Original Paper

Effects and Interactions of Prenatal Ethanol Exposure, a Post-Weaning High-Fat Diet and Gender on Adult Hypercholesterolemia Occurrence in Offspring Rats

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Key Words

Hypercholesterolemia • Prenatal ethanol exposure • High-fat diet • Gender • Interaction • Intrauterine growth retardation

Abstract

Background/Aims: Prenatal ethanol exposure (PEE) could induce intrauterine programming of hypothalamic-pituitary-adrenal axis-associated neuroendocrine metabolism, resulting in intrauterine growth retardation and susceptibility to adult hypercholesterolemia in offspring. This study aimed to analyse the effects and interactions of PEE, a post-weaning high-fat diet (HFD) and gender on the occurrence of adult hypercholesterolemia in offspring rats. **Methods:** Wistar female rats were treated with ethanol (4 g/kg.d) at gestational days 11–20. The offspring were given a normal diet or HFD after weaning, and the blood cholesterol metabolism phenotype and expression of hepatic cholesterol metabolism related genes were detected in 24-week-old offspring. Furthermore, the interactions among PEE, HFD, and gender on hypercholesterolemia occurrence were analysed. **Results:** PEE increased the serum total cholesterol (TCH) and low-density lipoprotein-cholesterol (LDL-C) levels and decreased the serum high-density lipoprotein-cholesterol (HDL-C) level in adult offspring rats; the changes in female offspring were greater than those in males. At the same time, the mRNA expression levels of hepatic cholesterol metabolic enzymes (apolipoprotein B (ApoB) and 7 α -hydroxylase (CYP7A1))—were increased, while the mRNA expression levels of the scavenger receptor B1 (SR-B1) and LDL receptor (LDLR) were decreased. Furthermore, a three-way ANOVA showed there were interactions among PEE, post-weaning HFD and gender. For PEE offspring, a post-weaning HFD aggravated the elevated hepatic ApoB and CYP7A1 expression and reduced SR-B1 and LDLR expression; the changes in hepatic SR-B1 and CYP7A1 expression were greater

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in female HFD rats than in males. **Conclusion:** Our findings suggest that a post-weaning HFD could aggravate offspring hypercholesterolemia caused by PEE and that this mechanism might be associated with hepatic cholesterol metabolic disorders that are aggravated by a post-weaning HFD; hepatic cholesterol metabolism was more sensitive to neuroendocrine metabolic alterations by PEE and a post-weaning HFD in the female offspring than in the male offspring.

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Introduction

Hypercholesterolemia is a type of disease with the characteristics of increased levels of both total cholesterol (TCH) and low-density lipoprotein-cholesterol (LDL-C) and decreased levels of high-density lipoprotein-cholesterol (HDL-C). Additionally, hypercholesterolemia is an important risk factor for atherosclerosis and coronary heart disease [1]. Epidemiological studies have found that the incidence rate of hypercholesterolemia for females is higher than that for males [2, 3]. In recent years, intrauterine growth retardation (IUGR) and macrosomia have been linked to some adult onset diseases. Specifically, IUGR could be a potential cause of adult hypertension and chronic kidney disease, and the related potential mechanisms have been studied [4-6]; macrosomia can cause the development of childhood obesity and type 2 diabetes and/or cardiovascular diseases in the later stage of life [7]. Traditionally, the incidence of hypercholesterolemia is mainly related to the postnatal environment [8, 9]; however, studies have demonstrated that IUGR is also an independent risk factor for hypercholesterolemia [10, 11].

It is known that the liver is involved in cholesterol synthesis, output, reverse transport and transformation functions. Within these functions, 3-hydroxy-3-methyl-glutaryl -CoA reductase (HMGCR) is the rate-limiting enzyme of cholesterol synthesis, apolipoprotein B (ApoB) is involved in the output of hepatic cholesterol to the peripheral tissues, and scavenger receptor B1 (SR-B1) and LDL receptor (LDLR) both mediate the reverse cholesterol transport of hepatic cholesterol via HDL and LDL/very low-density lipoprotein (VLDL), respectively. 7 α -Hydroxylase (CYP7A1) is mainly responsible for the transformation of cholesterol into bile acid, which clears cholesterol away from the body [12]. Because the liver is the largest cholesterol metabolic organ, hepatic cholesterol metabolic disorders can directly cause blood cholesterol disorders and are involved in many types of cerebrovascular diseases. It has been reported that increased blood cholesterol level that both occurs after the birth of offspring, and is caused by an adverse prenatal environment may be related to the changes in hepatic cholesterol metabolism [10, 13, 14]. Therefore, the changes in hepatic cholesterol metabolism in IUGR offspring are worthy of great attention [10, 15].

Ethanol is a common ingredient in the diet of human beings and widely exists in soft drinks, food, toiletries, automobile exhaust, and so forth. Statistically, approximately 7% of pregnant women consume alcoholic beverages, and up to 50% of women within the reproductive age indulge in excessive drinking in some countries [16, 17]. Many studies have shown that prenatal ethanol exposure (PEE) can affect foetal development and cause adverse pregnancy outcomes, such as IUGR and foetal alcohol syndrome [18-20]. Our previous study also demonstrated that PEE can induce intrauterine programming of the hypothalamic-pituitary-adrenal (HPA) axis-associated neuroendocrine metabolism, resulting in IUGR and susceptibility to adult hypercholesterolemia in offspring rats [21].

It is known that the occurrence of foetal-derived metabolic diseases is influenced by multiple postnatal environmental factors, including a high-fat diet (HFD) and gender. Epidemiological investigations have shown that a HFD can increase susceptibility to lipid metabolic disorders and gender-related obesity, with females being more likely to be obese than males [22]. However, the effects and interactions of PEE, a post-weaning HFD and gender on adult hypercholesterolemia occurrence in offspring rats have not been reported until now. In the present study, first, the changes in the serum cholesterol phenotype and expression of hepatic cholesterol metabolic-related genes were detected in IUGR rats from the

PEE group, and then multivariate analysis and three-way ANOVA were employed to analyse the interactions of PEE, a post-weaning HFD and gender on serum cholesterol phenotype and hepatic cholesterol metabolic function. This study could provide theoretical support to reveal the influencing factors and their interactions on foetal-derived hypercholesterolemia.

Materials and Methods

Chemicals and reagents

Ethanol (analytical pure grade and chromatographic pure grade) was purchased from Zhen Xin Co., Ltd. (Shanghai, China) and Kemiou Chemical Reagent Co., Ltd. (Tianjin, China). Isoflurane was purchased from Baxter Healthcare Co. (Deerfield, IL, USA). TCH, LDL-C and HDL-C kits were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Reverse transcription (lot number: RR047A) and real-time quantitative PCR (RT-qPCR) (lot number: AK8301) kits were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). GeXP multiplex gene expression analysis kits were purchased from Beckman-Coulter Inc. (Fullerton, CA, USA). The oligonucleotide primers for rat RT-qPCR genes (PAGE purification) and GeXP multiplex gene expression analyses (HPLC purification) were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Other chemicals and agents were of analytical grade.

Animals and treatment

Specific pathogen-free Wistar rats (No. 2012-2014, certification number: 42000600002258, licence number: SCXK (Hubei)) weighing 209 ± 12 g (female) and 258 ± 17 g (male) were obtained from the Experimental Center of the Hubei Medical Scientific Academy (Wuhan, China). Animal experiments were performed at the Center for Animal Experiment of Wuhan University (Wuhan, China), which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). The Committee on the Ethics of Animal Experiments of the Wuhan University School of Medicine approved the protocol (permit number: 14016). All the animal experimental procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Chinese Animal Welfare Committee.

Animals were housed in metal cages with wire-mesh floors in an air-conditioned room under standard conditions (room temperature: 18–22°C; humidity: 40–60%; light cycle: 12-h light-dark cycle; 10–15 air changes per hour) and were allowed free access to rat chow and tap water. All the rats were acclimated one week before experimentation, and two female rats were placed together with one male rat overnight in a cage for mating. The appearance of sperm in vaginal smears confirmed mating, and the day of mating was taken as gestational day (GD) 0. Pregnant rats were transferred to individual cages and were randomly divided into the control and PEE groups ($n=8$ for each group). The PEE group was given 4 g/kg.d ethanol by gavage administration [23] from GD11 until term delivery (GD20-21), while the control group was given the same volume of distilled water. The pregnant rats were maintained until normal delivery. At parturition, the dams and their offspring were fed *ad libitum*. On postnatal day 1 (PD1), 8 pups from each litter were randomly selected to assure adequate and standardized nutrition until weaning [24]. At postnatal week 4 (PW4), 32 pups from 8 different mothers were randomly selected from each group (16 male and 16 female IUGR pups from the PEE group, 16 male and 16 female normal pups from the control group), and all the pups were fed a normal or HFD *ad libitum* before being sacrificed. The standard rodent chow purchased from the Experimental Centre of Hubei Medical Scientific Academy contained 21% kcal from protein, 68.5% kcal from carbohydrate and 10.5% kcal from fat. The HFD used previously reported by our laboratory [25] and contained 88.0% corn flour, 11.5% lard, and 0.5% cholesterol, which provided 18.9% kcal from protein, 61.7% kcal from carbohydrates and 19.4% kcal from fat. The groups were named as follows: MVN, male with vehicle and normal diet; MEN, male with PEE and normal diet; MVH, male with vehicle and HFD; MEH, male with PEE and HFD; FVN, female with vehicle and normal diet; FEN, female with PEE and normal diet; FVH, female with vehicle and HFD; and FEH, female with PEE and HFD.

At PW24, all the offspring rats were anaesthetized with isoflurane and decapitated in a room separate from where the other animals were kept. Trunk blood was collected, and serum was prepared by centrifugation at $17,205 \times g$ for 15 min at 4°C; the samples were then stored at -80°C until being used for the detection of the serum LDL-C and HDL-C concentrations. Livers from each group were immediately frozen in liquid nitrogen followed by storage at -80°C for subsequent analyses.

Analysis of serum samples

The concentrations of serum TCH, LDL-C and HDL-C were measured by their respective biochemical assay kits following each of the manufacturers' protocols.

Total RNA extraction, reverse transcription, and RT-qPCR

The mRNA expression of HMGCR, ApoB, SR-B1, LDLR, CYP7A1 and glyceraldehyde phosphate dehydrogenase (GAPDH) were analysed by RT-qPCR. Detailed protocols for RNA extraction and reverse transcription have been published elsewhere [18]. The primers and procedure of RT-qPCR are shown in Table 1.

RT-qPCR was performed using an ABI Step One RT-PCR thermal cycler (ABI Stepone, NY, USA) in a 10- μ L reaction mixture. To quantify the gene transcripts more precisely, the mRNA level of the housekeeping gene GAPDH was measured and used as a quantitative control. Each sample was normalized against the GAPDH mRNA content.

Statistical analysis

SPSS 18 (SPSS Science Inc., Chicago, Illinois) and Prism (GraphPad Software, La Jolla, CA, USA) were used for data analysis. All the data presented are expressed as the mean $\pm$ S.E.M. We used three-way ANOVA to analyse the effects and interactions among PEE, post-weaning HFD and gender. A Student's two-tailed *t*-test was used to compare the mean values of the various groups where applicable. Statistical significance was designated at $P < 0.05$.

Results

We analysed the four groups of rats according to the test substances and female and male diet as follows: ①The PEE effect under normal diet: M(F)VN-M(F)EN; ②The PEE effect under HFD: M(F)VH-M(F)EH; ③The HFD effect of control group: M(F)VN-M(F)VH; and ④The HFD effect of PEE group: M(F)EN-M(F)EH. At the same time, to simplify the description of the experimental results, we only described the positive results with significant changes here.

Changes in the serum cholesterol phenotype

In the male groups (Fig. 1A-1C): MVN vs. MEN: The concentrations of serum TCH and LDL-C in the MEN group were increased compared with those in the MVN group ($P < 0.01$); MVH vs. MEH: The concentration of serum TCH in the MEH group was increased compared with that in the MVH group ($P < 0.01$), and the serum HDL-C concentration was decreased ($P < 0.01$); MVN vs. MVH: The concentrations of serum TCH and LDL-C in the MVH group were increased compared with those in the MVN group ($P < 0.01$), and the concentration of HDL-C

Table 1. Primer sequences and the optimal real-time quantitative PCR conditions. HMGCR: 3-hydroxy-3-methyl-glutaryl-CoA reductase; ApoB: apolipoprotein B; SR-B1: scavenger receptor B1; LDLR: low-density lipoprotein receptor; CYP7A1: 7 α -hydroxylase; GAPDH: glyceraldehyde phosphate dehydrogenase

Gene	Forward primer	Reverse primer	Annealing
HMGCR	AGGTGAAGAGCTGGCTTGAA	GGATGTAGAGGTTGCGTCCT	63°C, 30 s
ApoB	ATGGTGCATGAGTATGCCAA	ATCCCACTTTTGGGAGGTTC	63°C, 30 s
SR-B1	GCTCACCTACCAGGAATCAA	CACAGCAATGGCAGGACTA	63°C, 30 s
LDLR	ATTGGCTATGAGTGCCTATGT	AGAGCAGAAACCCTATGGAA	60°C, 30 s
CYP7A1	CTGTGTTCACTTTCTGAAGCCATG	CCCAGGCATTGCTCTTTGAT	60°C, 30 s
GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA	60°C, 30 s

Fig. 1. Effects of prenatal ethanol exposure (PEE, 4 g/kg.d) on the concentrations of serum total cholesterol (TCH), high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) in the offspring rats. A: Male TCH; B: Male HDL-C; C: Male LDL-C; D: Female TCH; E: Female HDL-C; F: Female LDL-C. MVN, male with vehicle and normal diet; MEN, male with PEE and normal diet; MVH, male with vehicle and high-fat diet (HFD); MEH, male with PEE and HFD; FVN, female with vehicle and normal diet; FEN, female with PEE and normal diet; FVH, female with vehicle and HFD; FEH, female with PEE and HFD. Mean $\pm$ S.E.M, n=8. * P <0.05, ** P <0.01 vs. vehicle; # P <0.05, ## P <0.01 vs. normal diet. Significance values were calculated by Bonferroni corrected two-tailed Student's t -test.

was decreased compared with that in the MVN group (P <0.01); MEN vs. MEH: The concentrations of serum TCH and LDL-C in the MEH group were increased compared with those in the MEN group (P <0.01, P <0.05), and the concentration of serum HDL-C was decreased compared with that in the MEN group (P <0.05).

In the female groups (Fig. 1D-1F): FVN vs. FEN: The concentration of serum LDL-C in the FEN group was decreased compared with that in the FVN group (P <0.01); FVH vs. FEH: The concentration of serum TCH was increased in the FEH group compared with that in the FVH group (P <0.01); FVN vs. FVH: The concentrations of serum TCH and LDL-C were increased in the FVH group compared with those in the FVN group (P <0.01), while the concentration of HDL-C was decreased (P <0.01); FEN vs. FEH: The concentrations of serum TCH and LDL-C were increased in the FEH group compared with those in the FEN group (P <0.01), and the serum HDL-C concentration was decreased (P <0.01).

Changes in the hepatic cholesterol metabolic-related genes

In the male groups (Fig. 2A-2E): MVN vs. MEN: The hepatic ApoB mRNA level was increased in the MEN group compared with that in the MVN group (P <0.01); MVH vs. MEH: The hepatic CYP7A1 mRNA level was increased in the MEH group compared with that in the MVH group (P <0.05); MVN vs. MVH: The hepatic ApoB and SR-B1 mRNA levels were increased in the MVH group compared with those in the MVN group (P <0.01), while the LDLR mRNA level was decreased (P <0.01); MEN vs. MEH: The hepatic SR-B1 and CYP7A1 mRNA levels were increased in the MEH group compared with those in the MEN group (P <0.01), while the mRNA expression levels of HMGCR and LDLR were decreased (P <0.01).

In the female groups (Fig. 2F-2J): FVN vs. FEN: The hepatic LDLR mRNA level was decreased in the FEN group (P <0.05); FVH vs. FEH: The hepatic LDLR and CYP7A1 mRNA

Fig. 2. Effects of prenatal ethanol exposure (PEE, 4 g/kg.d) on the mRNA expression of hepatic cholesterol metabolic genes in offspring rats. A: Male 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR); B: Male apolipoprotein B (ApoB); C: Male scavenger receptorB1 (SR-B1); D: Male low-density lipoprotein receptor (LDLR); E: Male 7 α -Hydroxylase (CYP7A1); F: Female HMGCR; G: Female ApoB; H: Female SR-B1; I: Female LDLR; J: Female CYP7A1. MVN, male with vehicle and normal diet; MEN, male with PEE and normal diet; MVH, male with vehicle and high-fat diet (HFD); MEH, male with PEE and HFD; FVN, female with vehicle and normal diet; FEN, female with PEE and normal diet; FVH, female with vehicle and HFD; FEH, female with PEE and HFD. GAPDH, glyceraldehyde phosphate dehydrogenase. Mean $\pm$ S.E.M, n=8. * P <0.05, ** P <0.01 vs. vehicle; # P <0.05, ## P <0.01 vs. normal diet. Significance values were calculated by Bonferroni corrected two-tailed Student's t-test.

levels were increased in the FEH group compared with those in the FVH group (P <0.01); FVN vs. FVH: The hepatic HMGCR, ApoB, SR-B1 and CYP7A1 mRNA levels were increased in the FVH group compared with those in the FVN group (P <0.01, P <0.05), while the LDLR mRNA level was decreased (P <0.01); FEN vs. FEH: The hepatic ApoB, LDLR and CYP7A1 mRNA levels were increased in the FEH group compared with those in the FEN group (P <0.01).

Table 2. Three-way ANOVA of prenatal ethanol exposure (PEE), post-weaning high-fat diet (HFD) and gender for cholesterol metabolic-related factors. Total cholesterol (TCH); high-density lipoprotein-cholesterol (HDL-C); low-density lipoprotein-cholesterol (LDL-C); 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR); apolipoprotein B (ApoB); scavenger receptorB1 (SR-B1); low-density lipoprotein receptor (LDLR); 7 α -Hydroxylase (CYP7A1). Mean $\pm$ S.E.M., n=8. The cutoff F-value for the statistical significance at the level of P<0.05 is 4.010 and P<0.01 is 7.170, respectively. “/” means F-value is less than 4.010

Factors	Indexes							
	TCH	HDL-C	LDL-C	HMGCR	ApoB	SR-B1	LDLR	CYP7A1
PEE	P<0.05	P<0.01	P<0.01	P<0.1	P<0.05	P<0.05	P<0.01	P<0.01
	F=2.21	F=0.04	F=1.14	F=1.73	F=2.91	F=4.33	F=4.87	F=174.03
HFD	P<0.05	/	/	P<0.05	P<0.01	P<0.05	P<0.05	P<0.01
	F=0.29	F=40.77	F=10.32	F=1.56	F=103.97	F=243.13	F=127.87	F=154.04
Gender	/	P<0.05	P<0.05	/	/	P<0.01	P<0.05	P<0.01
	F=17.78	F=0.02	F=54.91	F=6.97	F=3.66	F=10.12	F=2.14	F=0.06
PEE*HFD	/	/	P<0.01	P<0.05	P<0.05	P<0.05	P<0.01	P<0.01
	F=0.01	F=0.08	F=1.72	F=3.89	F=9.24	F=11.94	F=14.89	F=261.02
Gender*PEE	/	/	/	/	/	P<0.05	/	P<0.01
	F=2.20	F=5.78	F=11.21	F=0.68	F=0.63	F=0.02	F=0.66	F=7.32
HFD*Gender	P<0.05	P<0.05	P<0.01	P<0.01	/	P<0.01	/	P<0.01
	F=2.67	F=0.71	F=13.99	F=28.59	F=0.87	F=8.29	F=19.71	F=187.01
PEE*HFD*Gender	/	/	/	/	P<0.01	/	P<0.01	/
	F=0.07	F=0.11	F=1.55	F=0.09	F=11.54	F=0.38	F=21.53	F=1.11

Interactions among PEE, post-weaning HFD and gender was analysed by three-way ANOVA

In this section, we analysed the interactions among PEE, HFD and gender based on three-way ANOVA, including single-factor, dual-factor and three-factor analyses (Table 2). Subsequently, we applied the interactive effect profiles to directly reflect the relationship between any two factors (Figs. 3 and 4), with larger acute angles of two intersecting lines indicating stronger interactive effects. If the two lines were close to parallel, there was no interactive effect. To simplify the results description, we only described some positive results here.

Interactions among PEE, post-weaning HFD and gender in the cholesterol phenotype

Regarding the concentration of serum TCH (Fig. 3A, Table 2), the results of the three-way ANOVA showed that, the level in the PEE group was increased compared with that in the vehicle (PEE $P<0.05$), and the level in the HFD group was increased significantly compared with that in the normal diet group (HFD $P<0.05$). Interactions existed between HFD and gender ($P<0.05$), and the specific manifestation was that PEE and HFD could both increase the serum TCH level. However, after HFD consumption, the degree of increase in females was more significant than that in males.

Regarding the concentration of serum HDL-C (Fig. 3B, Table 2), the level in the PEE group was significantly decreased compared with that in the control group (PEE $P<0.01$), and the level in females was higher than that in males (Gender, $P<0.01$). Interactions existed between HFD and gender ($P<0.05$), and the specific manifestation was that PEE and HFD could both decrease the serum HDL-C level. However, after HFD consumption, the degree of decrease in females was more significant than that in males.

Regarding the concentration of serum LDL-C (Fig. 3C, Table 2), the level in the PEE group was increased compared with that in the vehicle (PEE $P<0.01$), and the level in females was higher than that in males (Gender, $P<0.05$). Interactions existed between PEE*HFD and HFD*gender, and the specific manifestation was that HFD could aggravate the degree

Fig. 3. Interactional analysis among prenatal ethanol expression (PEE), post-weaning high-fat diet and gender on the concentrations of serum total cholesterol (TCH), high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) in the offspring rats. A: the interaction in serum TCH concentration; B: the interaction in serum HDL-C concentration; C: the interaction in serum LDL-C concentration. Mean $\pm$ S.E.M., $n=8$. A significant interaction between two factors $P < 0.05$ or $P < 0.01$.

of increase of the serum LDL-C level caused by PEE; after HFD consumption, the degree of increase in females was more significant than that in males.

Interactions among PEE, post-weaning HFD and gender in hepatic cholesterol metabolic-related genes

Regarding the hepatic HMGCR levels (Fig. 4A, Table 2), the results of three-way ANOVA showed that, the level in the PEE group exhibited an increasing trend compared with that in the vehicle (PEE $P < 0.1$), and the level in the HFD group was increased compared with that in the normal diet group (HFD $P < 0.05$). Interactions existed between PEE*HFD and HFD*gender ($P < 0.01$), and the specific manifestation was that PEE could increase the hepatic HMGCR level, while HFD could aggravate the increasing effect of PEE on HMGCR; after HFD consumption, the female HMGCR level increased more significantly.

Regarding hepatic ApoB (Fig. 4B, Table 2), the levels in the PEE group and HFD group were both significantly increased compared with those in the respective control groups (PEE $P < 0.05$; HFD $P < 0.01$). Interactions existed between PEE*HFD and PEE*HFD*gender ($P < 0.05$, $P < 0.01$); the specific manifestation was that PEE and HFD could both increase the hepatic ApoB level, while HFD could aggravate the increasing degree of the ApoB level caused by PEE. After HFD consumption, the degree of increase in females was more significant than that in males.

Fig. 4. Interaction analysis among prenatal ethanol expression (PEE), post-weaning high-fat diet and gender on the mRNA expression of hepatic cholesterol metabolic genes in the offspring rats. A: the interaction in HMG CoA reductase (HMGCR); B: the interaction in apolipoprotein B (ApoB); C: the interaction in scavenger receptor B1 (SR-B1); D: low-density lipoprotein receptor (LDLR); E: 7 α -Hydroxylase (CYP7A1). Mean $\pm$ S.E.M, n = 8. A significant interaction between two factors $P < 0.05$ or $P < 0.01$.

Regarding hepatic SR-B1 (Fig. 4C, Table 2), the levels in the PEE and HFD groups were both decreased compared with those in their respective control groups ($P < 0.05$), and the levels in females were lower than those in males (Gender, $P < 0.01$). Interactions existed among PEE*HFD, Gender*PEE and HFD*gender ($P < 0.01$, $P < 0.05$), and the specific manifestation was that HFD could aggravate the degree of decrease of the hepatic SR-B1 level caused by PEE; the degree of decrease in females was more significant than that in males.

Regarding hepatic LDLR (Fig. 4D, Table 2), the level in the PEE group was decreased compared with that in the vehicle group (PEE $P < 0.01$). Interactions existed between PEE*HFD and PEE*HFD*gender ($P < 0.01$), and the specific manifestation was that HFD could aggravate the decreasing degree of the hepatic LDLR levels caused by PEE; the degree of decrease in females was more significant than that in males.

Regarding hepatic CYP7A1 (Fig. 4E, Table 2), the levels in the PEE group and HFD group were both increased significantly compared with those in their respective control groups (PEE $P < 0.01$; HFD $P < 0.01$), and the levels in females were higher than those in males (Gender $P < 0.01$). Interactions existed among PEE*HFD, Gender*PEE and HFD*gender ($P < 0.01$), and the specific manifestation was that HFD could aggravate the increasing degree of the hepatic CYP7A1 level caused by PEE; the degree of increase in females was more significant than that in males.

Discussion

PEE offspring with a normal diet or post-weaning HFD displayed adult hypercholesterolemia

Epidemiological investigations have shown that IUGR is a specific and important inducer of metabolic disorders, such as obesity, diabetes and cardiac- and cerebrovascular diseases [26-28]. Animal experiments have also demonstrated that an adverse intrauterine environment could cause increased serum cholesterol levels and enhanced susceptibility to metabolic syndrome in adult IUGR offspring. For example, serum TCH concentrations are increased in offspring rats with PEE [21]. Similarly, serum TCH concentrations in prenatal food-restricted offspring are also increased, and even hepatic cholesterol accumulation has been found [10, 29]. Some epidemiological investigations have found that the incidence of serum cholesterol disorder is increased in 20-year-old adults with low birth weight and is accompanied by increased serum LDL-C and decreased HDL-C levels [30]. Animal experiments have demonstrated that high-caloric food and HFD after birth can increase the susceptibility to metabolic diseases, hypercholesterolemia and coronary atherosclerosis in IUGR offspring [31, 32].

Our previous study found that PEE can lead to foetal overexposure to maternal corticosterone [20] and altered HPA axis-related neuro endocrine metabolic programming, leading to glucose and lipid metabolic changes and increasing the susceptibility to metabolic syndrome after birth [18]. In the present study, we found that the serum TCH levels in IUGR offspring were increased when they were fed a normal diet or post-weaning HFD, indicating that the susceptibility of adult offspring to hypercholesterolemia might be caused by PEE.

Hypercholesterolemia in IUGR offspring by PEE is related to hepatic cholesterol metabolic disorders

IUGR has been widely accepted as a risk factor for cholesterol disorders, but its underlying mechanism remains unclear. An adverse prenatal environment probably causes serum cholesterol disorders of offspring by influencing hepatic cholesterol metabolism [11]. It was found that serum TCH was increased in IUGR offspring rats [14], expression levels of hepatic HMGCR and CYP7A1 were increased, and epigenetic modification changes in hypomethylation and hyperacetylation were found [13]. Also, mRNA expression of LDLR, which help carry cholesterol back to the liver from the peripheral circulation, was decreased [33]. These results indicated that the abnormal serum cholesterol phenotype of offspring rats caused by the adverse prenatal environment was related to the changes in hepatic cholesterol metabolism. Some other researchers have found that exogenous glucocorticoid (corticosterone) can significantly promote HMGCR expression [34], indicating that glucocorticoid may be related to changes in hepatic cholesterol metabolism.

In this study, we found that mRNA expression levels of hepatic HMGCR and ApoB were increased under a normal diet, while those of SR-B1 and LDLR were decreased in the PEE group. This finding indicates that hypercholesterolemia in adult offspring with PEE

is probably related to the increased hepatic cholesterol synthetic function and decreased reverse-transport function, and the neuroendocrine metabolic programming caused by foetal overexposure to maternal glucocorticoid might mediate these processes.

Interactions exist among PEE, post-weaning HFD, and gender regarding the cholesterol phenotype and metabolism

The sex-specific differences in animal models of developmental programming have been well described in the literature [35], and it has been reported that ethanol or HFD can independently affect cholesterol metabolism with gender differences [36-38]. However, these studies are not associated with PEE, and there is still no related research concerning the interactions among PEE, post-weaning HFD, and gender. In this study, we used three-way ANOVA to analyse the interactions among PEE, post-weaning HFD, and gender. These results showed several interactions among these three factors in hepatic ApoB and LDLR expression (Table 2), indicating that PEE and post-weaning HFD might influence the hepatic cholesterol metabolism of PEE offspring in a gender-specific manner.

First, the interactions between PEE and post-weaning HFD were observed in the serum LDL-C concentration and in the hepatic HMGCR, ApoB, SR-B1, LDLR and CYP7A1 expression (see table 2). Post-weaning HFD could aggravate the increase in the serum LDL concentration as well as in hepatic HMGCR, ApoB and CYP7A1 expression caused by PEE and further inhibit the hepatic LDLR and SR-B1 expression. This phenomenon might be associated with HFD, directly leading to hepatic cholesterol metabolic disorders [39]. On the other hand, our previous research demonstrated that the neuroendocrine metabolic programming caused by PEE could be aggravated by post-weaning HFD, which increased the susceptibility to metabolic syndrome and non-alcoholic fatty liver disease (NAFLD) in adult offspring [18]. Combined with the results of this study, post-weaning HFD could aggravate PEE-induced hypercholesterolemia and hepatic cholesterol metabolic disorders in adult IUGR offspring rats.

Second, we also found interactions between PEE and gender in hepatic SR-B1 and CYP7A1 expression (see table 2). The specific manifestation was that the decrease in hepatic SR-B1 expression and increase in CYP7A1 expression in the female PEE group were more obvious than those in the male PEE group. These results indicate that the hepatic cholesterol metabolism of female offspring may be easily affected by PEE. This finding is similar to the results of several epidemiological studies, with the most classic cases being those of the Netherland and Chinese famines [40, 41]. The intrauterine nutrition restriction caused by the famine involved in the studies was similar to PEE, and all of these adverse prenatal environments could lead to IUGR, with female more easily developing lipid accumulation and obesity. Particularly, the survival of adult female with metabolic syndrome exposed to the Chinese famine in their early lives was significantly higher than that of adult males [40]. It is likely that an adverse intrauterine environment is better able to affect the neuroendocrine programming of lipid metabolic-related genes of female fetuses *in utero*. This gender difference may be related to sex hormones, and the different effects of oestrogen and androgen on hepatic cholesterol metabolism might play important roles [42, 43]. For example, low oestrogen levels can cause hypercholesterolemia by inhibiting the expression of hepatic CYP7A1 [43]. Our previous research also found that the PEE-associated neuroendocrine metabolic programming alteration was more significant in female offspring [44], and the increase in metabolic syndrome susceptibility in female offspring was also obvious [18]. This finding indicates that PEE could lead to hypercholesterolemia and hepatic cholesterol metabolic disorder with gender disparity in adult IUGR offspring, and the effects are more obvious in female offspring.

Finally, our results showed the interaction between post-weaning HFD and gender regarding the serum TCH, HDL-C and LDL-C concentrations as well as hepatic HMGCR, SR-B1, and CYP7A1 expression (see table 2). After HFD consumption, the serum TCH concentration and hepatic HMGCR and CYP7A1 expression of female offspring were more significantly increased than those of male offspring, while the serum HDL-C concentration and hepatic SR-B1 expression were decreased more significantly in female offspring. For the IUGR offspring

caused by the prenatal xenobiotic exposure, the incidence of NAFLD in female offspring was higher than that in male offspring [45], which is consistent with our previous research; PEE can lead to an increase in NAFLD susceptibility in female IUGR offspring under HFD after birth [18]. The gender difference in hepatic cholesterol metabolic changes caused by HFD is probably related to HFD impacting the insulin pathway in a gender-specific manner [46]. In addition, it was reported that the neuroendocrine disorders of the HPA axis caused by HFD were more serious in female rats, which might be related to the more significantly decreased glucocorticoid receptor levels in the hypothalamus of female rats [47]. Thus, we speculate that post-weaning HFD could affect the serum cholesterol concentration and hepatic cholesterol metabolism in a gender-specific manner, which was displayed as a more obvious change in female.

Epigenetic modifications might play a role in IUGR-originally programmed metabolic dysfunctions [48]. A previous study reported that food restriction during pregnancy enhances hepatic HMGCR mRNA expression in adult offspring and that hypomethylation and hyperacetylation occur in the HMGCR gene promoter [13]. This finding suggests that the alterations in hepatic cholesterol metabolism with epigenetic modifications result in an abnormal blood cholesterol levels. Therefore, further study is needed on the epigenetic mechanisms in future work.

Conclusion

In this study, we demonstrated that there were interactions among PEE, post-weaning HFD, and gender. A post-weaning HFD could aggravate hypercholesterolemia and the hepatic cholesterol metabolic disorder caused by PEE, and PEE and a post-weaning HFD could influence offspring cholesterol metabolism with a gender disparity, which was displayed as greater changes in female offspring than in male offspring. This research provides a theoretical and experimental basis for the mechanism and exploration of influencing factors of foetal-derived hypercholesterolemia. Furthermore, this study expands our understanding of the influence of the postnatal nutrient environment on foetal-derived diseases.

Disclosure Statement

All authors state no conflicts of interest.

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