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Prenatal ethanol exposure induces susceptibility to premature ovarian insufficiency

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Abstract

Prenatal ethanol exposure (PEE) adversely affects the offspring reproductive system. We aimed to confirm the susceptibility to premature ovarian insufficiency (POI) in female PEE offspring and elucidate its intrauterine programming mechanism. The pregnant Wistar female rats were intragastrically administered with 4 g/kg × day of ethanol from gestational day (GD) 9 to 20. Offspring reproductive parameters were detected on GD20, postnatal week (PW) 6 and PW12. The PEE fetuses showed a decreased number of oocytes, increased ovarian cell apoptosis and upregulated expression levels of ovarian insulin-like growth factor 1 (IGF1) signalling pathway and steroidogenic enzymes. The proportion of atretic follicles in adult rats was increased, while the number of anti-Müllerian hormone-positive antral follicles was decreased. The serum oestradiol (E₂) levels were decreased, but the follicle stimulation hormone levels were elevated. The ovarian *Igf1* signalling pathway was transformed from activation during puberty to relative inhibition in adulthood, and the expression levels of ovarian steroidogenic enzymes were inhibited in adulthood. Furthermore, we treated the human granulosa cell line KGN with different ethanol concentrations (15, 30, 60, 120 mM) and found that the expression of *IGF1* signalling pathway components, *3β-HSD* and *P450arom*, as well as the production of E₂, was increased. After IGF1 siRNA transfection, *P450arom* expression and E₂ production were downregulated. These results suggest that PEE induces POI susceptibility in adult females, which may be caused by over-activation of the foetal ovarian *Igf1* signalling pathway and steroidogenesis under PEE, resulting in accelerated early development of folliculogenesis and depletion of primordial follicles.

Key Words

- ▶ prenatal ethanol exposure
- ▶ premature ovarian insufficiency
- ▶ folliculogenesis
- ▶ steroidogenesis

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Introduction

Premature ovarian insufficiency (POI) refers to the clinical syndrome of ovarian activity decline in women aged less than 40 years. The clinical features of POI are menstrual disorders accompanied by hypergonadotropism and hypoestrogenism (ESHRE Guideline Group 2016), gradually progressing to premature ovarian failure (POF). POI is related to the accelerated depletion of primordial follicles, whose pool originates from the intrauterine period and could be easily disturbed by external factors, thus affecting the number of follicles throughout the reproductive lifespan (Findlay *et al.* 2015). Gene defects, autoimmune dysfunction and iatrogenic interventions are the known causes of POI, but 50% of the cases are not identified. A follow-up study found that adolescent girls prenatally exposed to polychlorinated biphenyls showed abnormal menstruation and elevated levels of gonadotropins (Yang *et al.* 2005). Rodent studies found that the intake of N-acetyl-para-aminophenol during pregnancy resulted in an irregular oestrous cycle, reduction of primordial follicles, premature absence of the corpus luteum (CL) and reduced fertility among female offspring (Arendrup *et al.* 2018). Female offspring rats of maternal undernutrition showed disruption in the estrous cycle, elevated gonadotropin levels, decreased oestradiol (E_2) levels and irregular ovulation (Khorram *et al.* 2015). Additionally, prenatal exposure to the environmental pollutant benzopyrene led to a disturbed estrous cycle, premature depleted primordial follicles and decreased fertility of female offspring mice (Vabre *et al.* 2017). The studies above suggest that the occurrence of POI could have a foetal origin.

Ethanol, which comprises many daily necessities, is a xenobiotic that is easily accessible to individuals. A growing number of women at childbearing age have reported long-term drinking habits, and up to 40% of women in some countries drink alcohol during pregnancy (Nykjaer *et al.* 2014). Thus, perinatal health problems caused by PEE are becoming a growing concern. PEE affects not only the pregnant women but also foetal development, causing spontaneous abortion, premature delivery, foetal alcohol syndrome and intrauterine growth retardation (IUGR) (Spohr *et al.* 1993, Nykjaer *et al.* 2014), with long-term impact on the offspring's reproductive function (Wilson & Handa 1997, Dupont *et al.* 2012). A previous study showed that PEE can disrupt the development and function of the hypothalamic–pituitary–ovarian (HPO) axis in females,

leading to dysfunction of the estrous cycle and sexual hormone secretion patterns (Gawalek & Sliwowska 2015). Furthermore, ovarian development is impaired in IUGR foetuses, with a significant reduction in the proportion of primordial follicles (de Bruin *et al.* 1998). Female IUGR offspring caused by maternal undernutrition undergo POI in adult life (Khorram *et al.* 2015). However, scant studies have reported on whether PEE can lead to POI in IUGR offspring and the underlying mechanism of the foetal origin.

Both local factors and the neuroendocrine axis play important roles in ovarian oocyte maturation and follicular development. Among them, an important local regulatory factor is insulin-like growth factor 1 (IGF1), which not only can participate in the activation of primordial follicles and regulate the number of apoptotic follicles but can also alter the expression levels of steroidogenic enzymes through triggering the phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) pathway (Shah *et al.* 2018). The HPO axis is mainly involved in the development and functional regulation of ovarian follicles after birth, because it has not been fully developed during the intrauterine period (Picut *et al.* 2015). Epidemiological data have shown that the serum IGF1 levels in POI women were significantly higher than those in healthy women (Hartmann *et al.* 1997). Animal studies have found that IGF1 can stimulate primordial follicle activation in the rat ovary (Stubbs *et al.* 2013), while abnormal primordial follicle maintenance is the pathophysiological basis for POI (Saatcioglu *et al.* 2016). Other studies have found that prepubertal ethanol-exposed rats have elevated ovarian *Igf1* mRNA expression but decreased protein levels, accompanied by decreased serum E_2 levels (Srivastava *et al.* 1999). Furthermore, ethanol can affect ovarian IGF1 expression, thereby interfering with follicular development and steroid synthesis (Srivastava *et al.* 2007). We speculate that PEE could affect the ovarian structural and functional development of the offspring, and then increase the incidence risk of POI, which may occur by elevating the expression of the IGF1 signalling pathway.

To determine whether PEE can cause POI susceptibility in female offspring, we observed ovarian structural and functional development before and after birth in an established IUGR female rat model by PEE (Huang *et al.* 2018). Additionally, to elucidate the underlying mechanism, we used the human granulosa cell line from the perspective of the IGF1 signalling pathway, which would provide experimental evidence for the early prevention and reduction of the foetal origins of POI.

Materials and methods

Animal and treatment

Specific pathogen-free adult Wistar female (mean body weight: 209 ± 12 g) and male (mean body weight: 258 ± 17 g) rats (certification no. 42000600002258, license no. SCXK (E) 2012-2014) were provided by the Experimental Centre of the Hubei Medical Scientific Academy (Wuhan, China). The experiment was performed at the Centre for Animal Experiment of Wuhan University (Wuhan, China), which has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All animal experimental procedures were approved by the Animal Welfare Committee of Wuhan University (License No: 14016) and were performed according to the Guidelines for the Care and Use of Laboratory Animals of the Chinese Animal Welfare Committee.

Adult female Wistar rats (F0 generation) were mated with male Wistar rats and were kept in the same room with an environmental temperature of $18\text{--}22^\circ\text{C}$, a relative humidity of 40–60%, a 12-h/12-h light–darkness cycle, 10–15 times air changes per hour and free access to food and water. Mating was confirmed by the presence of sperm in the morning vaginal smear, recorded as gestational day (GD) 0. The pregnant rats were randomly divided into the control and PEE groups. From GD9 to GD20, the PEE group was intragastrically administered with $4\text{ g/kg} \times \text{day}$ of ethanol (analytical pure grade; Sinopharm Chemical Reagent Co. Ltd., Shanghai, China; 10009218) (Fig. 1), while the control group was administered an equal volume of distilled water (Shen *et al.* 2014). An epidemiological survey showed that pregnant women who consumed 13.6 bottles of alcoholic beverages per week might cause foetal alcohol syndrome, and the ethanol intake was equivalent to $3.0\text{--}4.3\text{ g/kg}$ (May *et al.* 2005). Related studies found that when a person intakes 3–5 bottles of beer, the average blood ethanol concentration reaches 33 mM

(Gohlke *et al.* 2005), and the blood ethanol concentration reaches approximately 20–170 mM during moderate-to-severe alcoholism in humans (Pantazis *et al.* 1992). Our previous study reported that the blood concentrations of ethanol in PEE maternal and female foetal rats were 87 and 58 mM, respectively (Shen *et al.* 2014). Therefore, the exposure dose of ethanol used in the present study could reasonably simulate the exposure of individuals in real life.

On GD20, a subgroup of pregnant rats was anaesthetized by isoflurane (Baxter Healthcare Co., Deerfield, IL, USA; 10019-360-40) inhalation, and the foetuses were removed by caesarean section. The number of pregnant rats was 16 in each group. The IUGR diagnosis was based on two standard deviations of the foetal rat body weight lower than the mean weight of the control group (Wang *et al.* 2017a). The IUGR incidence was calculated based on the number of IUGR foetuses per litter compared with the total number of pups in the litter (Yu *et al.* 2018). Foetal blood was pooled by female grouping per dam and was centrifuged for storage. The right ovaries of five female foetuses in each group were randomly selected and fixed in freshly prepared Bouin's solution for 24 h and then were dehydrated with alcohol and embedded in paraffin for morphological analysis. The remaining foetal rats were collected from the litter and centrifuged for serum. The remaining foetal ovaries were quickly frozen in liquid nitrogen and stored at -80°C until analysis.

The remaining pregnant rats naturally produced F1 generation rats. The number of pregnant rats was eight in each group. After weaning, all F1 females were fed with standard laboratory feed *ad libitum*. Two subgroups of F1 female rats were fasted overnight to avoid the effects of ingestion on the hormone levels (Yan *et al.* 2014, Tsoulis *et al.* 2016) were euthanized following isoflurane anaesthesia at postnatal week (PW) 6 or PW12 in diestrus or metestrus ($n=8$ from eight different litters at each time point), and the ovaries, hypothalami and blood samples were collected. At PW12, the remaining female offspring were mated with normal male rats to produce F2 generation rats. The pregnancy rate, neonatal body weight, number of pups per litter, male-female ratio and litter death rate were recorded and calculated. The pregnancy rate was calculated as the number of pregnant females/number of females housed with one male $\times 100$. The litter death rate was calculated as the dead pups/number of pups per litter $\times 100$.

Vaginal patency is an important indicator of female rats entering puberty. The vaginal patency of F1 female

Figure 1

The schematic illustration of animal treatment. From gestational day (GD) 9–20, the pregnant Wistar rats were administered intragastrically with $4\text{ mg/kg} \times \text{day}$ of ethanol. The offspring rats were killed at GD20, postnatal week (PW) 6 and PW12, respectively.

rats was observed every day from the weaned day until complete opening (Lv *et al.* 2018). The normal estrous cycle of Wistar rats is 4–5 days (Serrano *et al.* 2017). The estrous cycle was examined daily at 08:00h starting from PW10 over two cycles and was determined by cytological examination of vaginal smears (Lv *et al.* 2018). Four stages were used for categorization: proestrus (predominance of nucleated epithelial cells), oestrus (anucleated cornified cells), metestrus (equal portions of leukocytes, epithelial and cornified cells) and diestrus (predominance of leukocytes).

Haematoxylin-eosin (HE) staining and immunohistochemistry (IHC)

The right ovaries of five rats in each group at three time points were randomly selected for sectioning. The fixed ovaries were processed, paraffin-embedded, serially sectioned at 5 µm and stained with HE. Every fifth section of the entire ovary was analysed and photographed using an Olympus AH-2 light microscope (Olympus). Only oocytes or follicles with a visible nucleus were counted to avoid multiple counts of the same one, except for atretic follicles. Healthy follicles showed no deformation of pyknotic nuclei or cell layers. Classification of the follicle stage was performed based on the granulosa cell layer morphology, and the presence or absence of an antral cavity, follicles with degeneration of oocytes and/or granulosa cells, retraction of the granulosa cell layer from oocytes or nuclear blebbing was counted as atretic follicles, as previously described (Chan *et al.* 2018). The number of primordial follicles, primary follicles, preantral follicles, antral follicles, atretic follicles and CL was calculated on every fifth section of the entire ovary. The thicknesses of the granulosa cell layers in antral follicles were measured in five different sections to evaluate the development of antral follicles.

IHC analysis was performed by using a DAB staining kit (GeneTech Co. Ltd., Shanghai, China; GK347011) to determine the expression levels of Ki67 (1:200; Abcam plc.; ab16667) and cleaved-caspase 3 (1:50; R&D Systems; AF835) in foetal ovaries and anti-Müllerian hormone (AMH) (1:200; Sigma-Aldrich; SAB2108646) in ovaries at PW6 and PW12. The paraffin cross-sections of ovarian tissues were rehydrated and washed by boiling the slides in 0.01 M citrate buffer (pH 6.0) at 95°C for 10 min, and then were incubated with primary antibody overnight at 4°C. Negative controls were prepared by replacing the primary antibody with nonimmune rabbit immunoglobulin G. All the images were analysed using Image Pro Plus software

(version 6.0; Media Cybernetics, Silver Spring, MD, USA). The intensity of Ki67 and cleaved-caspase 3 staining was determined by measuring the mean optical density in five random fields per section, and five sections from different ovaries per group were used for quantification. The evaluation of AMH-positive primary, preantral and antral follicles occurred according to the intensity and distribution of staining as previously defined (Ozcan *et al.* 2015), and five different fields from each section were analysed.

KGN cell culture and siRNA transient transfection

The human granulosa cell line KGN (Centre for Translational Medicine and Jiangsu Key Laboratory of Molecular Medicine, Nanjing, China) was cultured in red phenol-free Dulbecco's modified Eagle medium nutrient mixture F12 (DMEM/F12) (Gibco, Thermo Fisher Scientific; C11330500BT) containing 10% foetal bovine serum (Gibco; 10099-141) and 0.1% penicillin/streptomycin at 37°C with 5% CO₂. The MTS cell proliferation colorimetric assay (Promega Co.; G358B) was performed to evaluate the effects of ethanol treatment on KGN cell viability according to the manufacturer's instructions. Chemically synthesized IGF1 siRNA (sense: 5'-CCUCCUCGCAUCUCUUCUATT-3'; antisense: 5'-UAGAAGAGAUGCGAGGAGGTT-3') was obtained from Suzhou GenePharma Co., Ltd. (Suzhou, China). KGN cells were seeded into six-well plates at 1 × 10⁵ cells/well 1 day before IGF1 siRNA transfection to reach the 50% confluency. After the transfection procedures were performed using Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific; L3000150) for 24 h according to the manufacturer's instructions, the transfection mixture medium was replaced by medium with or without ethanol (120 mM) for 48 h, and then the cells were harvested for further analyses. Negative control (NC) siRNA (sense: 5'-UUCUCCGAACGUGUCACGUTT-3'; antisense: 5'-ACGUGACACGUUCGGAGAATT-3') was used to assess nonspecific gene-silencing effects.

Quantification of hormone levels

The foetal serum levels of E₂ were measured using an ELISA kit (R&D Systems; KGE014; minimum detectable concentration: 2.14 pg/mL; intra-assay coefficient of variation (CV) <6.0%; inter-assay CV <7.1%). The radioimmunoassay (RIA) kits for rat E₂, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were purchased from Beijing North Institute of

Biological Technology (Beijing, China). The levels of E₂ in the serum of postnatal rats and supernatant of KGN cells were detected by RIA (sensitivity <5 pg/mL; intra-assay CV <10%; inter-assay CV <15%). The serum levels of FSH and LH in the postnatal rats were measured by RIA (sensitivity <1.0 mIU/mL; intra-assay CV <10%; inter-assay CV <15%). The Rat AMH ELISA kit (Cusabio Biotech Co. Ltd., Wuhan, China; CSB-E11162r) was used to quantify the serum levels (detection range: 0.2–15 ng/mL; intra-assay CV <15%; inter-assay CV <15%). All test procedures were performed following the manufacturer's instructions.

RNA isolation and real-time quantitative PCR (RT-qPCR)

Total RNA extraction from the ovary, hypothalamic and KGN cells was performed using TRIzol reagent (Invitrogen Co.; 15596018) and following the manufacturer's protocol. The reverse transcription reagent kit used was purchased from TaKaRa Biotech Co. Ltd. (Beijing, China; RR047A). The tissues obtained from each littermate were pooled for homogenization as one sample. The total RNA quantity and quality were measured using the NanoDrop 2000 system (Bio-Rad Co.), and the total RNA concentration was adjusted to 1000 ng/μL. Complementary DNA was synthesized from 1 μg of total RNA according to the kit protocol and was stored at –20°C for testing. Primers were designed using Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, CA, USA), and the sequences were queried using the NCBI BLAST database (Supplementary Table 1, see section on [supplementary data](#) given at the end of this article). All the primers were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China). SYBR Green dye was purchased from Applied Biosystems (ABI; 4472908). Each PCR was performed in a 10 μL volume using the ABI StepOne RT-PCR Thermal Cycler (Thermo Fisher Scientific). To more accurately quantify the gene transcripts, the mRNA level of the housekeeping gene GAPDH was detected and used as the quantitative control. The transcript levels of the target genes at each time point were expressed relative to the GAPDH transcript levels. Each sample was normalized against the GAPDH mRNA level.

Western blotting

Protein was extracted from the KGN cells using RIPA buffer (Applygen Technologies Inc, Beijing, China; C1053) containing protease inhibitors (Complete Mini; Roche; 11836153001) and phosphatase inhibitors

(PhosSTOP; Roche; 04906845001). The protein concentrations were quantified using the BCA Protein Assay Kit (Beyotime; P0011). Protein (15 μg) extracts were separated by 12% SDS-PAGE and were transferred to PVDF membranes (Immobilon; Merck Millipore; IPVH00010) according to the manufacturer's instructions. The membranes were blocked with 5% nonfat dried milk for 1 h and were then incubated with primary antibodies at 4°C overnight. The following antibodies were used: anti-GAPDH (1:5000; ABclonal; AC002), anti-IGF1 (1:1000; Novus Biologicals; SPM406), anti-AKT (1:1000; CST; 4691s) and anti-phospho-AKT (Ser473) (1:2000; CST; 4060). After incubation and washing, the membranes were incubated with secondary antibodies (anti-mouse and anti-rabbit conjugated with horseradish peroxidase) at a 1:5000 dilution for 1 h and were detected using an enhanced chemiluminescent detection kit (Bridgen, Beijing, China; D046). Densitometric analyses were performed using ImageJ software (National Institutes of Health).

Statistical analysis

Quantitative data were expressed as the mean ± standard error of the mean (S.E.M.). All statistical analyses were performed using the SPSS 20.0 statistics package (SPSS) and Prism 6.0 (GraphPad Software). The mean weights of each litter were used for the statistical analysis as for the weight of the fetuses. The IUGR incidence of each litter was analysed using the arcsine square root transformed before the *t*-test evaluations. The mean values were compared using Student *t*-test or one-way ANOVA followed by the *post hoc* Dunnett's-*t*-test. The threshold for significance was set as *P* < 0.05.

Results

Animal experiments

Effects of PEE on body weight and ovarian morphology of foetal rats

We observed changes in the body weight and ovarian morphology of female foetal rats on GD20. Compared with the control group, PEE fetuses had a lower body weight and a higher IUGR incidence (*P* < 0.01, [Fig. 2A](#) and [B](#)). HE staining of the foetal ovary in the PEE group showed a decreased number of oocytes per unit area (10,000 μm²) (*P* < 0.05, [Fig. 2C](#) and [D](#)). IHC results showed that the mean optical density of positively stained cells of Ki67 in the foetal ovary was reduced (*P* < 0.05, [Fig. 2E](#) and [F](#)), while the cleaved-caspase 3 was increased

Figure 2

Effects of prenatal ethanol exposure (PEE) on body size, body weight and ovarian morphology in female foetal rats. (A and B) Foetal bodyweight and intrauterine growth restriction (IUGR) incidence. Mean $\pm$ S.E.M., $n = 12$. (C) Foetal ovary morphology (haematoxylin-eosin staining, HE $\times 400$); (D) Number of oocytes per unit area ($10,000 \mu\text{m}^2$); (E and G) Immunohistochemistry of Ki67 and cleaved-caspase 3 (immunohistochemistry, IHC $\times 400$) in the foetal ovary; (F and H) mean optical density of nuclei-stained cells with Ki67 and cleaved-caspase 3 protein expression. Mean $\pm$ S.E.M., $n = 5$. * $P < 0.05$, ** $P < 0.01$ vs control. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0063>.

($P < 0.01$, Fig. 2G and H). These results indicate that PEE can cause low birth weight and a high IUGR incidence in foetal rats and abnormal morphological development of the ovary in the IUGR offspring, characterized by abnormal folliculogenesis, reduced cell proliferation and increased apoptosis.

Effects of PEE on the levels of ovarian IGF1 signalling pathway components, steroidogenic enzymes and E_2 in foetal rats

To investigate the mechanism underlying the foetal origin of foetal ovarian development abnormalities caused by PEE, we examined the expression of ovarian IGF1 signalling pathway components, steroidogenic enzymes and serum E_2 levels in foetal rats. Compared with the control, the mRNA expression level of *Igf1* was significantly upregulated ($P < 0.01$), and the expression levels of its receptor *Igf1r* and downstream gene *Akt2* were also increased in the PEE female foetuses ($P < 0.01$, Fig. 3A). Furthermore, the mRNA expression levels of 3β -HSD

and 17β -HSD1 were increased ($P < 0.05$) and that of 17β -HSD2 was decreased ($P < 0.01$, Fig. 3B), but no significant change was found in the foetal serum E_2 levels in the PEE group ($P < 0.05$, Fig. 3C). These data suggest that the foetal ovarian IGF1 signalling pathway and steroid synthetic function are activated by PEE.

Effects of PEE on the body weight, estrous cycle and reproductive function of offspring rats after birth

To determine whether the ovarian development abnormalities caused by PEE will persist after birth and have a programming effect, we observed the body weight of female offspring rats at different time points after birth. Compared with the control, the birth weight of PEE group was significantly lower ($P < 0.01$), while the body weight was increased at PW6 and decreased at PW12 ($P < 0.01$, Fig. 4A). The body weight gain rate of the PEE group also increased significantly at PW6 ($P < 0.01$, Fig. 4B). These results suggest that the female offspring of PEE

Figure 3

Effects of prenatal ethanol exposure (PEE) on the levels of ovarian insulin-like growth factor 1 (IGF1) signalling pathway-related genes, steroidogenic enzymes and serum oestradiol (E_2) concentration in female foetal rats. (A) mRNA expression of ovarian IGF1 signalling pathway-related genes; (B) mRNA expression of ovarian steroidogenic enzymes; (C) serum E_2 concentration. Mean $\pm$ S.E.M., $n = 8$ for mRNA expression (6 pairs of foetal ovaries from two litters were pooled for homogenization into one sample); $n = 4$ for E_2 concentration (blood from 3 to 4 litters were pooled into one sample). * $P < 0.05$, ** $P < 0.01$ vs control. AKT1/2, protein kinase B1/2; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD1/2, 17β-hydroxysteroid dehydrogenase 1/2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IGF1R, insulin-like growth factor 1 receptor.

exhibit catch-up growth in the early postnatal period (especially at PW6).

We observed the estrous cycle of female offspring beginning from PW10. Compared with the control, the oestrus period of PEE was significantly shortened ($P < 0.05$, Fig. 4C); however, with the onset of puberty, the time of vaginal patency showed no significant difference (Supplementary Fig. 2A). We also tested the fertility of adult female offspring. The results showed no significant difference in the pregnancy rate of F1 females and neonatal body weight, number of pups per litter, male-female ratio and litter death rate of F2 females (Supplementary Fig. 2E, F, G, H and I). These data suggest that PEE can cause irregular cycles in the female offspring, but has no significant effect on the onset of puberty and reproductive function.

Effects of PEE on ovarian morphology and follicular development of offspring rats after birth

We continued to observe ovarian development from puberty to adulthood. Compared with the control, PEE showed no significant changes in the ovarian weight at PW6 and PW12 (Fig. 5A), while the ovary index increased significantly at PW12 ($P < 0.05$, Fig. 5B), suggesting that the ovarian weight of PEE female offspring showed catch-up growth after birth and the ovarian growth was faster than the overall development of the body.

Furthermore, we observed changes in ovarian morphology. Although the total numbers of follicles and the numbers of CL at PW6 and PW12 ovary were not significantly different from those of the control (Supplementary Fig. 2B and C), the proportion of atretic follicles at PW12 was increased ($P < 0.01$, Fig. 5C). Additionally, the thickness of granulosa cells of the antral follicle at PW6 was significantly thinner ($P < 0.05$, Fig. 5D and E), and this difference persisted at PW12 ($P < 0.05$, Fig. 5F and G). The serum AMH levels were similar between groups at PW6 and PW12 (Supplementary Fig. 2D), and the IHC results showed that the numbers of follicles positive for AMH were similar at PW6 (Fig. 5H and I), but the number of AMH-positive antral follicles was reduced significantly at PW12 ($P < 0.05$, Fig. 5J and K). These results suggest that PEE affects the development of the follicle structure and ovarian reserve.

Effects of PEE on the ovarian IGF1 signalling pathway and E_2 synthesis in offspring after birth

To investigate the mechanism of abnormal ovarian development in the PEE female offspring rats, we examined the levels of ovarian IGF1 signalling pathway components, steroidogenic enzymes and serum E_2 levels in rats after birth. The mRNA expression of IGF1 signalling pathway components (including *Igf1r*, *Akt1* and *Akt2*) in the PEE group increased significantly at PW6 ($P < 0.01$, Fig. 6A) but did not change at PW12. The mRNA

Figure 4

Effects of prenatal ethanol exposure (PEE) on the body weight, body weight gain rate and estrous cycle in female offspring rats after birth. (A) Body weight; (B) body weight gain rate and (C) oestrous cycle. Mean $\pm$ S.E.M., $n = 8$. * $P < 0.05$, ** $P < 0.01$ vs control.

Figure 5

Effects of prenatal ethanol exposure (PEE) on ovary weight, ovary weight index, ovarian histological morphology in female offspring rats after birth. (A) Ovary weight; (B) ovary index; (C) cross-section follicle component at postnatal week (PW) 6 and PW12; (D and F) antral follicle at PW6 and PW12 (haematoxylin-eosin staining, HE $\times 400$); (E and G) thickness of granulosa cells of rat antral follicle at PW6 and PW12; (H and J) immunohistochemistry of rat ovary anti-Müllerian hormone (AMH) at PW6 and PW12 (immunohistochemistry, IHC $\times 200$); (I and K) number of follicles positive for rat ovary AMH at PW6 and PW12. Mean $\pm$ S.E.M., $n = 5$ for HE and IHC. * $p < 0.05$, ** $p < 0.01$ vs control. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0063>.

expression levels of *Sf-1* and *P450c17* were increased at PW6 ($P < 0.01$, $P < 0.05$); however, the expression levels of ovarian steroidogenic enzyme-related genes at PW12, including *StAR*, *3 β -HSD*, *17 β -HSD1/2*, and *P450arom*, were inhibited ($P < 0.01$, $P < 0.05$), while only the expression level of *P450scc* was increased ($P < 0.05$, Fig. 6B). The serum E_2 level of the PEE group showed no change at

PW6 but was much lower than that of the control at PW12 ($P < 0.05$, Fig. 6C). These results suggest that the expression of IGF1 signalling pathway components and steroidogenic enzymes in the ovary of PEE female offspring rats is increased during puberty (PW6), but the oestrogen synthetic function is significantly inhibited in adulthood (PW12).

Figure 6

Effects of prenatal ethanol exposure (PEE) on the ovarian insulin-like growth factor 1 (IGF1) signalling pathway-related genes, steroidogenic enzymes and serum oestradiol (E_2) concentration in female offspring rats after birth. (A) mRNA expression of ovarian IGF1 signalling pathway-related genes; (B) mRNA expression of ovarian steroidogenic enzymes; (C) serum E_2 concentration. Mean $\pm$ S.E.M., $n = 8$. * $P < 0.05$, ** $P < 0.01$ vs control. 17 β -HSD1/2, 17 β -hydroxysteroid dehydrogenase type 1/2; 3 β -HSD, 3 β -hydroxysteroid dehydrogenase; AKT1/2, protein kinase B1/2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IGF1R, insulin-like growth factor 1 receptor; P450arom, cytochrome P450 aromatase; P450c17, steroid 17-hydroxylase; P450scc, cytochrome P450 cholesterol side chain cleavage; Sf-1, steroidogenic factor 1; StAR, steroidogenic acute regulatory protein.

Effects of PEE on the HPO axis of offspring rats after birth

We also observed the changes in the HPO axis in the female offspring rats after birth following PEE. Compared with the control, the mRNA expression levels of *GnRH* and *Kiss1* in the hypothalamus of PEE offspring rats did not change significantly at PW6 and PW12 (Fig. 7A). The serum LH levels were similar at PW6 and PW12 (Fig. 7C), but the serum FSH level and FSH/LH ratio were increased significantly at PW12 ($P < 0.05$, $P < 0.01$, Fig. 7B and D), accompanied by decreased downstream ovarian *Fshr* mRNA and E_2 levels ($P < 0.01$, $P < 0.05$, Figs 6C and 7A). These results suggest that the HPO axis of female PEE offspring is activated in adulthood and participates in the negative feedback regulation of ovarian steroid hormone

synthesis, resulting in excessive release of FSH secreted by the anterior pituitary gland.

In vitro experiments

Effects of ethanol on KGN cell viability and E_2 production

To demonstrate the direct toxic effects of ethanol on ovarian granulosa cells, we performed *in vitro* experiments using the human KGN cell line. According to our previous study, the blood concentrations of PEE maternal and female foetal rats were 87 and 58 mM, respectively (Shen *et al.* 2014). Therefore, we treated the KGN cells with 15, 30, 60 and 120 mM ethanol. The MTS results showed that no cytotoxic effect in each concentration group after 72 h

Figure 7

Effects of prenatal ethanol exposure (PEE) on the hypothalamic-pituitary-ovarian (HPO) axis in female offspring rats after birth. (A) mRNA expression of HPO axis-related genes; (B, C and D) Serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) concentration, as well as FSH/LH concentration ratio. Mean $\pm$ S.E.M., $n = 8$. * $P < 0.05$, ** $P < 0.01$ vs control. FSHR, follicle-stimulating hormone receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GnRH, gonadotropin releasing hormone; KISS-1, Kisspeptin-1.

Figure 8

Effects of ethanol exposure on cell supernatant oestradiol (E_2) concentration, insulin-like growth factor 1 (IGF1) signalling pathway-related genes and steroidogenic enzymes in KGN cells. (A and B) E_2 concentration in cell supernatant; (C, D, E and F) mRNA expression of IGF1 signalling pathway-related genes; (G and H) mRNA expression of 3β -HSD and *P450arom*; (I and J) protein expression of IGF1 signalling pathway-related genes. Mean $\pm$ s.e.m., $n = 6$ for concentration and mRNA expression; $n = 3$ for protein expression. * $P < 0.05$, ** $P < 0.01$ vs control. 3β -HSD, 3β -hydroxysteroid dehydrogenase; AKT1/2, protein kinase B1/2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IGF1R, insulin-like growth factor 1 receptor; *P450arom*, cytochrome P450 aromatase; pAKT, phospho-AKT.

of ethanol treatment (data not shown). Furthermore, the E_2 levels increased or presented an increased trend after ethanol treatment at 120 mM for 24, 48 and 72 h ($P < 0.05$, $P = 0.06$, Fig. 8A). After treatment with four different concentrations of ethanol (15, 30, 60, 120 mM) for 48 h, the E_2 levels of the 60- and 120-mM ethanol groups were significantly elevated ($P < 0.01$, Fig. 8B) in a concentration-dependent manner. The results suggest that ethanol treatment does not affect the activity of KGN cells but promotes the E_2 synthesis.

Effects of ethanol on the IGF1 signalling pathway and steroidogenic enzymes in the KGN cells

We further explored the effects of ethanol treatment on KGN cells for 48 h and found that the mRNA expression of *IGF1* in the 15- and 120-mM ethanol groups ($P < 0.05$, $P < 0.01$, Fig. 8C), as well as that of *AKT2* ($P < 0.01$, Fig. 8F) and 3β -HSD ($P < 0.05$, Fig. 8G) in the 60- and 120-mM ethanol groups,

was increased, and the mRNA expression levels of *P450arom* in the 15-, 60- and 120-mM ethanol groups were also increased compared with that of the control ($P < 0.05$, $P < 0.01$, Fig. 8H). Next, the expression levels of IGF1 signalling pathway components and several steroidogenic enzymes showed concentration-dependent upregulation, corresponding to the above change in the E_2 levels (Fig. 8B). The protein expression of IGF1 in the 60- and 120-mM ethanol groups ($P < 0.05$, Fig. 8I) and pAKT/AKT in the 120-mM ethanol group ($P < 0.05$, Fig. 8J) were significantly elevated. These results suggest that ethanol can upregulate the IGF1 signalling pathway and E_2 synthesis in KGN cells.

Effects of ethanol on the IGF1 signalling pathway and E_2 synthesis in KGN cells transfected with IGF1 siRNA

We used IGF1 siRNA to verify whether IGF1 was involved in the KGN cell dysfunction by ethanol exposure. The results showed that the IGF1 mRNA and protein

expression levels were significantly decreased after IGF1 siRNA treatment compared with those in the NC siRNA group ($P < 0.01$, Fig. 9A and G), confirming the silencing efficiency. Additionally, the mRNA expression of downstream AKT2 in the IGF1 siRNA plus ethanol group was significantly lower than that of the NC siRNA plus ethanol group ($P < 0.01$, Fig. 9D). The protein expression levels of IGF1 and pAKT/AKT in the IGF1 siRNA plus ethanol group were significantly lower than those of the NC siRNA plus ethanol group ($P < 0.01$, Fig. 9G and H). The mRNA expression of 3β -HSD and $P450arom$, as well as the E_2 levels of the IGF1 siRNA plus ethanol group, were lower than those of the NC siRNA plus ethanol treatment group ($P < 0.01$, $P < 0.05$, Fig. 9E, F and I). These results

suggest that IGF1 participates in the ethanol-induced activation of the IGF1 signalling pathway and steroid synthetic function in KGN cells.

Discussion

Susceptibility to POI induced by PEE

The features of the POI animal model include the following (Zhang *et al.* 2016, Vabre *et al.* 2017): (1) a shortened, prolonged or stopped estrous cycle (Bernstein *et al.* 2014, Cruz *et al.* 2017); (2) increased FSH and/or LH levels, and decreased E_2 levels; (3) a decreased number of ovarian follicles and CL, a decreased ovulation rate and increased

Figure 9

Effects of 120 mM ethanol exposure for 48 h on the insulin-like growth factor 1 (IGF1) signalling pathway-related genes and steroidogenic enzymes in KGN cells transfected with IGF1 siRNA. (A, B, C and D) mRNA expression of IGF1 signalling pathway-related genes; (E and F) mRNA expression of 3β -HSD and $P450arom$; (G and H) protein expression of IGF1 signalling pathway-related genes; (I) Oestradiol (E_2) concentration in cell supernatant. Mean $\pm$ s.e.m., $n = 6$ for concentration and mRNA expression; $n = 3$ for protein expression. $*P < 0.05$, $**P < 0.01$ vs negative control (NC); $\#P < 0.05$, $\##P < 0.01$ vs NC + ethanol. 3β -HSD, 3β -hydroxysteroid dehydrogenase; AKT1/2, protein kinase B1/2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IGF1R, insulin-like growth factor 1 receptor; $P450arom$, cytochrome P450 aromatase; pAKT, phospho-AKT.

atretic follicles and (4) declined fertility. Among them, the sex hormone levels, ovarian structure and function are the core indicators of the establishment of the POI model (Zhang *et al.* 2016). In this study, we observed the following changes in PEE female offspring rats: (1) estrous cycle disorder, characterized by a shortened oestrus; (2) an elevated serum FSH with decreased E_2 in adulthood; (3) a significantly increased proportion of atretic follicles in the ovary in adulthood. Thus, our results suggest that female PEE offspring have features similar to POI after birth.

Over-activation of the IGF1 signalling pathway mediates PEE-induced foetal ovarian development abnormalities

It was found that exposure to chromium in the middle and late stages of pregnancy increased the apoptosis of germ cells in female offspring, reduced the number of oocytes, and accelerated the breakdown of the germ cell nests and formation of primordial follicles, leading to POI (Sivakumar *et al.* 2014). The time point GD20 selected in this study was the time that the rats were in the process of primordial follicle formation, and the results showed that the local structure of the PEE foetal ovary shows a decreased number of oocytes, the expression of Ki67 reflecting cell proliferation is decreased, and the expression of cleaved-caspase 3 reflecting the cell apoptosis is increased.

Studies have shown that IGF1 is directly involved in ovarian folliculogenesis (Silva *et al.* 2009) and initiates the transformation of primordial follicles into primary follicles (Stubbs *et al.* 2013). In the present study, the IGF1 signalling pathway was over-activated, speculating that its activation accelerated the normal follicular growth rate. Related studies have also suggested that the establishment of follicular pool at birth in rats would be disturbed by a detrimental maternal environment (Guzman *et al.* 2014). Exposure to monoethylhexyl phthalate in mice during pregnancy causes early ovarian ageing in the F1 generation due to accelerated follicular recruitment, resulting in depletion of the primordial follicle pool (Moyer & Hixon 2012, Zhang *et al.* 2015). It was reported that the PI3K/AKT signalling pathway is involved in the regulation of primordial follicle survival and recruitment, and activation of this pathway leads to oocyte development and follicle activation (Zheng *et al.* 2012). In the studies of other organ development (such as the eyeball), increased exogenous IGF1 accelerates visual cortical development, promoting the early maturity of visual acuity (Ciucci *et al.* 2007). These results suggest that PEE causes abnormal

development of female offspring and interferes with the initial stage of folliculogenesis, which might be caused by excessive activation of the IGF1 signalling pathway.

IGF1 not only participates in follicular development but also promotes the expression of steroidogenic enzymes and affects ovarian oestrogen synthesis (Silva *et al.* 2009). Oestrogen is known to play an important role in ovarian function and follicle growth (Zhang *et al.* 2017), and the biosynthesis of ovarian steroid hormones requires the action of various hydroxylases and aromatase enzymes. We observed that the mRNA expression of 3β -HSD and 17β -HSD1 in the ovary of PEE foetal rats was significantly increased. However, the expression of 3β -HSD was significantly reduced from 15.5 days after mating and was sustained until after birth under physiological conditions (Dutta *et al.* 2014).

In this study, the expression levels of steroidogenic enzymes in the PEE fetuses were abnormally activated, but no significant increase was found in the foetal serum E_2 level. The cause might be that on the one hand, the expression of $P450arom$ (a key enzyme for E_2 synthesis) was not elevated; on the other hand, foetal hormones were from multiple sources, including local production, maternal blood circulation and placenta and interference in any part will affect the E_2 level (Dutta *et al.* 2014). Furthermore, we found that the expression levels of $IGF1$, $AKT2$, 3β -HSD and $P450arom$ were increased in a concentration-dependent manner with ethanol treatment, and the E_2 level in the high concentration of ethanol group was increased *in vitro*. After IGF1 siRNA treatment, the protein expression of pAKT/AKT was decreased under ethanol, and the downstream $P450arom$ mRNA level was relatively decreased, accompanied by a relative decrease in the E_2 level. Because the KGN cell line is a useful cell type to explore steroidogenesis, growth and apoptosis of granulosa cells, it was used to verify the effects of maternal simazine exposure on the activity and proliferation of granulosa cells of offspring (Park *et al.* 2014). In the present study, although some limitations exist in the species and exposure window in the *in vitro* experiment, the interference of ethanol on the function of granulosa cells could also be validated preliminarily to a certain extent. These results suggest that the excessive activation of the IGF1 signalling pathway induced by PEE promotes the expression of steroidogenic enzymes, and the activated steroidal synthetic system might induce the potentially disordered endocrine environment in the ovary, likely affecting the physiological development of the foetal ovary.

Alterations of the IGF1 signalling pathway and steroidogenic enzymes are involved in ovarian development abnormalities in PEE female offspring after birth

A comparative study of various POI animal models showed that the body weight gain rates of the model groups under some inducers were lower (Zhang *et al.* 2016). In the present study, the body weight gain rate of female PEE offspring rats at PW6 was significantly higher, and then gradually slowed down; at PW12, the body weight was significantly reduced, but the ovarian index was increased. Several studies have confirmed that IUGR offspring caused by adverse environment during pregnancy will have catch-up growth in the early postnatal period—that is, a state in which growth is accelerated due to nutritional status or environmental improvement after birth and is associated with increased levels of IGF1 *in vivo* (Jensen *et al.* 2015, Myrie *et al.* 2017). In the present study, the IGF1 signalling pathway was converted from the activated state at PW6 to relative inhibition at PW12. These results suggest that the changes in IGF1 signalling pathway expression are involved in the compensatory development in the early childhood of PEE offspring, and local development of ovary was faster than that of the whole body.

In recent years, many clinical studies have regarded the serum AMH level as a sensitive indicator of POI (Du *et al.* 2016). More primordial follicles will be recruited to growing follicles under a deficient AMH level, which will accelerate the depletion of the ovarian follicle pool; therefore, the POI women always have significantly lower AMH levels than healthy women (Kruszynska & Slowinska-Srzednicka 2017). Our IHC results showed that the number of AMH-positive antral follicles in the adult PEE female offspring was reduced, indicating that the local AMH expression in the ovary was inhibited to some extent. However, the serum AMH levels did not decrease significantly as expected at this time, probably due to the limited number of small antral follicles that express AMH (Anderson 2012). Furthermore, ovarian granulosa cells are important for the survival of oocytes (Wang *et al.* 2017b). We observed that the thicknesses of ovarian granulosa cells in the PEE female offspring were continuously thinned from puberty to adulthood, and this phenomenon was also observed in other POI animal models (Zhang *et al.* 2016). Additionally, E₂ is produced by follicle granulosa cells, promoting follicle development and the survival of granulosa cells by reducing apoptosis (Billig *et al.* 1993), and the IGF1 signalling pathway stimulates the production

of E₂ and increases the expression of P450arom mRNA in granulosa cells (Chabrolle *et al.* 2009, Mani *et al.* 2010). In this study, the atretic follicle ratio of PEE female offspring was increased significantly at PW12, while the serum E₂ levels and expression of ovarian steroidogenic enzymes were decreased. These results suggest that the decreased function of ovarian steroidogenic enzymes in adulthood mediates the low oestrogen synthesis, which might be related to changes in the IGF1 signalling pathway, while the decreased E₂ level further affects the survival of granulosa cells and induces the thinning of the granulosa cell layer.

HPO axis feedback regulates abnormal ovarian development in PEE female offspring

Regulation of the hypothalamus–pituitary axis also plays an important regulatory role in ovarian development. During the intrauterine period, the function of the HPO axis is not fully established. The maturity of the female reproductive function initiates from puberty, and the HPO axis is fully established at this moment to activate puberty (Sliwowska *et al.* 2016). Animal studies have confirmed that PEE can change the development and function of the HPO axis in offspring, delay the initiation of puberty, reduce the feedback secretion of gonadotropin on E₂ and lead to early estrous cycle disorder (Sliwowska *et al.* 2016). A disordered estrous cycle indicates that the normal

Figure 10

Intrauterine programming mechanism of susceptibility to premature ovarian insufficiency (POI) in adult female rat offspring induced by prenatal ethanol exposure. E₂, oestradiol; FSH, follicle-stimulating hormone; IGF1, insulin-like growth factor 1.

regulatory function state of the HPO axis is disturbed (Goldman *et al.* 2007), as observed in this study. Although the serum FSH level was significantly increased in adult PEE offspring, the serum E₂ level did not increase, and *Kiss1* and *GnRH* mRNA expression in the hypothalamus did not change significantly. Therefore, no feedback was observed at the hypothalamic level, indicating that negative feedback dysfunction occurred among the hypothalamus, pituitary and gonads, and the ovarian response to pituitary gonadotropin was impaired (Tsoulis *et al.* 2016) that, in turn, affected the normal reproductive endocrine function of the HPO axis. These results suggest that PEE can inhibit the establishment of the HPO axis function in the female offspring, indirectly enhancing the susceptibility to POI.

Conclusion

PEE could increase the susceptibility to adult POI in the female offspring rats, accompanied by abnormal ovarian structural and functional development. The mechanism may be related to over-activation of the intrauterine ovarian IGF1 signalling pathway and steroidal synthetase system induced by ethanol. These changes accelerate folliculogenesis, and affect the levels of steroid hormones, resulting in increased atretic follicles after birth (Fig. 10). This study provides some evidence to explore abnormal ovarian development and the intrauterine origin mechanism of POI in drinking during pregnancy, which has guiding significance to advocate the maintenance of healthy living habits in the gestational period.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0063>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

D X and H W designed the experiments; Y N, F L, G F, W Z and Y C carried out the experiments; F L and L P prepared the experimental animals; Y N, D X, J Y and H W analysed the experimental results; N Y and D X wrote the manuscript and F L, Y W, and G F revised the manuscript. All the work was performed under the guidance of J Y and H W.

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