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Intergenerational genetic programming mechanism and sex differences of the adrenal corticosterone synthesis dysfunction in offspring induced by prenatal ethanol exposure

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HIGHLIGHTS

- Low expression of P450scc mediates the inhibition of steroid hormone synthesis in PEE fetal adrenal glands.
- GR/SF1/HDAC1-regulated epigenetic changes mediate intrauterine inhibition of P450scc.
- PEE adult offspring shows sex differences in adrenal steroid hormone synthesis function injury.
- The H3K9ac of P450scc mediates the intergenerational inheritance of adrenal steroid hormone synthesis function abnormity in PEE adult offspring.

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ABSTRACT

We previously found that prenatal ethanol exposure (PEE) induced adrenal dysplasia in offspring, which was related to intrauterine maternal glucocorticoid overexposure. This study investigated the intergenerational genetic effect and sex differences of PEE-induced changes in the synthetic function of adrenal corticosterone in offspring, and to clarify the intrauterine origin programming mechanism. Wistar pregnant rats were gavaged with ethanol (4 g/kg bw/d) from gestation day (GD) 9–20, and F1 generation was born naturally. The F1 generation female rats in the PEE group were mated with normal male rats to produce F2 generation. Serum and adrenal glands of fetal rats and F1/F2 adult rats were collected at GD20 and postnatal week 28. PEE increased the serum corticosterone level, while diminishing the expression of adrenal steroid synthases of fetal rats. Moreover, PEE enhanced the mRNA expression of GR and HDAC1, but inhibited the mRNA expression of SF1 and reduced the H3K9ac level of P450scc in the fetal adrenal gland. In PEE adult offspring of F1 and F2 generation the serum corticosterone level, the H3K9ac level of P450scc and its expression were decreased in males but were increased in females. In NCI-H295R cells, cortisol reduced the production of endogenous cortisol, down-regulated SF1, and up-regulated HDAC1 expression by activating GR, and decreased H3K9ac level and expression of P450scc. In conclusion, PEE could induce adrenal dysplasia in offspring with sex differences and intergenerational genetic effects, and the adrenal insufficiency in male offspring was related to the induction of low functional genetic programming of P450scc by intrauterine high corticosterone through the GR/SF1/HDAC1 pathway.

Abbreviations: PEE, prenatal ethanol exposure; CORT, corticosterone; GR, glucocorticoid receptor; SF-1, steroidogenic factor-1; HDACs, histone deacetylases; StAR, steroidogenic acute regulatory protein; P450scc, cytochrome P450 cholesterol side chain cleavage enzyme; 3β-HSD, 3β-hydroxysteroid dehydrogenase; P450c11, steroid 11β-hydroxylase; P450c21, steroid 21-hydroxylase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IGF1, insulin-like growth factor 1; H3K9ac, histone 3 acetylated lysine 9; IgG, immunoglobulin G; IUGR, intrauterine growth retardation; GD, gestational day; PW, postnatal week.

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1. Introduction

Alcohol (ethanol) is one of the most widely abused substances in the world (Zimatkin et al., 2006). According to the clinical practice guidelines, including the National Institute for Health and Clinical Excellence (NICE) and some major scientific societies, pregnant women should not drink alcohol at any time during pregnancy (2011). However, it is estimated that 15–40 percent of women worldwide drink alcohol during pregnancy (Lamy and Thibaut, 2010; Lanting et al., 2015). According to a U.S. Center for Disease Control (CDC) surveillance report (2015–2017), the incidence of current drinking was 11.5 % and binge drinking in the past 30 days was 3.9 % in pregnant women between18–44 years old (Denny et al., 2019). Studies reported that alcohol consumption during pregnancy was associated with multiple developmental toxicities in offspring, including intrauterine growth retardation (IUGR) (Martinez-Galiano et al., 2019; Murphy et al., 2013). Our previous studies showed that prenatal ethanol exposure (PEE) can also directly inhibit the development and the synthesis of corticosterone (CORT) in fetal rats' adrenal glands (Huang et al., 2018). PEE caused low basal activity and high-stress sensitivity of male offspring rats' adrenal glands (Huang et al., 2015), which led to susceptibility to a variety of metabolic diseases in adult rats (Xia et al., 2014). However, the molecular mechanism of abnormal synthesis of fetal CORT caused by PEE has not yet been clarified.

It has been reported that there may be sex differences in developmental toxicity in offspring caused by prenatal ethanol exposure. Haley et al. studied cortisol levels in 5–7 month-old infants born to low-frequency drinking mothers during pregnancy and found no significant change in cortisol level in male babies, while cortisol level in female babies was significantly increased (Haley et al., 2006). This result indicated that PEE could cause dysfunction of CORT synthesis in infants and had sex differences. In addition, it has also been reported that prenatal ethanol, caffeine, and nicotine exposure can affect the development of offspring continuously into adulthood and can be inherited. Luo et al. (2014) and Kou et al. (2017) reported that prenatal caffeine and ethanol exposure changed the neuroendocrine metabolic processes related to the HPA axis in offspring rats through intrauterine high glucocorticoid (GC), and such GC-dependent phenotypic changes were inherited to the F2 generation (Kou et al., 2017; Luo et al., 2014). We also found that prenatal nicotine exposure caused chondrodysplasia in offspring rats (Xie et al., 2018) and abnormal glucose and lipid metabolism in rat liver (Hu et al., 2020b), and such injury phenotypes were transmitted to the F2 generation. These studies suggested that prenatal adverse environmental exposures, including PEE, had intergenerational genetic effects on offspring developmental toxicity. However, whether the PEE-induced adrenal developmental toxicity had intergenerational genetic effects and sex differences in adult offspring has not been reported yet.

Cortisol physiologically promotes fetal organ maturation and progeny growth. In the adrenal gland, cortisol is synthesised from cholesterol by a series of steroid hormone synthetases. Among them, the cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc) is one of the rate-limiting enzymes in this process (Han et al., 2014). It has been reported that the human P450scc gene is regulated by tissue-specific orphan nuclear receptor steroidogenic factor 1 (SF1) (Gizard et al., 2002). SF1 in the adrenal gland increases the activity of the P450scc promoter and induces its expression (Monte et al., 1998). Our previous study showed that PEE inhibited the expression of SF1 and P450scc in adrenal glands of male fetal rats, and decreased CORT levels (Huang et al., 2015). These results suggested that PEE might inhibit the synthesis of steroid hormones in offspring adrenal glands by inhibiting the expression of SF1and P450scc. “Intrauterine programming alteration” mean that the function and structure of an organ are permanently altered by adverse environmental effects early in life (Linner and Almgren, 2020). Epigenetic modifications mediate the effects of an adverse environment in early life on long-term health, resulting in continuous changes in mRNA expression and organ function (Tobi et al., 2018). The epigenetic modification and the expression change of specific genes play a central role in intrauterine programming (Goyal et al., 2019; Vaschetto and Editor, 2019). Studies have shown that arsenic exposure increased the histone 3 lysine 9 methylation 3 (H3K9me3) level of P450scc in rat testis, thereby inhibited the expression of P450scc and reduced testosterone levels (Alamdar et al., 2019). Given the important role of P450scc in adrenal steroid hormone synthesis, we hypothesized that epigenetic modification of P450scc might mediate adrenal developmental programming and intergenerational inheritance.

In this study, we aimed to observe the intergenerational genetic effects and sex differences of offspring adrenal dysplasia caused by PEE. Furthermore, we investigated that PEE regulated the expression of glucocorticoid receptor (GR), SF1, histone deacetylases (HDACs) and P450scc, and level of P450scc epigenetic modification. This study will be helpful to clarify the mechanism and biological significance of PEE-induced adrenal developmental toxicity and to provide a theoretical and experimental basis for the analysis of the origin of adrenal development in adult diseases.

2. Materials and methods

2.1. Chemicals and reagents

Ethanol (1000918) and isoflurane (21022101) were supplied by Zhen Xin Co., Ltd. (Shanghai, China) and Baxter Healthcare Co. (Deerfield, IL, USA) respectively. Rat CORT ELISA kit (61K080) and human cortisol ELISA kit (RE52061) were purchased from Assaypro LLC. (Saint Charles, Missouri, USA) and ILB International GmbH (Hamburg, Germany) severally. The antibody of P450scc (A16363) and anti-H3K9 acetylation (H3K9ac) (A7255) was obtained from Abclonal Technology Co., Ltd. (Wuhan, China). Antibodies such as immunoglobulin G (IgG) (ab172730) were bought from Abcam Technology Co., Ltd. (Cambridge, UK). Reverse transcription kit (DRR047A) and real-time quantitative polymerase chain reaction (RT-qPCR) kit (Q223) were supplied by Takara Biotechnology Co., Ltd. (Dalian, China). Cytotoxicity assay MTS kit (PH1700) was purchased from Cayman Chemical Co. (Ann Arbor, Michigan, USA). DNA purification kit (Q5314) was bought from TIANGEN Biotech Co., Ltd. (Beijing, China). Proteinase K (ST533) and mifepristone (RU486) (ODR4395) were purchased from Kori Biotech Co., Ltd. (Wuhan, China). SF1 overexpressed plasmid pcDNA3.1(+)(Y8655) and HDAC1 siRNA were ordered from Biosci Biotech Co., Ltd. (Wuhan, China). All chemicals and biological reagents were analytical grades.

2.2. Animals and treatments

Specific pathogen free (SPF) Wistar rats (No. 2018–2020, license number: SCXK (Hubei), certification number: 42000600002258) were provided by Hubei Provincial Center for Disease Control (Wuhan, China). The weight of male and female rats was 278 ± 22 *g*, and 205 ± 15 *g*, respectively. Animal experiments were carried out at the Animal Experiment Center of Wuhan University (Wuhan, China), an institution accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). The whole experimental scheme was approved by the Ethics Committee of Animal Experiment of School of Medicine, Wuhan University (License No: 14016). All

animal testing procedures followed the guidelines of the Chinese Animal Welfare Committee.

F0 generation: Wistar rats were placed in standard cages and given a normal diet after a week of acclimatization. Two female and one male rat were housed overnight. The appearance of the sperm on the vaginal smear was observed the next morning, and the day on confirmation of copulation was regarded as gestation day (GD) 0. In accordance with our previous study (Shen et al., 2014), the PEE pregnant rats (n = 20) were administrated ethanol intragastrically at 4 g/kg bw/d during GD9-20, and the control group (n = 20) were given the same amount of distilled water. On GD20, the pregnant rats were anesthetized with 3% isoflurane and euthanized. Pregnant rats were selected with 10–14 live fetuses from each group (n = 12 for each group). Blood samples were collected from the fetus and serum was separated after blood coagulation. All fetal adrenal glands were stripped, and five fetal adrenal glands were randomly collected from each group and were fixed in 4% paraformaldehyde (Aspen Tech, Inc., Beijing, China), paraffin-embedded, and sectioned. The remaining fetal adrenals collected from littermates were pooled, immediately frozen in liquid nitrogen, and stored at −80 °C for further analyses.

F1 generation: 8 pregnant rats delivered naturally at term. In order to balance the nutrition during lactation, the 1-week-old pups were screened according to the following principles: ①pregnant rats with litter size ≥10 were selected and the litter size of pups were normalized to 10; ②the control group keeps non-IUGR pups, the PEE group keeps IUGR pups; ③the ratio of male and female offspring was kept at 1:1. The rats were weaned at postnatal week 4 (PW4). One male and one female were randomly selected from each litter and all selected offspring rats were assigned to the control group and PEE group (n = 8) according to the prenatal exposure. At PW28, the offspring rats were euthanized with 3% isoflurane anesthesia after one night of fasting. Serum and adrenal samples were collected as described above.

F2 generation: At PW12, the F1 PEE females were mated with the control males (CE), while the control males were mated with control females (Control) to generate F2 offspring. There is no inbreeding. The euthanasia of the rat, and the fixation and collection of adrenal samples were similar to those performed on F1 offspring. Fig. 1 shows the flowchart of animal treatment.

2.3. Cell culture and treatment

NCI-H295R cells (ATCC, Manassas, VA) were cultured in DMEM medium (Gibco, NY, USA) containing 10 % fetal bovine serum (Gibco, NY, USA), penicillin G (100 U/mL), streptomycin (100 μg/mL) under 37℃ homothermic conditions with 5% CO_2. The cells were treated with cortisol (0, 150, 300, 600, and 1200 nM) or ethanol (0, 15, 30, and 60 mM) for 24 h, then harvested for cortisol concentration and gene expression analysis. The absorption intensity was measured at 490 nm with a microplate reader (TECAN, Australia) to evaluate the cytotoxicity of ethanol and cortisol on NCI-H295R cells detected by MTS.

Next, we treated NCI-H295R cells with 600 nM cortisol and 2.5 μM glucocorticoid receptor (GR) inhibitor RU486 for 24 h to confirm the inhibitory effects of cortisol were mediated by upregulating and activating GR. In addition, RNA interference technology was used to knockdown HDAC1. NCI-H295R cells were transiently transfected with the siRNAs targeting human HDAC1 (sequences: sense: 5′-GGAGAUCCCUAAUGAGCUUTT-3′; antisense: 5′-AAGCUCAUUAGGGAUCUC CTT-3′) or with nontargeting control siRNA using 10 μL of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) at a final concentration of 20 nM. After 12 h, the cells were treated with the fresh medium containing 600 nM cortisol. The cells were harvested 24 h later for further analysis. To overexpress SF1, NCI-H295R cells were transfected with 5 μg plasmid pcDNA3.1(+) vector in combination with 5 μL of Lipofectamine 3000 and 10 μL of P3000 (Invitrogen, Carlsbad, CA, USA). After 12 h, the cells were treated with cortisol as described above.

2.4. CORT or cortisol level measurement

The concentration of serum CORT, cortisol intracellular, or in the culture medium was measured by ELISA kit following the protocol of the manufacturer (He et al., 2016).

2.5. Immunohistochemistry (IHC) measurements

The IHC procedures were conducted by streptavidin-peroxidase (SP)-conjugation. The paraffin-embedded tissues were sectioned, then dewaxed with xylene and dehydrated with gradient ethanol. The tissues were sealed with sheep serum working fluid and then incubated overnight with diluted primary antibody (P450scc 1:100) at 4℃. Phosphate buffer saline (PBS) buffer was used as negative control instead of primary antibody. Biotin-labeled secondary antibodies were incubated at 37℃ for 30 min. After counterstaining with hematoxylin, dehydrating, and drying, then the tissue sections were sealed. All images were captured using an Olympus AH-2 Light Microscope (Olympus, Tokyo, Japan). The analysis of the digital images was performed using Olympus software (Olympus, Tokyo, Japan).

2.6. Total RNA extract and RT-qPCR assay

Trizol reagent was used to isolate total RNA. The concentration and purity of the isolated total RNA were determined by a

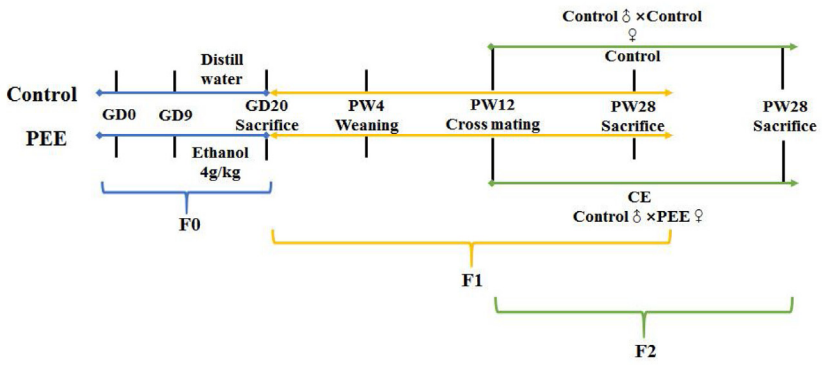

Fig. 1. Schematic illustration of animal treatment. PEE, prenatal ethanol exposure; GD, gestational day; PW, postnatal week; CE, the F1 control males mated with the F1 PEE females.

spectrophotometer (NanoDrop 2000C, Thermo), and the total RNA concentration was adjusted to 1 μg/μL. Total RNA was reverse-transcribed using a first-strand cDNA synthesis kit. cDNA was amplified using a one-step RT-qPCR reaction (Takara Biotechnology Co., Ltd., Dalian, China). Primers were designed using Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, CA), and the sequences of each of the designed primers were queried using the NCBI BLAST database for homology comparison. Primers of GR, SF1, HDAC1-10, steroidogenic acute regulatory protein (StAR), P450scc, 3β-hydroxysteroid dehydrogenase (3β-HSD), steroid 11β-hydroxylase (P450c11), steroid 21-hydroxylase (P450c21), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are presented in Table 1.

2.7. Chromatin immunoprecipitation-polymerase chain reaction (ChIP-PCR)

The homogenate of adrenal tissues or scraped cells was fixed with 1% formaldehyde for 15 min at 37°C to cross-link DNA and its associated proteins. The H3K9ac level of P450scc was detected following the protocol of the manufacturer (Hu et al., 2020c). The sequences of the primers spanning the P450scc binding region are as follows: Rat: TGAAGGGCAGTGGTCTTAGG (forward) and GCCCAAACGCAGAGAAAGAA (reverse), human: AAGCAGGAGAAT GGCTTGAA (forward) and AGGGCCAGGAATTCAAAACT (reverse).

2.8. Statistical analysis

SPSS 19 (SPSS Science Inc., Chicago, Illinois) and Prism 8.0 (Graph Pad Software, La Jolla, CA, USA) was used to perform data analysis. Quantitative data were expressed as mean ± S.E.M. Two-tailed Student's *t*-test was used for comparisons between control and treatment groups, and studies involving more than two groups, data were evaluated with one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. PEE decreased CORT level and the expression of steroid hormone synthases in F1 fetal adrenal glands

First of all, in the fetal adrenal glands of F1 generation rats, we observed the level of CORT and mRNA expression of the steroid hormone synthetase system including StAR, P450scc, 3β-HSD, P450c11, P450c21. As shown in Fig. 2, the content of CORT in the adrenal gland of both male and female fetal rats was significantly decreased in the PEE group ($P<0.05$, Fig. 2A, D). In PEE fetal rats, the mRNA expression of StAR, P450scc, 3β-HSD, and P450c21 in male and the mRNA expression of StAR, P450scc, 3β-HSD, P450c11, and P450c21 in female was significantly decreased ($P<0.05$, $P<0.01$, Fig. 2B, E). Meanwhile, PEE significantly inhibited the protein expression of P450scc in both male and female fetal adrenal glands ($P<0.01$, Fig. 2C, F). These results indicated that PEE could inhibit the adrenal expression levels of steroid hormone synthases system and CORT contents in both male and female fetal rats.

3.2. PEE regulates the expression of GR/SF1/HDAC1 in F1 fetal adrenal glands and inhibits histone acetylation of P450scc

Next, we investigated the possible mechanism of adrenal dysfunction in fetal rats induced by PEE. We found that the serum CORT level was significantly enhanced in both male and female PEE groups ($P < 0.05$, $P < 0.01$, Fig. 3A, D). The expression of adrenal GR was elevated, while the mRNA level of adrenal SF1 was significantly reduced in the PEE groups ($P < 0.05$, Fig. 3B, E). Moreover, the H3K9ac levels of P450scc in the fetal adrenal of the PEE group were lower than that of the control group ($P < 0.05$, $P < 0.01$, Fig. 3C, G). Since HDACs are the main enzymes for histone acetylation modification, we screened the expression of HDACs family at the mRNA level and found that the mRNA expression of HDAC1 in the adrenal gland of both male and female fetal rats was significantly increased ($P < 0.05$, $P < 0.01$, Fig. 3D, H). These results indicated that PEE increased the level of serum CORT and enhanced

Table 1
Oligonucleotide primers and PCR conditions for real-time quantitative PCR.

Species	Genes	Forward primer	Reverse primer	Annealing (°C)
Rat	GAPDH	GCAAGTTCAATGGCACAG	AAGTTCTTCCTGGCCGGTAT	63 (30 s)
	GR	CACCCATGACCCTGTCAGTC	AAAGCCTCCCTCTGCTAACC	63 (30 s)
	SF1	CCAGTACGGCAAGGAAGA	GAGGCTGAAGAGGATGAGGA	63 (30 s)
	StAR	GGGAGATGCCTGAGCAAAGC	GCTGGCGAACTCTATCTGGGT	60 (30 s)
	P450scc	GCTGCCTGGGATGTGATTTTC	GATGTTGGCCTGGATGTTCTTG	60 (30 s)
	3β-HSD	TCTACTGCAGCACAGTTGAC	ATACCCTTATTTTTGAGGGC	58 (30 s)
	P450c11	AGCAAGAACACACCACGTCT	GGAGGACACTCCACTTACAGC	60 (30 s)
	P450c21	AGCAAGAACACACCACGTCT	TGAGAGAGCACACTGACAGG	60 (30 s)
	HDAC1	TCTCCAAACACCACGTCACC	GTGTCCGTGTCCACAAGCAT	60 (30 s)
	HDAC2	CGCTCCTTTTCACTGCTTACG	ACAGGCAAGCCCATGAGTAG	60 (30 s)
	HDAC3	GGTTGCACCTGGTAAGTCCA	TGCAACTCTAGGGGTAGGGA	60 (30 s)
	HDAC4	GGGCCATTAGTGCAGACCAT	GGGATGCAGTTAGGTTGCCT	60 (30 s)
	HDAC5	TGGGGACACAGAGCCACTTA	AAGCTGATCCCAGAAGCATGG	60 (30 s)
	HDAC6	GCTCAGGTAAAGCCCACCAA	GGGATTGTCACAGGTTGCTTG	60 (30 s)
	HDAC7	ACCTGAGGGACAGTGAGCTA	GCCCCAGAGGATTCAGTAGT	60 (30 s)
	HDAC8	GCATTTACATTGTGGGGCCAG	TGTGCTACATGCGGGAACTT	60 (30 s)
	HDAC9	TTAGGTCCCCAGGCAGTTCT	ATCACAGCAGTTCAAGAACCAAA	60 (30 s)
	HDAC10	GCTCTGGGCACTCAATTTCG	TCTAGACAGAAGTAGGGGGCTG	60 (30 s)
Human	GAPDH	GCAACTAGGATGGTGTGGCT	TCCCATTCCCCAGCTCTCATA	60 (30 s)
	GR	AAGGTCTGCGCTACACAGTT	TGCCTTTCCCTGGAATTCTG	60 (30 s)
	SF1	GGCAGAGACAAAATCCATCCG	GTTTAGTTGTGGTCCGGCCT	60 (30 s)
	HDAC1	TCTCGAGCCTGAGAGGATGAA	TCAGGGCAAAAGTGCAGACA	60 (30 s)
	P450scc	TACTGAGGGTCTAGGAAGTGGT	ACCAGGAGGGACAGTACGTT	60 (30 s)

GR, glucocorticoid receptor; SF1, steroidogenic factor 1; HDAC, histone deacetylase; StAR, steroidogenic acute regulatory protein; P450scc, cytochrome P450 cholesterol side chain cleavage enzyme; 3β-HSD, 3β-hydroxysteroid dehydrogenase; P450c11, steroid 11β-hydroxylase; P450c21, steroid 21-hydroxylase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

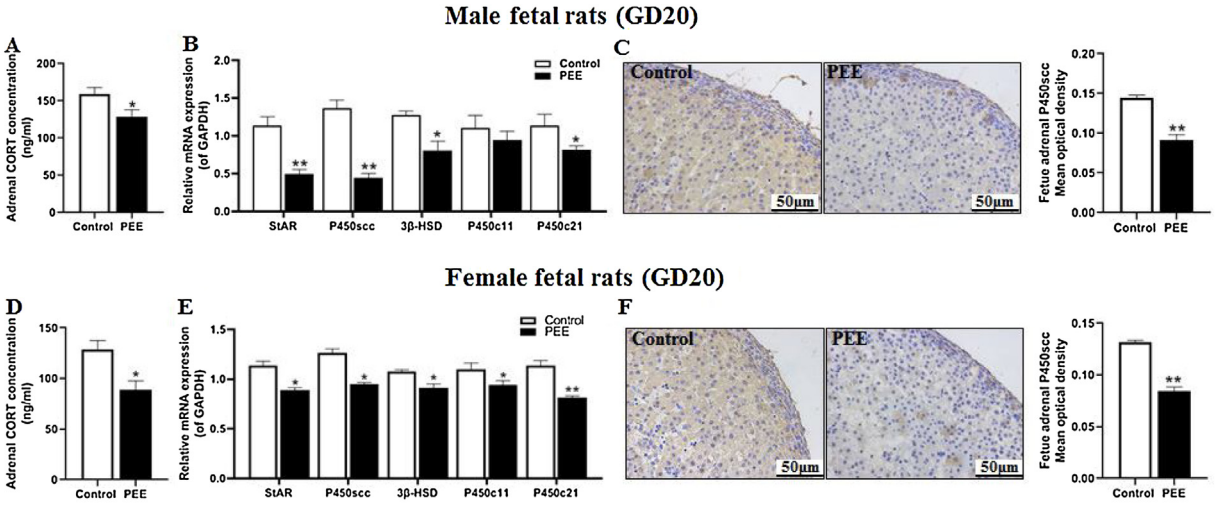

Fig. 2. Effects of PEE on adrenal CORT concentration and steroid synthases expression in male and female fetal rats at gestational day 20. (A, D) adrenal corticosterone concentration in females and males, $n = 6$. (B, E) StAR, P450scc, 3β-HSD, P450c11, and P450c21 mRNA expression in females and males, $n = 6$, six pairs of fetal adrenals from two littermates were pooled for homogenization into one sample. (C, E) P450scc protein expression (IHC staining, ×400) $n = 5$ (yellow represents P450scc positive cells), sections of each group were selected and five random fields of each section scored. *P* value was calculated by Independent Samples *t*-test. Mean ± S.E.M., $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control. PEE, prenatal ethanol exposure; CORT, corticosterone; StAR, steroidogenic acute regulatory protein; P450scc, cytochrome P450 cholesterol side-chain cleavage enzyme; 3β-HSD, 3β-hydroxysteroid dehydrogenase; P450c11, steroid 11β-hydroxylase; P450c21, steroid 21-hydroxylase.

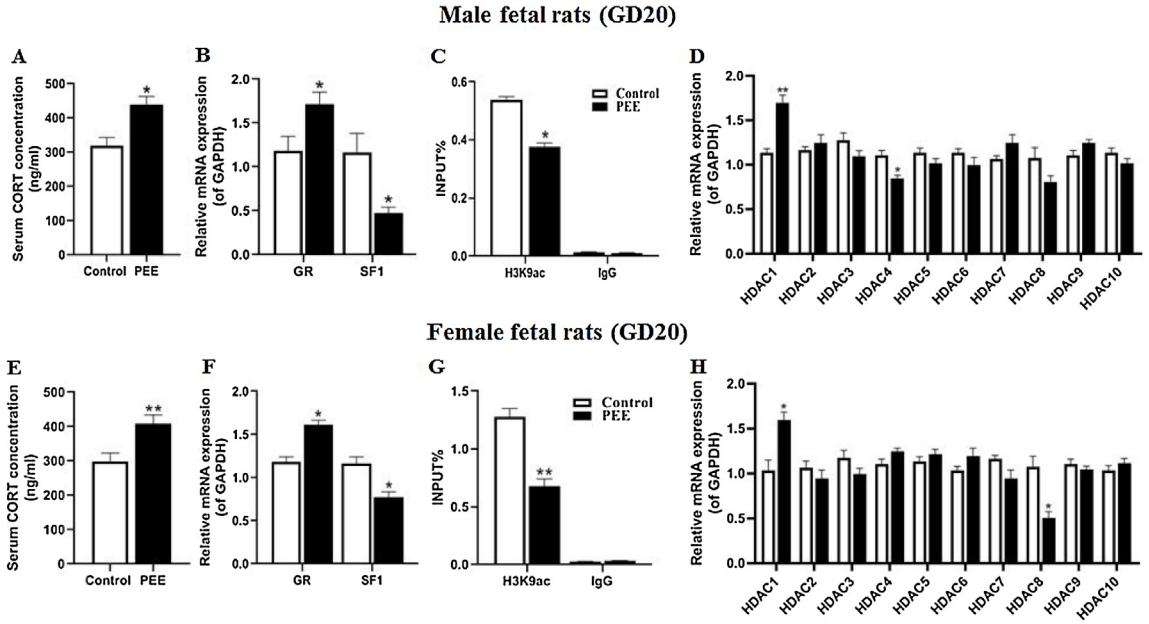

Fig. 3. Effects of PEE on serum CORT concentration, GR, SF1, HDACs expression, and H3K9ac level of P450scc in male and female fetal rats at gestational day 20. (A, D) serum CORT concentration in males and females, $n = 12$. (B, D, F, H) GR, SF1, and HDACs mRNA expression in males and females, $n = 6$, six pairs of fetal adrenals from two littermates were pooled for homogenization into one sample. (C, G) H3K9ac level of P450scc in male and female fetal rats, $n = 3$. *P* value was calculated by Independent Samples *t*-test. Mean ± S.E.M., $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control. PEE, prenatal ethanol exposure; CORT, corticosterone; GR, glucocorticoid receptor; SF1, steroidogenic factor 1; HDAC, histone deacetylase; H3K9ac, histone 3 acetylated lysine 9.

the expression of adrenal GR and HDAC1, while reducing the expression of SF1 and the H3K9ac level of P450scc in both male and female fetal rats.

3.3. Effects of PEE on serum CORT level, adrenal P450scc expression, and histone acetylation level of P450scc in F1 and F2 generation adult rats

In order to observe the effects of PEE on the adrenal CORT synthesis function of adult offspring and the intergenerational genetic phenomenon, we detected the level of serum CORT and mRNA expression of steroid hormone synthase system in male and female adult offspring of F1 and F2 generation. The results showed that compared with the control group, the serum CORT levels of male F1 and F2 generations in the PEE group were decreased ($P < 0.05$, $P < 0.01$, Fig. 4A, E). The mRNA expression of adrenal StAR, P450scc, and P450c21 in the F1 generation and adrenal P450scc, 3β-HSD, and P450c21 in the F2 generation was significantly decreased in the PEE group ($P < 0.05$, $P < 0.01$, Fig. 4B, F). Immunohistochemical results showed that adrenal protein

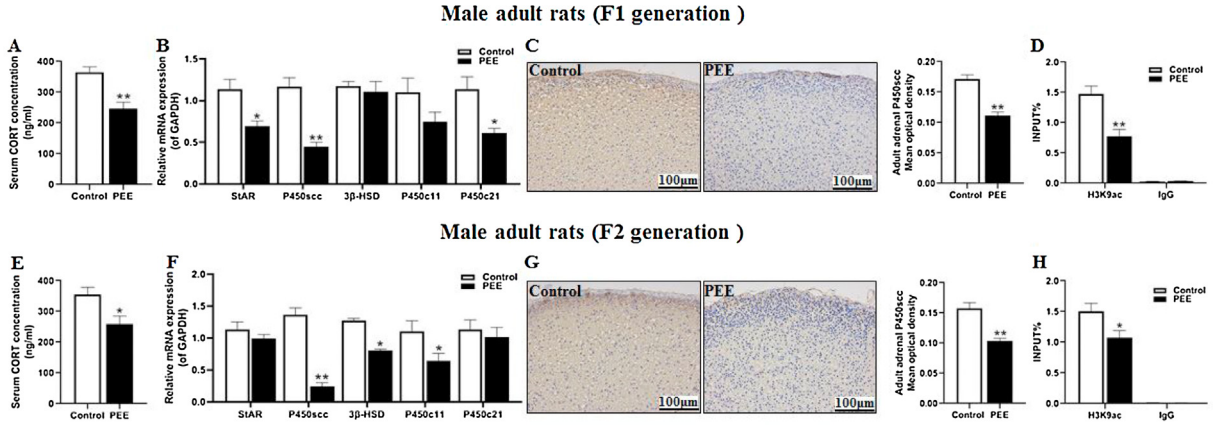

Fig. 4. Effects of PEE on serum CORT concentration, steroid synthases expression, and H3K9ac level of P450scc in male F1 and F2 generation rats at postnatal week 28. (A, D) serum CORT concentration in males, $n = 12$. (B, F) StAR, P450scc, 3β-HSD, P450c11, and P450c21 mRNA expression in males, $n = 8$. (C, G) P450scc protein expression (immuohistochemical staining, ×200), $n = 5$ (yellow represents P450scc positive cells), sections of each group were selected and five random fields of each section scored. (D, H) H3K9ac level of P450scc in F1 and F2 male rats, $n = 3$. P value was calculated by Independent Samples t-test. Mean ± S.E.M., $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control. PEE, prenatal ethanol exposure; CORT, corticosterone; StAR, steroidogenic acute regulatory protein; P450scc, cytochrome P450 cholesterol side-chain cleavage enzyme; 3β-HSD, 3β-hydroxysteroid dehydrogenase; P450c11, steroid 11β-hydroxylase; P450c21, steroid 21-hydroxylase; H3K9ac, histone 3 acetylated lysine 9.

expression of P450scc was significantly decreased in PEE male F1 and F2 generation rats ($P < 0.01$, Fig. 4C, G). ChIP-PCR results showed that PEE reduced the H3K9ac level of P450scc of male F1 and F2 generation rats ($P < 0.05$, $P < 0.01$, Fig. 4D, G). These results indicated that PEE could reduce the H3K9ac level of adrenal P450scc and its expression in the male adult F1 and F2 generation rats, and inhibit the function of CORT synthesis (Fig. 4).

We also observed the effect of PEE on adrenal CORT synthesis and the intergenerational genetic phenomenon in female offspring. In female F1 and F2 generation, compared with the control group, the serum CORT levels offspring were increased in the PEE group ($P < 0.05$, Fig. 5A, E). The mRNA expression of adrenal StAR, P450scc, and 3β-HSD in the F1 generation and adrenal P450scc, 3β-HSD, and P450c11 in the F2 generation was significantly increased in the PEE group ($P < 0.05$, $P < 0.01$, Fig. 5B, F). Immunohistochemical results showed that adrenal protein expression of P450scc was significantly decreased in PEE female F1 and F2 generation rats ($P < 0.01$, Fig. 5C, G). ChIP-PCR results showed that PEE increased the H3K9ac level of P450scc of female F1 and F2 generation rats ($P < 0.05$, $P < 0.01$, Fig. 5D, H). These results indicated that PEE could induce the H3K9ac level of adrenal P450scc and its expression in the female adult F1 and F2 generation rats, and promote the function of CORT synthesis.

3.4. Effects of ethanol and CORT on cortisol level and expression of P450scc in NCI-H295R cells

In order to investigate whether the inhibitory effects of PEE on fetal CORT synthesis were caused by ethanol or cortisol, we conducted cytotoxicity, P450scc expression, and cortisol synthesis tests on NCI-H295R cells. NCI-H295R cells treated with ethanol (0, 15, 30, 60 mM) and cortisol (0, 150, 300, 600, 1200 nM) for 24 h showed no obvious cytotoxicity (Fig. 6A, D). Ethanol increased the mRNA expression of P450scc and cortisol production ($P<0.01$, Fig. 6B, C). In contrast, cortisol inhibited the mRNA expression P450scc and intracellular cortisol levels in a concentration-dependent manner ($P<0.05$, $P < 0.01$, Fig. 6E, F). These results suggested that CORT (rather than ethanol) was responsible for the inhibitory effect of PEE-induced fetal adrenal steroid synthesis.

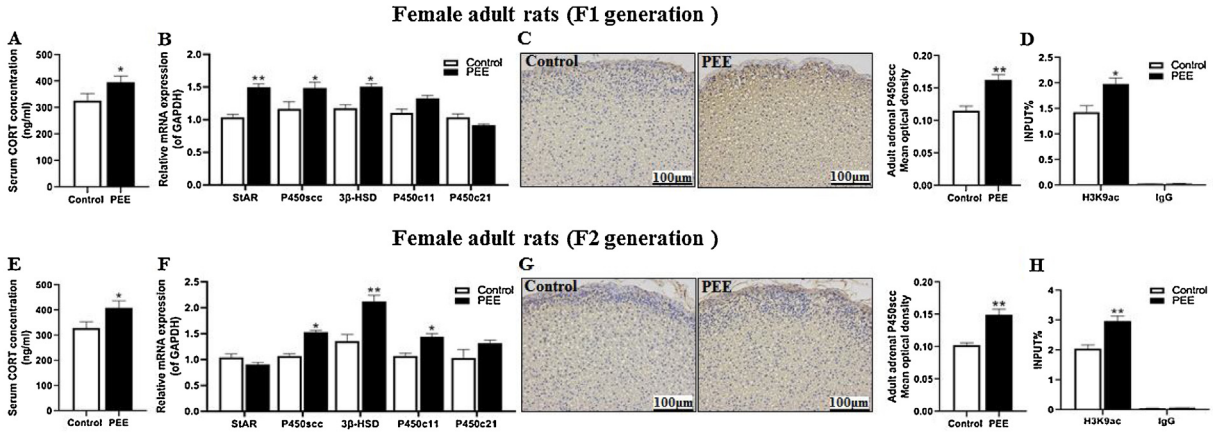

Fig. 5. Effects of PEE on serum CORT concentration, steroid synthases expression, and H3K9ac level of P450scc in female F1 and F2 generation rats at postnatal week 28. (A, D) serum CORT concentration in males, $n = 12$. (B, F) StAR, P450scc, 3β-HSD, P450c11, and P450c21 mRNA expression in males, $n = 8$. (C, G) P450scc protein expression (immunohistochemical staining, ×200), $n = 5$ (yellow represents P450scc positive cells), sections of each group were selected and five random fields of each section scored. (D, H) H3K9ac level of P450scc in F1 and F2 male rats, $n = 3$. P value was calculated by Independent Samples t-test. Mean ± S.E.M., $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control. PEE, prenatal ethanol exposure; CORT, corticosterone; StAR, steroidogenic acute regulatory protein; P450scc, cytochrome P450 cholesterol side-chain cleavage enzyme; 3β-HSD, 3β-hydroxysteroid dehydrogenase; P450c11, steroid 11β-hydroxylase; P450c21, steroid 21-hydroxylase; H3K9ac, histone 3 acetylated lysine 9.

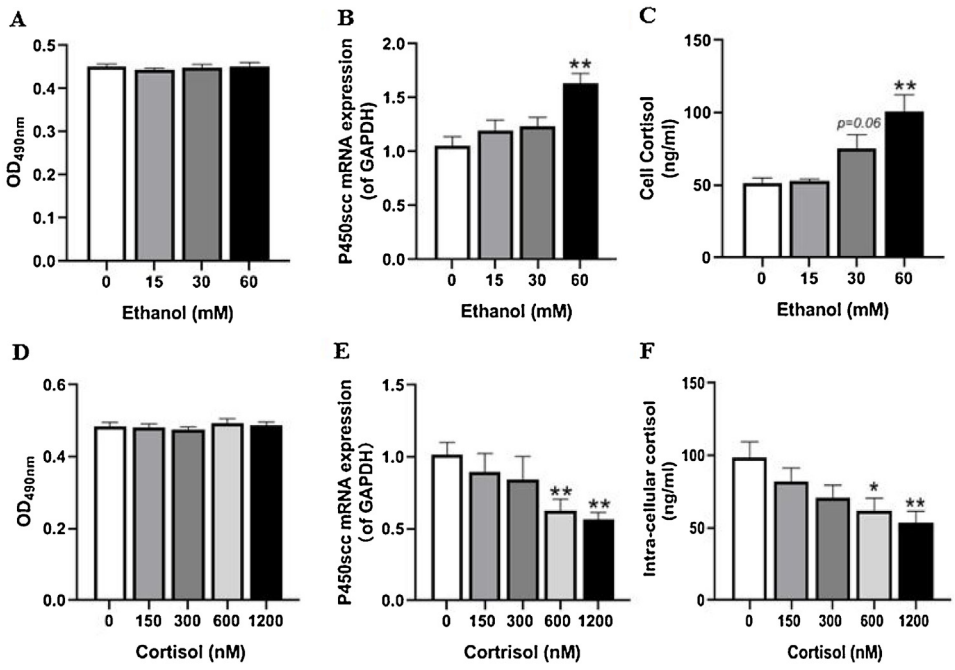

Fig. 6. Effects of ethanol and cortisol on cortisol production and P450scc expression in NCI-H295R cells. (A, D) cytotoxicity of ethanol and corticosterone by MTS assays, *n* = 6. (B, E) P450scc mRNA expression, *n* = 6. (C, F) cell cortisol and inter-cellar cortisol, *n* = 6. *P* value was calculated by ANOVA. Mean ± S.E.M., $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control. P450scc, cytochrome P450 cholesterol side-chain cleavage enzyme.

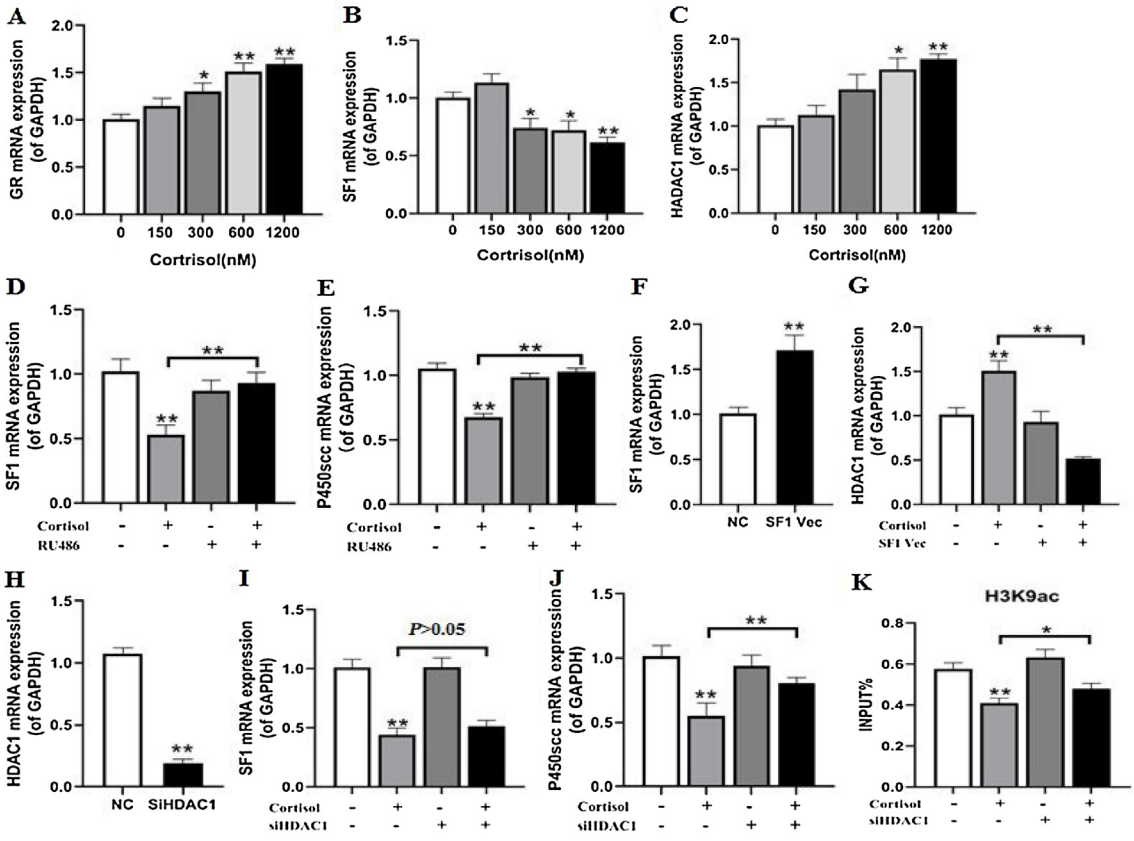

Fig. 7. Effects of RU486, HDAC2 siRNA, and SF1 pcDNA3.1(+) plasmid vector on the expression of SF1, HDAC1 and P450scc, and H3K9ac level of P450scc in NCI-H295R cells. (A) GR mRNA expression in NCI-H295R cells, n = 6. (B, D, E, I) SF1 mRNA expression in NCI-H295R cells, *n* = 6. (F, G, H) HDAC1 mRNA expression in NCI-H295R cells, *n* = 6. (C, J) P450scc mRNA expression in NCI-H295R cells, *n* = 6. (K) H3K9ac level of P450scc in NCI-H295R cells, *n* = 3. *P* value was calculated by Independent Samples *t*-test (F, H) and ANOVA(A-E, I-K). Mean ± S.E.M., $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* respective control. GR, glucocorticoid receptor; SF1, steroidogenic factor 1; HDAC, histone deacetylase; P450scc, cytochrome P450 cholesterol side-chain cleavage enzyme; H3K9ac, histone 3 acetylated lysine 9.

3.5. Influence of GR/SF1/HDAC1 signaling pathway on H3K9ac level and expression of P450scc

As mentioned above, *in vivo*, we found that PEE inhibited the H3K9ac level of P450scc and its expression by regulating GR/SF1/HDAC1 pathway in fetal adrenal glands. To further elucidate the molecular mechanism, we treated NCI-H295R cells with different concentrations of cortisol (0, 150, 300, 600, and 1200 nM) and observed the expression changes of these factors. We found that cortisol promoted the mRNA expression of GR and HDAC1 and inhibited the mRNA expression of SF1 in a concentration-dependent manner ($P < 0.05$, $P < 0.01$, Fig. 7A–C).

To verify the effect of GR on SF1 and P450scc, we investigated whether the inhibitory effect of cortisol on SF1 and P450scc was reversed by the GR inhibitor RU486 (2.5 μM) ($P < 0.01$, Fig. 7D, E). These results suggested and confirmed that it was GR mediated the inhibitory effect of cortisol on SF1 and P450scc. In addition, to verify the role of SF1, we overexpressed SF1 with SF1 pcDNA3.1(+) lipid vector. The results showed that the mRNA expression of SF1 was significantly increased after transfection with SF1 lipid vector ($P < 0.01$, Fig. 7F), and the overexpression of SF1 significantly attenuated the induction effect of cortisol on HDAC1 ($P < 0.01$, Fig. 7G). It was suggested that SF1 was involved in regulating HDAC1 expression. To further verify that H3K9ac of P450scc was regulated by HDAC1, we transfected HDAC1 siRNA into NCI-H295R cells. The results showed that HDAC1 siRNA could decrease the mRNA expression of HDAC1 ($P < 0.01$, Fig. 7H). Compared with the control, HDAC1 knockdown significantly ameliorated the mRNA expression and H3K9ac level of P450scc reduced by cortisol ($P < 0.05$, $P < 0.01$, Fig. 7J, K), but did not affect the expression of SF1 (Fig. 7I). These results suggested that cortisol reduced the H3K9ac level of P450acc and its expression through the GR/SF1/HDAC1 pathway.

4. Discussion

4.1. Low expression of P450scc mediates the inhibition of steroid hormone synthesis in PEE fetal adrenal glands

Alcohol consumption during pregnancy can cause fetal developmental toxicity and lead to birth defects and dysfunction, such as fetal alcohol syndrome (FAS) (Muggli et al., 2017; Popova et al., 2018). Epidemiological surveys showed that the daily alcohol consumption of the mothers whose children had FAS is approximately equivalent to 3.0–4.3 g/kg (Kwak et al., 2014), with blood ethanol concentrations ranging from 20 to 170 mM (Burd et al., 2012). Our previous studies showed that when ethanol intake reached 4 g/kg bw/d, the mean serum ethanol concentrations of maternal and fetal rats were 87 and 58 mM, respectively (Shen et al., 2014), which were close to the blood ethanol concentrations of human drinkers. Therefore, the amount of ethanol during pregnancy (4 g/kg bw/d) in this study had certain practical significance And we treated NCI-H295R cells with 15, 30, and 60 mM ethanol, which can reasonably cover the concentration of ethanol in fetal blood.

We previously showed that prenatal ethanol exposure increased maternal GC and induced damage to the placental barrier. Maternal GC transplacental entry into the fetus caused fetal rats overexposed to GC (Yu et al., 2018). And the high level GC inhibited the development of multiple organs, for example, hippocampus (Lu et al., 2018), testis (Liu et al., 2019), ovary (Ni et al., 2019), kidney (Ni et al., 2019), liver (Hu et al., 2020c), and cartilage (Ni et al., 2018). In addition, maternal GC overexposure can also affect the adrenal function of offspring. 4For example, PEE-induced maternal GC overexposure increased fetal blood CORT level, inhibited adrenal cortical cell proliferation and IGF1 signal, and decreased steroid synthesis (Huang et al., 2015, 2018). In this study, we found that the serum CORT level of PEE fetal rats was also increased, which was consistent with the above finding. *In vitro*, cortisol dose-dependently inhibited the expression of P450scc in NCI-H295R cells and reduced the level of endogenous cortisol. However, ethanol significantly increased the mRNA expression of P450scc and cortisol level in NCI-H295R cells. These results indicated that in the PEE rats model, CORT, rather than ethanol, reduced steroid production. And the maternal high GC caused by PEE induced the low expression of fetal adrenal P450scc, leading to the inhibition of steroid hormone synthesis in fetal adrenal glands.

4.2. GR/SF1/HDAC1-regulated epigenetic changes mediate intrauterine inhibition of P450scc

GC works by binding to intracellular GR. We previously reported that PEE induced maternally originated high level of GC in fetal blood and activated GR to induce abnormal development of fetal tissue, including testis (Liu et al., 2019) and liver (Hu et al., 2020b). In this study, PEE increased the GC level in fetal blood and induced the high expression and activation of GR in fetal adrenal glands. *In vitro*, the results also showed that cortisol increased the mRNA expression of GR in NCI-H295R cells and decreased the mRNA expression of SF1 and P450scc. We further demonstrated that the GR inhibitor RU486 reversed the inhibitory effect of cortisol on SF1 and P450scc. These results suggested that cortisol regulated the expression of SF1 and P450scc by activating GR. Studies showed that SF1 increased the expression of P450scc by binding to the reaction element sites in the promoter region of P450scc (Hu et al., 2004, 2001). In this study, PEE inhibited the expression of SF1 in fetal adrenal glands. At the cellular level, we also demonstrated that cortisol reduced the expression of SF1, and SF1 overexpression ameliorated the inhibitory effect of cortisol on P450scc. These results suggested that GC/GR down-regulates the expression of P450scc by inhibiting SF1.

Epigenetic modifications can be affected by adverse environmental factors in early life and permanently affect gene expression (Greco et al., 2011; Sahakian et al., 2017). H3K9 is an epigenetic marker of transcriptionally active chromatin (Qiao et al., 2015), and H3K9ac is closely associated with transcriptional activation (Oestreich and Moley, 2017). In this study, we found that the H3K9ac level of P450scc in PEE fetal adrenal was reduced, and similar results were also obtained in cortisol-treated NCI-H295R cells. Therefore, we proposed that inhibition of the H3K9ac level mediated the reduction of P450scc mRNA expression. Various epigenetic enzymes (including HDACs) are known to regulate epigenetic modification of P450scc. Therefore, we screened a series of HDACS expressions and found that the expression of HDAC1 in the fetal adrenal gland was increased in the PEE group. *In vitro*, we also found that cortisol promoted the mRNA expression of HDAC1, and HDAC1 knockdown ameliorated the inhibitory effect of cortisol on the H3K9ac level and expression of P450scc. In terms of the interaction between SF1 and HDAC1, our results showed that SF1 overexpression could mitigate the promoting effects of cortisol on HDAC1, while HDAC1 silencing did not affect SF1 expression. The results suggested that SF1 could affect HDAC1 expression. In conclusion, cortisol inhibited the H3K9ac level of P450scc through the GR/SF1/HDAC1 pathway, thus reduced the expression of P450scc.

4.3. Sex difference in injury to adrenal steroid hormone synthesis in PEE adult offspring

An increasing number of studies have shown that the programming changes of the key genes induced by PEE can lead to organ dysplasia and disease susceptibility in adult offspring

(Chen et al., 2020; Ni et al., 2019; Yu et al., 2020). As a key enzyme in the synthesis of steroid hormones, the expression changes of P450scc affect the function of adrenal steroid hormone synthesis. For example, arsenic exposure significantly inhibited the expression of P450scc in the testis, which in turn resulted in inhibition of testosterone synthesis in rats (Alamdar et al., 2019). We also found that the expression level of P450scc and the synthesis of CORT were decreased in PEE male fetuses and adult offspring. Therefore, we speculated that the low expression level of P450scc had a programming effect, which mediated the lower adrenal steroid hormone synthesis function in PEE male adult offspring. The sex-specific effects of alcohol intake during pregnancy on fetal development have attracted widespread attention. In this study, we found that the P450scc expression and serum CORT levels of F1 and F2 generations of PEE male adult offspring were decreased, while that of female offspring was increased. These results suggested that there were sex differences in the abnormal adrenal steroid hormone synthesis in PEE adult offspring.

In our previous series of studies, we propose "two-programming" mechanisms for PEE-induced developmental toxicity in offspring. "The first programming" is the low functional programming of key genes in tissues, that is, PEE causes the abnormal expression of key functional genes in tissues through the epigenetic mechanism from intrauterine to adulthood, thereby mediating multiple organ dysplasia and postnatal dysfunction. "The second programming" is the "GC-IGF1 axis" programming in which there is a negative correlation between blood GC level and tissue IGF1 expression. That is, maternal high GC caused by PEE inhibits the expression of the IGF1 pathway in tissues and participates in mediating tissue dysplasia. However, leaving the maternal high GC environment after birth, the IGF1 pathway is activated, which mediates "catch-up growth" and abnormal tissue function (Huang et al., 2015). Our previous studies also showed that developmental toxicity in male offspring was often associated with "the first programming", which was characterized by persistent low functional programming before and after birth (Liu et al., 2019; Lu et al., 2020). The "catch-up growth" is that IUGR fetuses accelerate at a high growth rate after a period of cause of growth retardation is removed, and then gradually decelerate until the original or normal growth channel is reached. Serum growth factors and genetically programmed cells are more likely to be involved in "catch-up growth" (Prader, 1978). And the "catch-up growth" of female offspring after birth is more obvious than the male offspring, and the mechanism is related to the "GC-IGF1 axis" programming change (Chen et al., 2020; Ni et al., 2019). In this study, we interestingly found that there were obvious sex differences in the aboid hormone synthesis of offspring caused by PEE. That is, the adrenal steroid hormone synthesis function of male offspring continued to be low before and after birth, and P450scc showed low functional programming. Conversely, in the female offspring, although the adrenal steroid hormone synthesis function was lower than normal prenatally. However, the female offspring showed "catch-up growth" after birth, and the expression of P450scc was also gradually increased after birth. The expression of P450scc in the PEE group was higher than that of the control group starting from PW12 and continued until PW28 (see Supplementary data). These sex differences persisted to the F2 generation. Although the mechanism of sex difference remains to be further elucidated, we hypothesized that the up-regulation of P450scc expression and steroid hormone synthesis function in the adrenal gland after the birth of female offspring may be related to the activation and regulation of the IGF1 axis.

We previously found that PEE can affect the development of the rat offspring's liver and pancreas by changing baseline CORT levels, which in turn affects glucose and lipid metabolism and showed sex differences(Hu et al., 2020c; Kou et al., 2017). PEE had long-term effects on the rat liver development and function of offspring, which was manifested that PEE reduced the level of blood CORT in F1 and F2 male adult offspring, resulting in the enhancement of hepatic lipid synthesis function. On contrast, PEE increased the level of blood CORT and inhibited the hepatic lipid synthesis in F1 and F2 female adult offspring (Hu et al., 2020c). PEE could also affect the rat pancreatic function of adult offspring, which showed that PEE decreased blood CORT, increased blood insulin level, and decreased blood glucose level in F1 and F2 male adult offspring (Kou et al., 2017; Xiao et al., 2019). The above results were consistent with the changes in blood GC levels of offspring rats in this study, that is, PEE resulted in a decrease in blood GC levels in male adult offspring, while an increase in blood GC levels in female adult offspring. In conclusion, PEE can affect the developmental programming and functional homeostasis of the offspring's adrenal glands, resulting in sex-specific changes in the blood GC levels of adult offspring. The sex-specific changes in the blood GC can cause GC-dependent changes in the glucose and lipid metabolism of the liver and pancreas, and ultimately lead to susceptibility to metabolic syndrome and related metabolic diseases (Hu et al., 2019; Shen et al., 2014).

4.4. The "contemporary programming" of P450scc mediates the intergenerational inheritance of adrenal steroid hormone synthesis function abnormality in PEE adult offspring

When maternal (F0) adverse environmental exposure induces consistent injury phenotypes in the F1 and F2 generations, this effect is called intergenerational inheritance (Fernandez-Twinn et al., 2015). For example, our study reported that prenatal dexamethasone exposure induced temporal lobe epilepsy (TLE) in the male F1 generation, which was inherited by the F2 generation (Hu et al., 2020a). Moreover, prenatal nicotine exposure caused chondrodysplasia in offspring (Xie et al., 2018) and abnormal liver glycolipid metabolism (Hu et al., 2020b) by inducing maternal GC overexposure, and could be transmitted to the F2 generation. In this study, the adrenal steroid hormone synthesis function and the expression of adrenal P450scc of F2 adult male and female offspring in the PEE group were decreased and increased, respectively and consistent with the performance of the F1 generation, which indicated that there was intergenerational heredity of abnormal steroid hormone synthesis function of offspring caused by PEE. Our latest research has found that the developmental toxicity in offspring adrenal glands caused by prenatal caffeine exposure has multi-generational genetic effects, and its mechanism may be related to the changes in the expression of "GC-dependent" steroid hormone synthase in the contemporary uterus (He et al., 2021). That is to say, contemporary fetal adrenal steroid hormone synthesis function is negatively correlated with intrauterine GC level. High intrauterine GC can inhibit the expression of contemporary fetal adrenal steroid hormone synthase. Conversely, if the intrauterine GC level is low, it will induce contemporary fetal adrenal steroid hormone synthase expression. In this study, the maternal high GC induced by PEE led to an increase in blood GC levels in F1 fetal rats, thereby inhibiting the development of the adrenal glands and the expression of P450scc in fetal rats. Since F1 males are mainly regulated by P450scc low-function programming, the adrenal function of F1 male rats before and after birth continues to be inhibited, and the level of blood GC decreases. In F1 female rats, due to "catch-up growth" after birth, blood GC increased at PW12 conception (see Supplementary data), which in turn induced an increase in blood GC levels in F2 fetal rats. The high GC environment in F2 fetal rats further inhibited the development of fetal adrenal glands and the expression of P450scc, resulting in the continuous suppression of P450scc expression in the adrenal glands of F2 male adult rats and

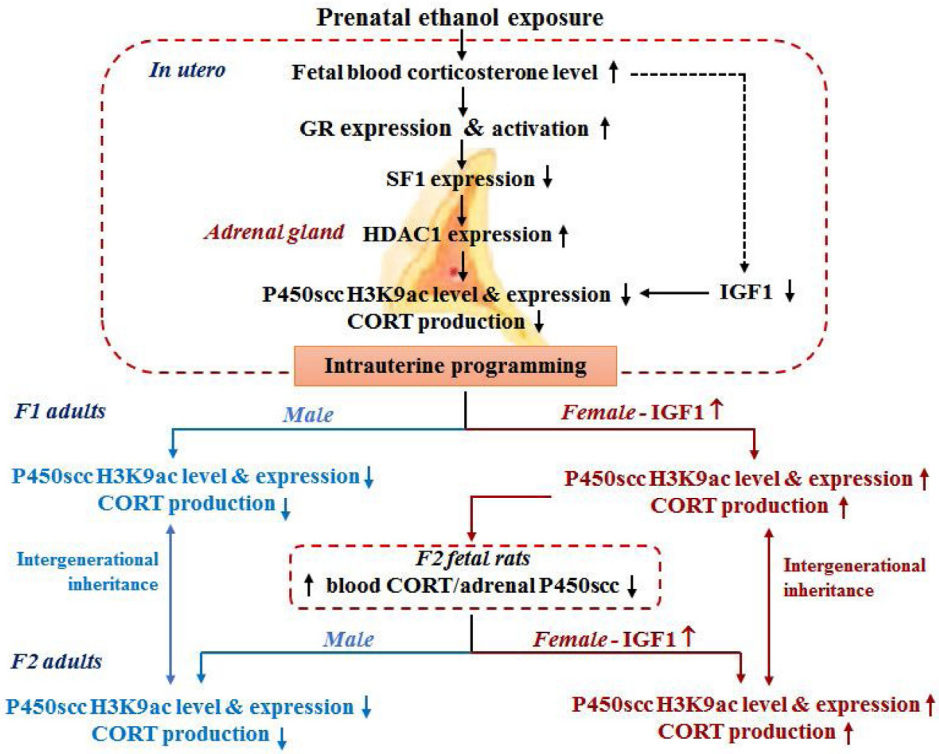

Fig. 8. Heritability and sex differences of adrenal dysplasia in adult offspring induced by prenatal ethanol exposure. GR, glucocorticoid receptor; SF1, steroidogenic factor-1; HDAC1, histone deacetylase 1; P450scc, cytochrome P450 cholesterol side-chain cleavage enzyme; H3K9ac, histone 3 acetylated lysine 9; IGF1: insulin-like growth factor 1.

the reduction of blood GC. In F2 female rats, due to "catch-up growth" after birth, the expression of P450scc in the adrenal glands increased, and the level of blood GC increased. Unfortunately, the F2 fetal rats were not collected previously, so our hypothesis cannot be confirmed at present. In conclusion, the intergenerational genetic phenomenon of steroid hormone synthesis function caused by PEE is related to the programming changes in P450scc expression probably caused by the "contemporary" maternal high GC levels in the uterus, while the sensitivity of male and female offspring to "two-programming" after birth leads to the sex difference of intergenerational inheritance (Fig. 8).

5. Conclusion

In conclusion, for the first time, this study shows that there are sex differences and intergenerational heredity of abnormal adrenal steroid hormone synthesis in PEE adult offspring, and the mechanism of intergenerational heredity was associated with maternal high GC mediated programming changes in P450scc expression. Intrauterine high GC induced by PEE caused changes in the GR/SF1/HDAC1 pathway in fetal rats, thereby reducing the H3K9ac level and expression of P450scc, and resulting in the decrease of steroid hormones synthesis in fetal adrenal glands. The low functional programming of P450scc continued to adult male offspring of F1 and F2 generation, resulting in low steroidal hormone synthesis. However, the adrenal glands of female adult F1 and F2 offspring showed high H3K9ac levels and expression of P450scc, leading to increased steroid hormone synthesis. This study provides a theoretical and experimental basis for clarifying the mechanism of PEE-induced adrenal dysfunction and analyzing the role of abnormal adrenal development in adult diseases.

Transparency document

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

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxlet.2021.08.007.

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