesterol side-chain cleavage (P450scc), cytochrome P450 17α-hydroxylase/17,20 lyase (P450c17) and cytochrome P450 aromatase (P450arom) (102). Many reports showed that exposure to endocrine disruptor chemicals during neonatal period could affect steroidogenesis in developmental ovary (3; 39; 49). However, the effect of PNP on steroidogenesis in ovaries of female rats is still unclear.

To gain better insight into the mechanism of the delayed effects on the reproductive system by single neonatal exposure of PNP, I investigate the ovary histology, circulating hormone levels and the gene expression before the puberty. Moreover, the vaginal opening and estrous cycle were also checked during and after the onset of puberty. Furthermore, the direct effect of PNP on the gene expression in the ovary was also examined in the PNP-cultured ovary.

5.2 Materials and methods

5.2.1 Animals

Wistar-Imamichi male and female rats were purchased from SLC (Shizuoka, Japan) and maintained at 23 ± 2 ˚C under a 14-hour lighting schedule (lights on 05:00 to 19:00 h). Food and tap water were given *ad libitum*. After mating and delivery, the female pups were used for the experiment. All procedures were permitted by the ethical committee of animal experimentation of Tokyo University of Agriculture and Technolog and carried out in accordance with the animal welfare regulations of Tokyo University of Agriculture and Technology (23-1).

5.2.2 Experiment design

5.2.2.1 Preliminary experiment:

Newborn female pups were assigned to one of the following neonatal treatment: controls given dimethyl sulfoxide (DMSO) vehicle alone as control or different doses of PNP (0.1 mg/kg, 1

mg/kg, 10 mg/kg, 100 mg/kg). Treatments were given on within 24 hours of delivery, postnatal day 0 (PND0), by subcutaneous (s.c.) injection in the nape of the neck. All compounds were dissolved in DMSO.

5.2.2.2 In vivo experiment

DMSO (control group) or 10 mg/kg PNP (PNP treatment group) were used in the *in vivo* experiment. The procedure was same as the preliminary experiment. The ovaries treated with 10 mg/kg PNP were collected at PND7, PND14 and PND21 (each treatment *n*=8). The vaginal opening was checked and the estrous cycle was examined until PND90.

5.2.2.3 In vitro experiment

To evaluate the acute effects of PNP, ovaries at PND0 were collected and cultured with or without 10 nM PNP in phenol red-free DMEM containing ITS supplement and antibiotic-antimycotic (Invitrogen). After 24h culture, ovaries were snap frozen and stored at -80°C for real time PCR (each treatment n=7).

5.2.3 Histology

Ovaries at PND21 were fixed in 4% paraformaldehyde, embedded in paraffin, serially sectioned at 6 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 was counted in the mid-portion of each ovary (20 sections per ovary, four ovaries per group). The composition of primordial follicles, secondary follicles and antral follicles was counted in control and PNP treated ovaries.

5.2.4 Radio-immunoassay

17β-estradiol in the serum samples was measured with the help of a double-antibody RIA system using ^{125}I-labeled radio ligands. Antisera against estradiol (GDN #244) were provided by Dr. G. D. Niswender (Animal Reproduction and Biotechnology, Colorado State University,

Fort Collins, CO, U.S.A.). The intra- and inter-assay coefficients of variation were 6.2% and 10.4% for estradiol.

Serum concentrations of LH and FSH were measured using an NIDDK rat RIA kits. 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 intra- and interassay coefficients of variations were 5.4 and 10.5% for LH, 5.4 and 13.1% for FSH, respectively.

Serum concentrations of immunoreactive (ir-)inhibin were measured using rabbit antiserum against bovine inhibin (TNDH-1) and ^{125}I-labeled 32-kDa bovine inhibin. The intra- and interassay coefficients of variation were 5.1 and 12.7%, respectively.

5.2.5 QRT-PCR analysis

Total RNA was extracted from ovaries using TRIzol Reagent (Invitrogen Co., CA, USA) according to the protocol. And cDNA was synthesized using PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan). The oligonucleotide primers for qRT-PCR analysis were designed using the Primer 3 program (Table 5-1). The PCR reactions were carried in a 10 µl volume using Ex TaqR Hot Start Version containing SYBR-Green I (Takara Bio) and performed with chrome4 Real-Time PCR System (Bio-Rad, CA) using the following conditions: 95°C for 30 sec, followed PCR reaction by 40 cycles of 95°C for 5 sec, 60°C for 30 sec, and dissociation protocol. The expression level of each target mRNA relative to gamma-tubulin mRNA was determined using the $2^{-\Delta\Delta Ct}$ method.

5.2.6 Statistical analysis

Statistical comparisons were made with the Student’s t-test or Two-way ANOVA followed by Bonferroni’s multiple range comparisons test using Prism 5 (Graphpad Software Inc., CA, USA). A value of $p<0.05$ was considered indication of statistical significance.

5.3 Results

5.3.1 High dose of PNP induced neonatal rats' death

The serial dose of PNP from 0.1 mg/kg to 100 mg/kg was injected into neonatal rats. All neonatal rats that were treated with 100 mg/kg died within one week after birth.

Due to this reason, 10 mg/kg were used for the following experiments.

5.3.2 Body weight and organs weight in PNP treated animals

There was no difference in the body weight between control and PNP treated animals from PND1 to PND45 (Fig. 5-1A). There also was no difference in the ovary weight (Fig. 5-1B), uterus weight (Fig. 5-1C) and adrenal gland weight (Fig. 5-1D) between control and PNP treated animals at PND30 and PND45.

5.3.3 Vaginal opening, estrous cycles and ovary histology in PNP treated animals

The follicle compositions in the ovaries at PND21 in control or PNP treated animals were examined (Fig. 5-2A and B, respectively). The percentage of primordial and primary follicles increased in the PNP treated ovaries, while the percentage of the antral follicles decreased in the PNP treated ovaries (Fig. 5-2C). Comparing with the control animals, the vaginal opening day was delayed in the PNP treated animals (Fig. 5-2D). However, no disrupted estrous cycle was observed in the PNP treated animals until PND90 (Fig. 5-2E).

5.3.4 Gene expression of gonadotropin receptors and steroidogenic enzymes and 17β-estradiol concentration in PNP treated animals

The expression of LH receptor (LHR) was higher in the ovary of PNP treated animals than that in control animals at PND14 (Fig. 5-3A), although there was no difference in the expression of FSH receptor (FSHR) between the ovaries of control and PNP treated animals (Fig. 5-3B). Similar to the expression of LHR, the expression of steroidogenic enzymes including StAR, P450scc, P450c17 and P450arom increased after neonatal exposure to PNP at PND14 (Fig. 5-3C-E and G, respectively). Moreover, the expressions of 3β-HSD and P450arom increased in the ovaries of PNP treated animals at PND21 (Fig. 5-3F and G, respectively). In consistent

with the gene expression of LHR and steroidogenic enzymes, the developmental changes of plasma 17β-estradiol concentration in control and PNP treated rats were also checked. There was a significant increase in 17β-estradiol at PND14 in PNP treated rats comparing with control rats (Fig. 5-3H).

5.3.5 Gene expression of inhibin/activin subunits and Inhibin concentration in PNP treated animals

The expression of inhibin α subunit in the ovary was no difference between control and PNP treated animals (Fig. 5-4A). However, the expressions of inhibin/activin subunits βA and βB were decreased at PND21 (Fig. 5-4B and C, respectively). Moreover, the circulating inhibin level was decreased at PND21 in PNP treated rats compared with that in control rats (Fig. 5-4D).

5.3.6 Circulating LH and FSH concentration in PNP treated rats

The circulating LH concentration was lower in the control rats than that in the PNP treated rats (Fig. 5-5A). No difference in the circulating FSH concentration between control and PNP treated rats was observed from PND7 to PND21 (Fig. 5-5B).

5.3.7 The effect of PNP on the expressions of gonadotropin receptors, steroidogenic enzymes and inhibin/activin subunits *in vitro*

Surprisingly, the expressions of luteinizing hormone receptor (LHR) and steroidogenic enzymes including Star, P450scc, P450c17, 3β-HSD and P450arom were not changed in the ovaries cultured with PNP (Fig. 5-6). The results indicate PNP may influence those genes expression by the indirectly way.

5.3.8 The effect of PNP on the expressions of estrogen receptors *in vivo* and *in vivo*

To find out the cause how PNP affects the expressions of LHR and steroidogenic enzymes in the developmental ovary, the expressions of estrogen receptors including ERα and ERβ were checked in the developmental ovaries and PNP cultured ovaries. The in vivo and in vitro results

showed the expression of ERα was not affected by PNP (Fig. 5-7A and C, respectively), however, the expression of ERβ was suppressed by PNP in the developmental ovaries at PND7 and PND14 and also in PNP cultured ovaries (Fig. 5-7B and C, respectively).

5.4 Discussion

Environment pollution has become one urgent hazards, which has a strong effect on the physiological functions of all living creatures including human and wildlife in this world (13; 15). PNP, as the degrades of pesticide and the substance in the diesel exhaust particles, has been proved to be toxic to laboratory animals (67; 76; 89). In the present study, 100 mg/kg PNP induced the neonatal rats' death, which confirmed that high dose of PNP is lethal for the animals. The oral lethal dose 50% (LD_{50}) of PNP for the adult rats is 220 mg/kg (2). Not surprisingly, PNP is much more toxic for neonatal animals. And the LD_{50} of PNP for the neonatal rats by subcutaneous injection is less than 100 mg/kg based on the present data. The present data demonstrate that PNP is much more toxic for neonatal animals.

Several studies concerning on the effect of PNP on the testis function have been reported. In the immature rats, PNP exposure induces the decreased plasma concentration of follicle stimulating hormone (FSH) and luteinizing hormone (LH) and increased plasma concentration of testosterone and inhibin (77). In addition, PNP exposure induces Leydig cell hyperplasia and disrupts the balance of androgen receptor and estrogen receptors expression (149). PNP can also induce oxidative stress in spermatogonia cells in male embryonic chickens (90). However, there is still blank on the effect of PNP on female reproduction. In this study, the body weight and organs' weight including the ovary, uterus and adrenal gland were not affected by the PNP at the dose of 10 mg/kg. However, the antral follicle ratio was decreased by PNP treatment, while the ratios of primordial and primary follicle was increased by PNP. Those results suggest that PNP may not influence body growth, but impair the follicle development in the ovary during the early period. Furthermore, the PNP treatment delayed the vaginal

opening day. Combining those results, it is suggested that PNP may retard female reproductive development via disturbing the follicle development in the pre-pubertal rats after neonatal exposure.

Gonadotropins including LH and FSH could regulate the estrogen production and follicle development via their receptors expressed in the ovary (32). A previous study showed that the elevated circulating levels of LH and FSH during PND15 were important for the follicle development (40). In contrast, PNP treatment sharply decreased the circulating LH concentration at PNP14. Considering the decreased antral follicle ratio in the PNP treated ovaries, the decreased LH concentration may be the cause for the impaired follicle development in the PNP treated rats. With the compensation of low LH level, the expression of LHR increased at PND14 in the PNP treated rats. Furthermore, the disrupted LHR and steroidogenic enzymes expressions in the PNP treatment group were consistent with the changes of circulating 17β-estradiol concentration. This data displayed PNP may disrupted 17β-estradiol production in the developmental vary via interfering with the expressions of LHR and steroidogenic enzymes. Those data indicate PNP may disrupt the steroidogenesis in the developmental ovary. And surprisingly, the *in vitro* data showed that PNP may not alter the gene expressions of those steroidogenic enzymes and LHR. Those results indicate that PNP may not influence the gene expressions of LHR and steroidogenic enzymes via directly acting on the ovary.

The previous reports in the uterotrophic assay showed that PNP has the estrogenic and anti-androgenic activity (75). Exposure to the estrogenic chemicals always alter the expression of estrogen receptors in the reproductive organs or non-reproductive organs (59; 69; 135). For example, exposure to methoxychlor during fetal and neonatal period disrupts the ERβ expression in the ovary (135). In the present study, both in vivo and in vitro results also showed that PNP could disrupted the expression of ERβ in the ovary. Estrogen receptors signaling

pathway plays an important role in the follicle development (30). Since ERβ mainly expressed in the granulosa cells, and ERβ is critical for granulosa cells functions and differentiation (24; 143), it supposed that the disrupted expressions of ERβ may also be one of the cause for the decreased antral follicles in the PNP treated ovaries.

Inhibins and activins are the two closely related proteins sharing the same subunits, which have the opposite functions in the regulations of hypothalamus-pituitary-gonad axis (26). In the present study, there was an decrease in the circulating inhibin concentration in PNP treatment groups, although there was no difference the expressions of inhibin subunit α in the ovary between control and PNP treatment groups. Moreover, the expressions of inhibin/activin subunits βA and βB were decreased at PND21 in the PNP treatments groups. Those data indicate that PNP may disrupt the balance between inhibins and activins in the developmental ovaries. Further confirmation need to be done in the future.

In summary, I showed that the neonatal exposure to PNP disrupted the expressions of gonadotropin receptors, steroidogenic enzymes and estrogen receptors. Moreover, the circulating levels of 17β-estradiol and LH were altered after PNP treatment. Therefore, the disruption after PNP treatment may affect the follicle development and delay the vaginal opening.

Table 5-1. Nucleotide sequences of the primers used for real-time PCR.

Gene	Forward	Reverse
LHR	5′-GCATTCAATGGGACGACTCT-3′	5′-GTAGGAAGACAGGGCGATGA-3′
FSHR	5′-TTTACTTGCCTGGAAGCGACTAA-3′	5′-CCCAGGCTCCTCCACACA-3′
Star	5′-GGGAGAGTGGAACCCAAATGT-3′	5′-CATGGGTGATGACTGTGTCTTTTC-3′
p450scc	5′-GGAGGAGATCGTGGACCCTGA-3′	5′-TGGAGGCATGTTGAGCATGG-3′
P450c17	5’-CCATCCCGAAGGACACACAT-3’	5’-CTGGCTGGTCCCATTCATTT-3’
3βHSD	5′-GCCCAACTCCTACAAGAAGATCAT-3′	5′-CTCGGCCATCTTTTTGCTGTATG-3′
P450arom	5’-GAACGGTCCGCCCTTTCT-3’	5’-TGGATTCCACACAGACTTCTACCA-3’
ERα	5’-CATCGATAAGAACCGGAGGA-3’	5’-AAGGTTGGCAGCTCTCATGT-3’
ERβ	5’-TATCTCCTCCCAGCAGCAGT-3’	5’-CTCCAGCAGCAGGTCATACA-3’
INHα	5′-GCTCTACCAGGGAGCATGAG-3′	5′-CACCTTCCTCCTAGCTGACG-3′
INHβA	5′-TTTCTGTTGGCAAGTTGCTG-3′	5′-CGGGTCTCTTCTTCAAGTGC-3′
INHβB	5′-GCCACGTATCCCTGACTTGT-3′	5′-CTGCTCCATGGTCTCTGTGA-3′
γ-Tubulin	5′-GCTGACCAGTGCACGGGT -3′	5′-AAACCTGGGGGGCTGGGT -3′

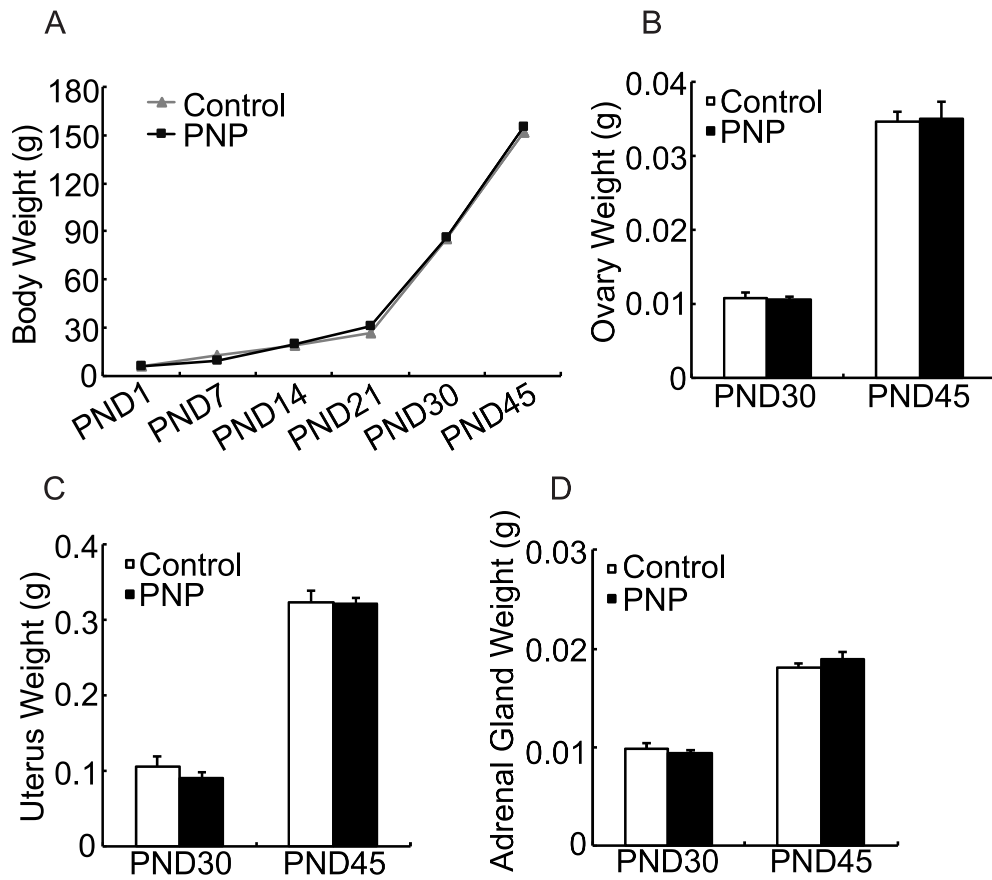


Fig. 5-1. (A) The body weight from PND1 to PND45 in control and PNP treatment animals. The ovary weight (B), uterus weight (C) and adrenal gland weight (D) during PND30 and PND45 in control and PNP treatment animals.

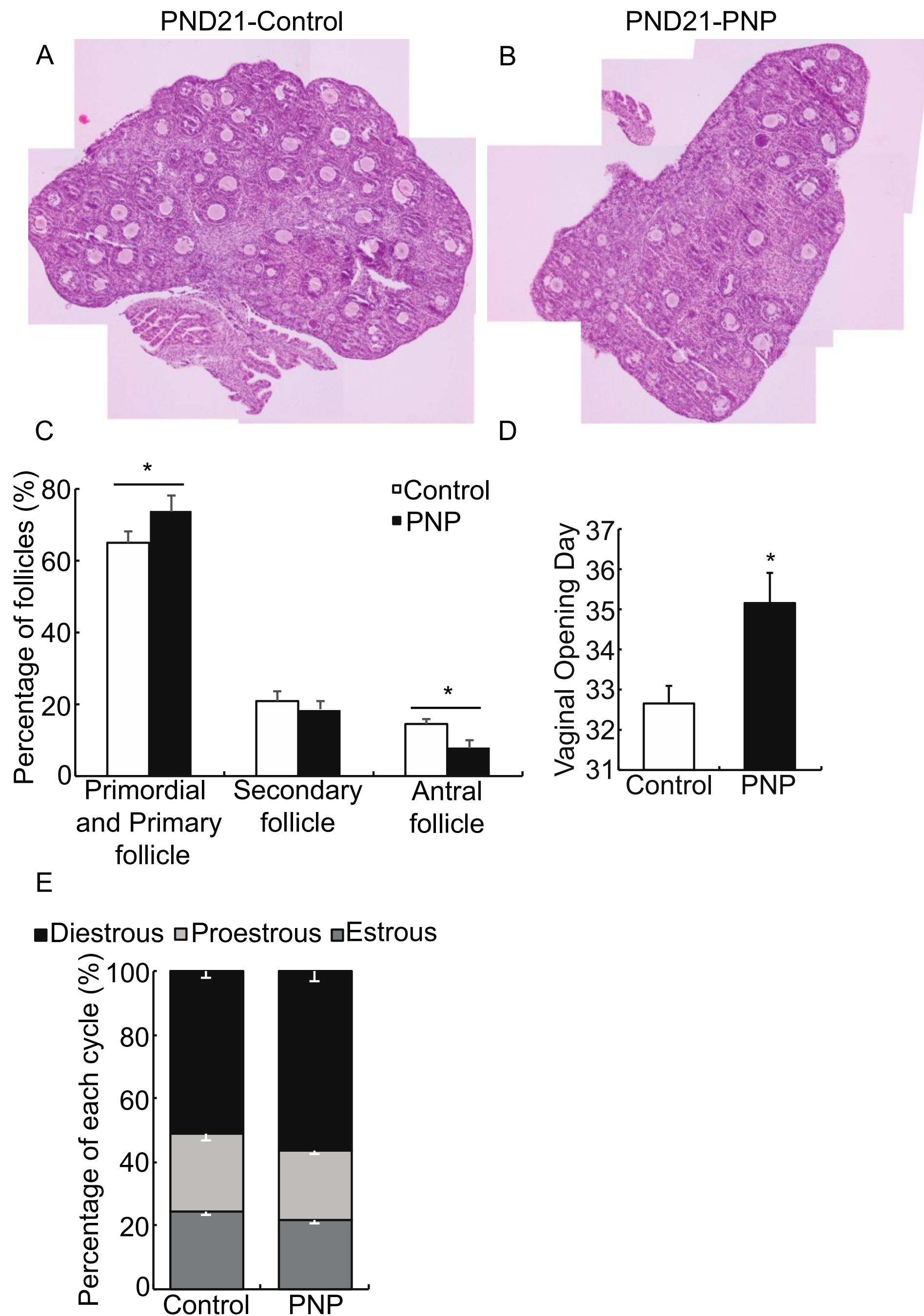


Fig. 5-2. Histology of rat ovary in control (A) and PNP treatment (B) groups at postnatal day 21. (C) The ratios of different types of follicles in rat ovaries at postnatal day 21. (D) The vaginal opening day in the control and PNP treatment animals. (E) The estrous cycle in the control and PNP treatment animals before PND90. Asterisks represent the significant difference (p<0.05).

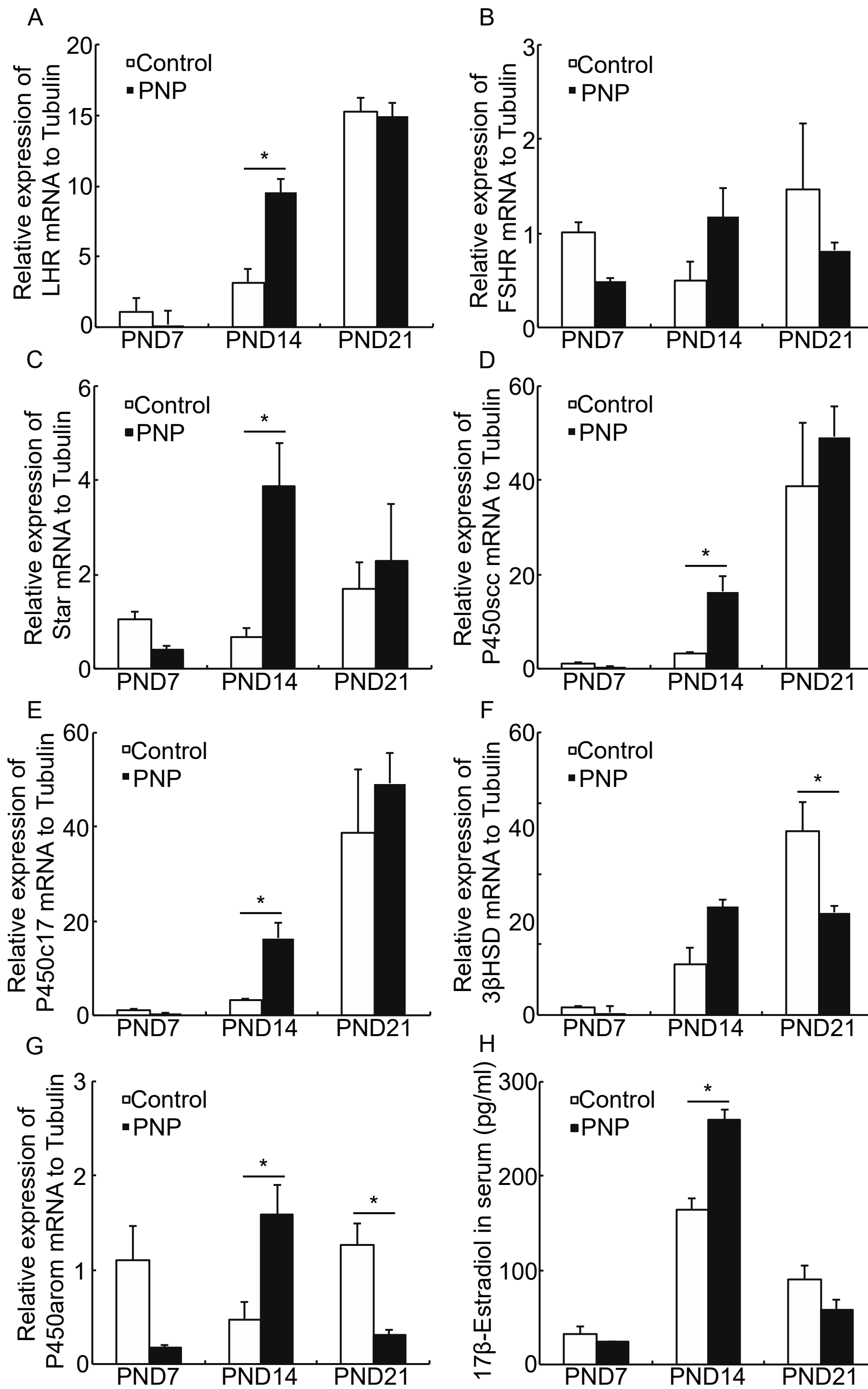


Fig. 5-3. The gene expressions of LHR (A), FSHR (B), Star (C), P450scc (D), P450c17 (E), 3βHSD (F) and P450arom (G) in the control and PNP treatment ovaries from PND7 to PND21. The circulating concentration of 17β-estradiol in the control and PNP treatment animals (H). Asterisks represent the significant difference (p<0.05).

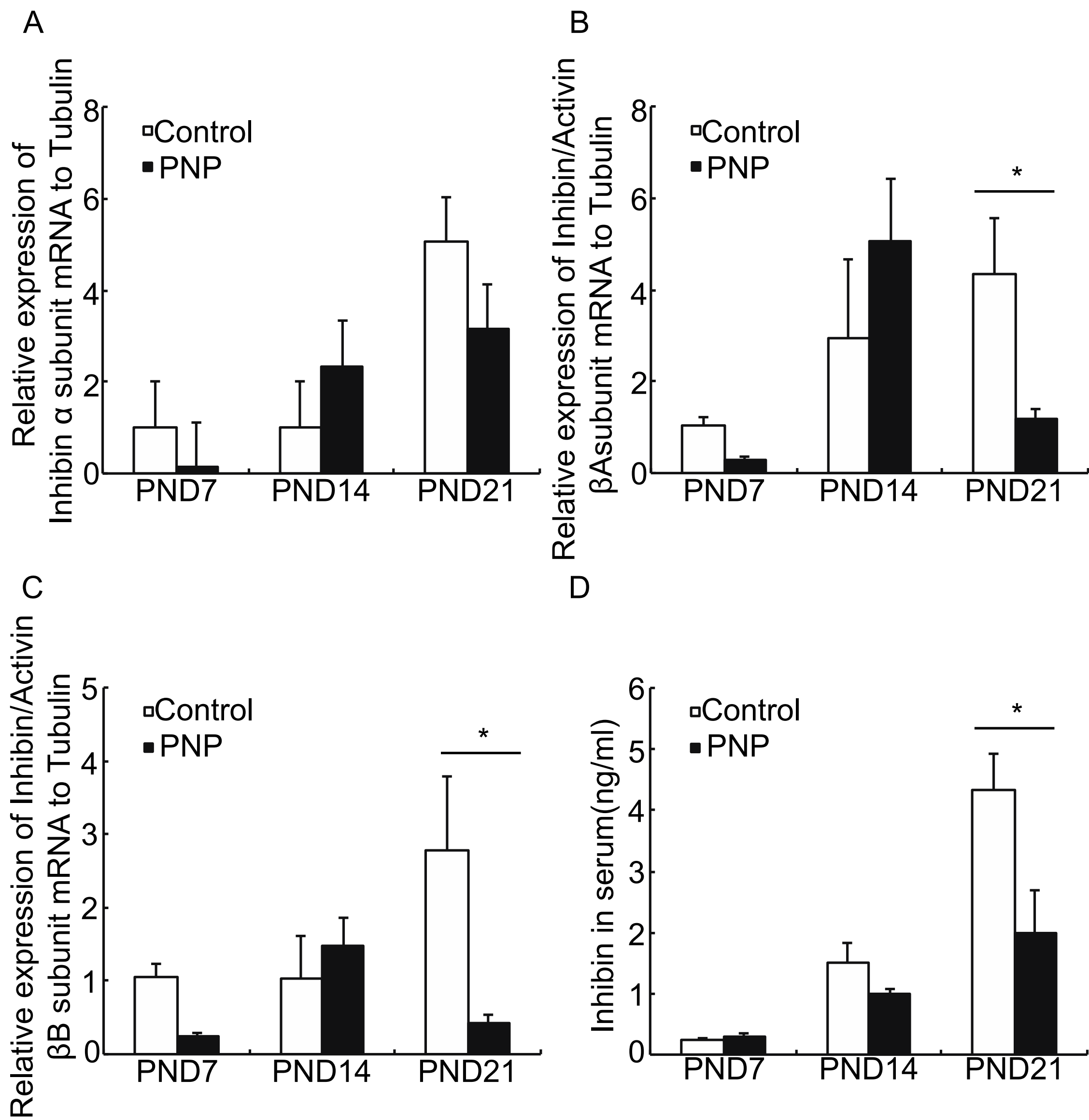


Fig. 5-4. The gene expressions of inhibin α (A), inhibin/activin βA (B) and inhibin/activin βB (C) in the control and PNP treatment ovaries from PND7 to PND21. The circulating concentration of inhibin in the control and PNP treatment animals. Asterisks represent the significant difference (*p*<0.05).

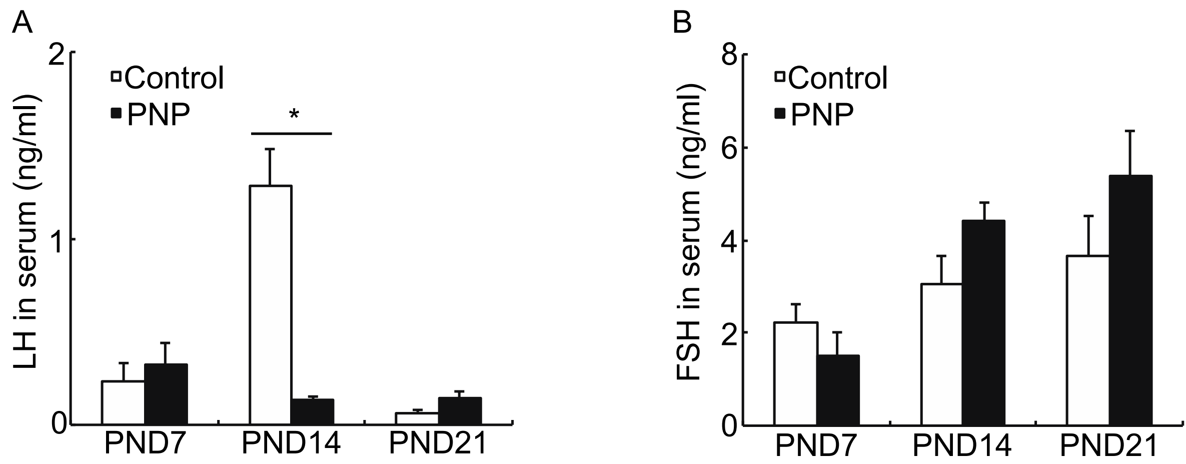


Fig. 5-5. The circulating concentration of LH (A) and FSH (B) in the control and PNP treatment animals. Asterisks represent the significant difference (p<0.05).

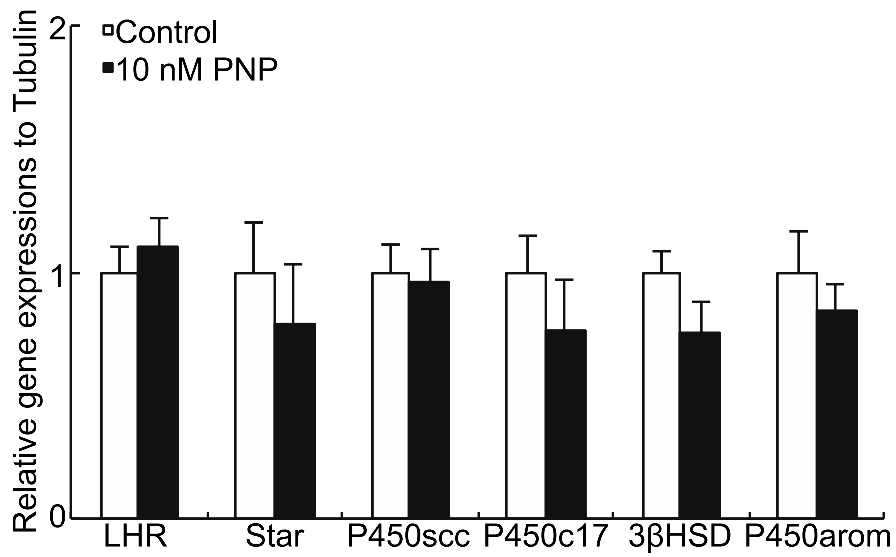


Fig. 5-6 The gene expressions of LHR and steroidogenic enzymes including Star, P450scc, P450c17, 3βHSD and P450arom in the cultured ovaries with or without 10 nM PNP treatment.

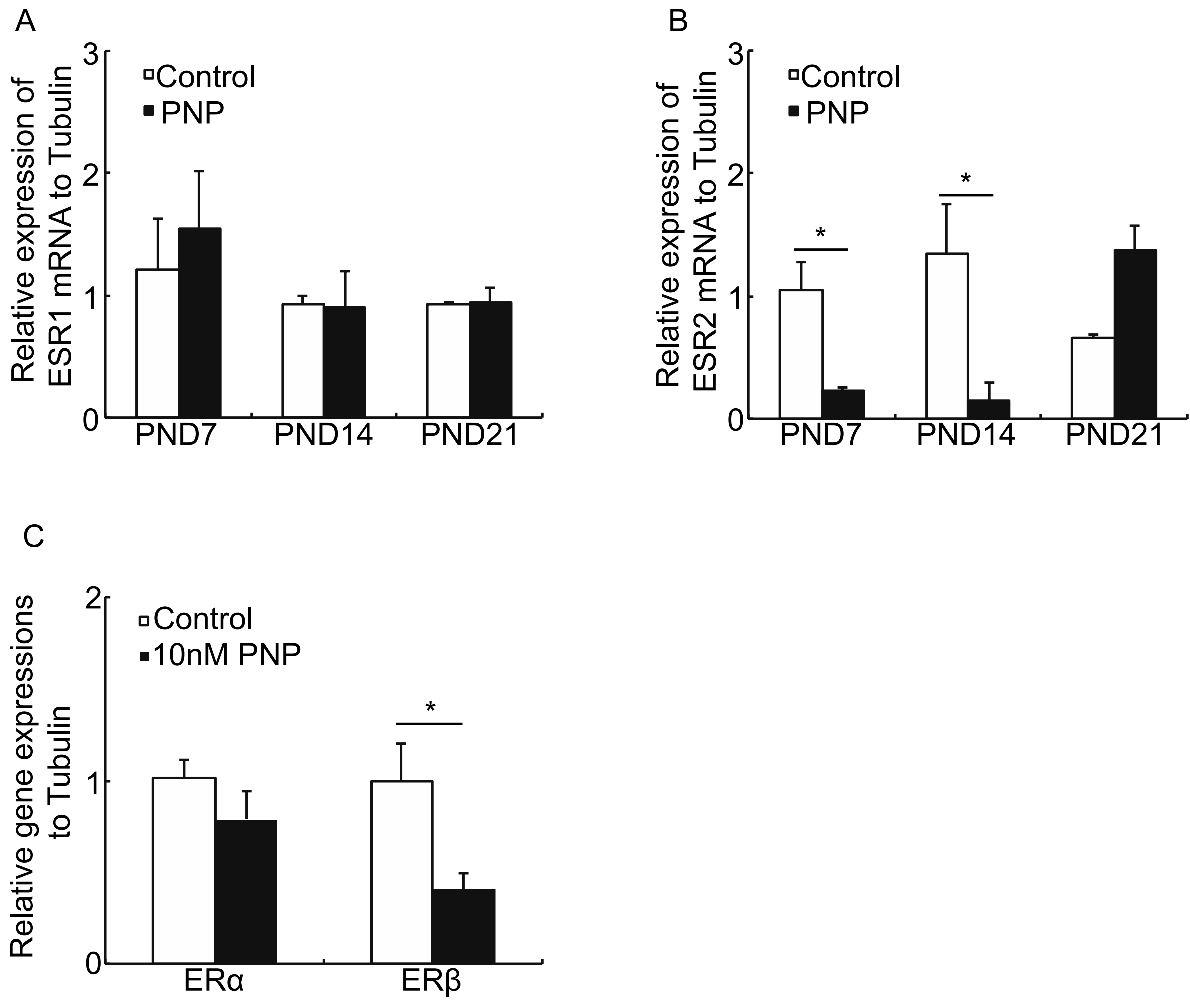


Fig. 5-7. The expressions of ERα (A) and ERβ (B) in the control and PNP treated ovaries from PND7 to PND21. The expressions of ERα and ERβ in the ovaries cultured with or without PNP (C). Asterisks represent the significant difference (p<0.05).

Chapter 6 Summary

Endocrine disrupting chemicals, shortly for EDCs, are the human-made or natural chemicals that can interfere with the human endocrine system. A variety of EDCs were found in the environment including water, food and air. Exposure to EDCs, especially during the fetal or neonatal period, may induce the toxic effects on development, growth, metabolism and reproduction or even endocrine cancer. However, the mechanisms underlying the toxic effects after neonatal exposure to EDCs are still unclear. To uncover the mechanisms for these toxic effects, the reproductive system was examined during the pre-pubertal and adult periods in the present study. after neonatal exposure to EDCs including 17α-ethynyl estradiol (EE) or 4-nitrophenol (PNP). The results are summarized as follows:

In Chapter 2, neonatal exposure to EE induced an abnormal estrous cycle during postnatal day (PND) 171–190 in low-dose and PND126–145 in high-dose group. At PND90 within normal estrous cycle, luteinizing hormone (LH) surge and ovarian hormones in serum level altered in the EE treated animals. Gene expression analysis demonstrated that the level of mRNA encoding luteinizing hormone/chorionic gonadotropin receptor (LHCGR) was higher in EE-treated ovaries than in control ovaries. Those results clearly showed that the single neonatal exposure to EE induced the ovarian dysfunction in the adulthood.

In Chapter 3, to uncover the cause for the delayed effect after neonatal exposure to EE, the reproductive parameters including ovary weight, ovarian steroidogenesis, and hormone profile were investigated in developmental female rats. The ovary weight decreased at PND14 and PND21 after neonatal exposure to EE. The expressions of P450arom increased at PND14 and the expressions of inhibin/activin subunits βA and βB decreased at PND21 in the EE treated ovaries. Consistent with the expressions of P450arom, the circulating levels of 17β-estradiol increased at PND14 in the EE treated animals. Moreover, the circulating level of luteinizing hormone (LH) increased at PND14 in EE treated animals. However, the expression of

kisspeptin reduced in the arcuate nucleus (ARC) of hypothalamus at PND14 in EE treated rats. Based on these data, it is suggested that neonatal exposure to EE altered the reproductive hormones in the developmental rats, which may result in the reproductive dysfunction in the adulthood.

In Chapter 4, to further investigate the mechanism for the delayed effect, the ovaries at PND1 were examined after neonatal exposure to EE. Microarray analysis identified the down-regulation of *Harakiri* (*Hrk*), one of apoptosis activator, in neonatal EE exposed ovaries, and real-time PCR analysis also showed the decrease of *Hrk* mRNA in ovaries treated with EE *in vitro*, as well as from neonatal EE exposed animals. The immunostaining analysis revealed that Hrk protein and cleaved caspase 3 were co-localized in the oocytes at PND1. TUNEL staining showed that oocyte number of positive staining in the EE exposed ovaries was less than in control ovaries at PND1. The abnormal follicle formation was observed at PND7 and PND21 of EE exposed ovaries. *Hrk* depletion from oocytes reduced the number of positive TUNEL staining. In addition, the down-regulation of *Hrk* mRNA expression was observed in ovaries treated with different estrogenic chemicals. Taken together, it can be concluded that EE may inhibit oocyte apoptosis in the neonatal ovary via suppressing the expression of *Hrk* and disrupting follicle formation and ovary function, which may induce the delayed effect in the adult.

In Chapter 5, the neonatal exposure to PNP showed the toxic effects that are different from neonatal exposure to EE. The vaginal opening was delayed after neonatal exposure to PNP, however, the estrous cycles seem normal until PND90. The ratio of primordial and primary follicles increased and the ratio of antral follicles decreased in the PNP treated ovaries at PND21. Moreover, the expressions of steroidogenic enzymes including StAR, P450scc, P450c17 and P450arom increased at PND14 in the PNP treated rats comparing with the control rats. Consisting of the steroidogenic genes expression, the concentration of estradiol-17β

showed a similar pattern. Furthermore, PNP could suppress the expression of estrogen receptor β (ERβ), but not estrogen receptor α (ERα) in PNP cultured ovaries or in ovaries of PNP treatment. These results suggest that PNP may directly affect the expression of ERβ in developmental rat ovaries, thence, causing the disrupted steroidogenesis during ovarian development and the delayed puberty timing in adulthood.

In conclusion, the neonatal exposure to EE and PNP may disrupt the follicle development and hormone profiles in the early stage of the postnatal period through the different pathways, and lead to the delayed effects in the adulthood.

Acknowledgements

I would like to express special thanks to my major supervisor, professor Gen Watanabe, who gave me the project advising and kind support and solicitude during my PhD study.

I would like to give my appreciation to associate professor Kentaro Nagaoka, who provide the support to my experiment details and help me to solve the experimental trouble during my PhD study.

I also would like to thank professor Kazuyoshi Taya, who gave the kind support and solicitude during I stay in Japan.

In addition, I would like to thank the Japan government which provide the scholarship to support my daily life in Japan. And this makes my life become easy in Japan.

Thanks to the lab members who help me to take care of the animals during my PhD work. In particular, I thank Mr. Yuto Yoshimoto, Ms. Yuko Shigeno and Ms. Natsumi Sakurai for their valuable assistance in breeding and careful animal care.

I am greatful to Dr. Yasuyuki Horii, Dr. Kento Usuda and Dr. Kazuki Fujii for kind friendship and the enjoyable drinking and discussion time.

Finally and specially, I would like to say thanks from my heart to my parents and my girlfriend for their strong support and encouragements to my study.

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