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Title page

Prenatal caffeine exposure-induced adrenal developmental abnormality of male offspring rats and its possible intrauterine programming mechanisms

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Abbreviations: AKT1: protein kinase B; CEBPα: CCAAT/enhancer binding protein α; CEBPβ: CCAAT/enhancer binding protein β; CORT: corticosterone; CR: corticoid receptors; GAPDH: glyceraldehyde phosphate dehydrogenase; GD: gestational day; GR: glucocorticoid receptor; HE: hematoxylin and eosin; HPA: hypothalamic-pituitary-adrenal; IUGR: intrauterine growth retardation; IGF1: insulin-like growth factor 1; IGF1R: IGF1 receptor; MS: metabolic syndrome; NAFLD: non-alcoholic fatty liver diseases; MR: mineralocorticoid receptor; P450scc: cytochrome P450 cholesterol side chain cleavage; PCE: prenatal caffeine exposure; PW: postnatal week; StAR: steroidogenic acute regulatory protein; TEM: transmission electron microscopy; 3β-HSD: 3β-hydroxysteroid dehydrogenase; 11β-HSD1: 11β-hydroxysteroid dehydrogenase type 1; 11β-HSD2: 11β-hydroxysteroid dehydrogenase type 2.

Abstract

Glucocorticoid (GC) is a major factor for fetal tissue maturation and fate decision after birth. We previously demonstrated that prenatal caffeine exposure (PCE) suppressed fetal adrenal steroidogenesis and resulted in adrenal dysplasia. However, whether these changes play a role until adulthood and its intrauterine programming mechanisms remain unknown. In the present study, rat model of intrauterine growth retardation (IUGR) was established by PCE, male fetus and adult offspring were sacrificed at postnatal day (PD) 1, PD7, PD35, PD100 and PD168, respectively. Results showed that PCE fetal weight decreased and IUGR rate increased, while serum corticosterone (CORT) level increased but insulin-like growth factor 1 (IGF1) level decreased. Fetal adrenal exhibited an enhanced GC-activation system (11β-hydroxysteroid dehydrogenases/corticoid receptors/CCAAT/enhancer binding proteins), an inhibited IGF1 pathway and steroid synthesis function. After birth, the serum CORT levels in the PCE offspring were increased in early period followed by falling in later stage, while the serum IGF1 levels change were opposite and accompanied by obvious catch-up growth. Furthermore, the adrenal GC-activation system was inhibited but the IGF1 signaling pathway was enhanced, resulting in a compensatory increase of adrenal steroidogenesis, presenting by the change trend of some steroidal synthetase were the same with that of IGF1 signaling pathway. Based on these findings, we proposed a "two-programming mechanisms" for PCE-induced adrenal abnormality: the "first programming" is a lower function of adrenal steroidogenesis, and prenatal and postnatal adrenal structural and functional abnormality triggered by the intrauterine GC-IGF1 axis programming-mediated by the GC-activation system that acts as "the second programming".

Keywords: Prenatal caffeine exposure; Adrenal steroidogenesis; Glucocorticoid-insulin-like growth factor 1 (GC-IGF1) axis

Introduction

Caffeine is a methylxanthine alkaloid present in coffee, tea, soft drinks, food and some drugs, which is one of the most frequently used pharmacologically active substances [1, 2]. Many experiments have demonstrated that prenatal caffeine exposure (PCE) could induce adverse effects on reproductive and embryo toxicity [3, 4], such as intrauterine growth retardation (IUGR) and miscarriage [5, 6]. Reports also showed that children who ingested food or drinks containing caffeine were highly susceptible to metabolic syndromes (MS), such as obesity and hypertension [7, 8]. Our previous studies had demonstrated that PCE in rodents could lead to IUGR fetuses' over-exposure to maternal glucocorticoids (GC) in rodents [9]. Meanwhile, the fetuses showed hypothalamic–pituitary–adrenal (HPA) axis development suppression, which showed a low basal activity and enhanced stress sensitivity, as well as GC-dependent alteration of blood glucose and lipid metabolic phenotypes after birth [10, 11]. Furthermore, these fetuses showed increased susceptibility to non-alcoholic fatty liver diseases (NAFLD) when fed with high-fat diet after birth [12]. Therefore, we proposed a HPA axis-associated neuroendocrine metabolic programming mechanism induced by PCE, which could lead to the offspring with high risk of developing MS [12].

The adrenal gland is the earliest and fastest developing organ of HPA axis, which has important significance to promote fetal growth and development by synthesizing GC [13]. Meanwhile, the adverse intrauterine environment could cause abnormal GC secretion of the adrenal, which could increase the risk of developing MS in offspring [14-16]. In our previous studies, PCE could inhibit the HPA axis development and

decrease steroidal synthetase expression and adrenal corticosterone (CORT) level [17]. However, whether adrenal dysfunction of PCE offspring rats possesses an intrauterine programming effect and whether this continues after birth remains unknown. GC function was related with the activity of the 11β-hydroxysteroid dehydrogenases (11β-HSDs) system, 11β-HSD1 reductively activated GC and 11β-HSD2 oxidative inactivated GC [18]. Corticoid receptors (CR) included mineralocorticoid receptor (MR) and glucocorticoid receptor (GR). Many studies showed that GC/CR could induce 11β-HSD1 expression and suppress 11β-HSD2 expression, and the change of 11β-HSDs/CR system expression could cause viscera dysplasia [19, 20]. CCAAT enhancer binding proteins (C/EBP) was one of the most important transcriptional factors that regulated downstream gene expression in collaboration with GC/CR [21]. Numerous experiments have demonstrated that GC can inhibit the insulin like growth factor 1 (IGF1) expression in many tissues and cells [22, 23]. Our previous studies indicated that fetuses' over-exposure to maternal GC induced by PCE may increase the expression of 11β-HSD1 and GR as well as reduce 11β-HSD2 and IGF1 expression in tissues, accompanied by dysfunction of multiple organs [9, 10]. The studies suggested that GC-activation system (11β-HSDs/CR/CEBPs) may mediate the growth inhibition of adrenal function induced by PCE.

The present study aims to explore the developmental toxicity of adrenal and its intrauterine programming mechanisms in IUGR offspring rats caused by PCE, mainly *via* detecting the changes of the steroidal synthetase system, IGF1 signaling pathway and GC-activation system before and after birth. This study will be beneficial in deeply understanding the intrauterine origin mechanisms of adrenal dysfunction and the development of GC-associated disorders in IUGR offspring.

Results

In fetuses

Fetal bodyweight, serum CORT level and adrenal steroidgenesis function

As shown in Fig. 1, the dams that received 30 and 120 mg/kg·d of caffeine exhibited a significant decrease of fetal mean bodyweight, which dropped to 93.7% and 75.1% of that in the control on GD20, respectively (P<0.01), and the corresponding IUGR rates were 39.4% and 96.7%, respectively (P<0.01). Meanwhile, the level of male fetal serum CORT was elevated in a dose-dependent manner, which were 1.34- (P<0.05) and 1.47-fold (P<0.01) to that of the control. Meanwhile, the dams that received 30 and 120 mg/kg·d of caffeine exhibited a significant decrease of the adrenal steroidal synthetase system, presenting by decreased mRNA expression levels of 3β-HSD, P450c21 and P450c11 (P<0.01). Meanwhile, the content of adrenal CORT in 120 mg/kg.d PCE male fetuses was reduced to 49.9% of the control (P<0.05). These results demonstrated that PCE could cause elevated serum CORT concentration and inhibited adrenal steroid synthesis in IUGR fetuses.

Fetal adrenal histological examination and cell proliferation

In the PCE (120 mg/kg·d) group, the male fetal adrenal cortex displayed thinner adrenal cortical zone, disorganized cell arrangement and lymphocyte infiltration, and the maximum cross-sectional area of fetal adrenal in the PCE group deceased to 43.8% of that in the control (P<0.01, Fig. 2A/B). Immunohistochemical analyses showed that adrenal Ki67 protein expressions in the PCE group were significantly decreased to 39.2% of that in the control group (P<0.01, Fig. 2C/D). Electron microscopy images revealed that the mitochondrial structure was damaged due to decreased tubular cristae structure as well as enhanced cavitation cells in the PCE group (Fig. 2E). Therefore, PCE could cause fetal adrenal tissue dysplasia in both morphology and function.

Fetal adrenal GC-activation system and IGF1 signaling pathway

As shown in the Fig. 3, the dams that received caffeine with 120 mg/kg·d exhibited an increased 11β-HSD1 expression (P<0.05, Fig. 3A) and decreased 11β-HSD2 expression (P<0.05, Fig. 3B), which resulted in 11β-HSD1/11β-HSD2 expression ratio increased (P<0.01, Fig. 3C). Meanwhile, the expression levels of MR, GR and C/EBPα in the PCE group were elevated in varying degrees (Fig. 3D-F) and C/EBPβ expression was decreased (P<0.05, P<0.01, Fig. 3G) in a dose-dependent manner, that resulted in C/EBPα to C/EBPβ expression ratio increase (P<0.05, P<0.01, Fig. 3H). Meanwhile, the serum IGF1 level and adrenal IGF1R and AKT1 expression in the PCE group markedly decreased in a dose-dependent manner compared to the control (P<0.05, P<0.01, Fig. 3I-L). These results above suggested that PCE could activate GC-activation systems (11β-HSDs/CR/CEBPs) and suppress IGF1 signaling pathways in fetal adrenal.

After birth

Offspring's bodyweight, serum CORT and IGF1 levels, and adrenal steroidogenesis function

As shown in Fig. 4A-D, the male PCE (120 mg/kg.d) offspring showed an obvious catch-up growth indicated by a continuous gain rate of bodyweight from PW1 to PW24 (P<0.05, P<0.01), although the bodyweight remained lower than that of their control counterparts (P<0.05, P<0.01). Furthermore, the serum CORT level of the PCE group was higher than that of the control during the early postnatal stage from PD1 to PD35, but then gradually decreased (PD60) and was close (PD100 and PD168) to the control at later time points (P<0.05, P<0.01) [11]. On the contrary, the serum IGF1 level of the PCE group was lower than that of the control from PD1 to PD35,

but then gradually increased when compared with those in the control group.

As shown in Fig. 4E-G, compared with their respective controls, the expression of adrenal StAR, P450scc and 3β-HSD in the PCE groups showed reduced to varying degrees at postnatal different time points. However, the expression of adrenal P450c21 and P450c11 in the PCE group, as shown in Fig. 4E-F, reduced from PD1 to PD35 but increase after PD100 to varying degrees when compared with their respective controls. Meanwhile, the level of adrenal CORT in PCE male offspring was close to the control.

The results indicated that PCE-induced IUGR offspring had a contrary change between serum GC and IGF1 concentrations after the birth of a period of time, and adrenal steroid synthetase system continued to lower expression but adrenal endogenous CORT production was close to the control.

Adrenal histological examination and cell proliferation

Adrenal histological analysis by HE staining showed a small number of cells with cytoplasmic swelling in the offspring adult adrenal in the PCE (120 mg/kg.d) group (Fig. 5A). Furthermore, the maximum cross-sectional area and Ki67 expression of the adult adrenal in the PCE group were 87.5% and 78.6% to those of the control, respectively (Fig. 5B,D).

Adrenal GC-activation system and IGF1 signaling pathway

As show in Fig. 6, compared with the control, the PCE (120 mg/kg.d) group exhibited increased expression of 11β-HSD2 (P<0.01), that resulted in decreased 11β-HSD1/11β-HSD2 expression ratio (P<0.01). Meanwhile, expression of MR and CEBPβ was significantly increased in the PCE group (P<0.01), and resulted in decreased C/EBPα to C/EBPβ expression ratio (P<0.01). Furthermore, the mRNA expression of genes involved in IGF1 signaling pathway (such as IGF1, IGF1R,

AKT1) were all markedly increased in the PCE group compared to the control (P<0.01). These results suggested that PCE induced IUGR adult offspring had inhibited GC-activation system and activated IGF1 signaling pathway of in the adrenals.

Discussion

PCE induced intrauterine low-functional programming of adrenal steroidogenesis in offspring rats

Based on the dose-conversion correlation between humans and rats (humans : rats=1 : 6.17) [24], the 30 mg/kg.d dose (low dose in this study) of caffeine exposure in rats is equivalent to 1.4-1.9 cups of coffee in human (a standard cup of coffee contains 100 mg to 200 mg of caffeine on average). Previous study has shown that the peak caffeine concentration in human blood can reach 4.2-26.0 μg/mL (20.6-133.9 μM) after intake of 250 mg caffeine-containing drinks [25]. In the present study, the caffeine concentrations in maternal and fetal blood after the 120 mg/kg.d dose (high dose) of caffeine exposure were 254±11 μM and 155±28 μM [12], respectively, which is achievable in the crowd in favor of coffee. Meanwhile, we found that PCE (30 and 120 mg/kg.d) caused a lower birth bodyweight and higher IUGR rate, decreased fetal adrenal maximum cross-sectional area and cortex Ki67 expression (43.8% and 39.2% of the control respectively), accompanied by disordered arrangement of cells, decreased tubular cristae structure and other pathological alterations. Furthermore, the expression of adrenal steroid synthetase system (including StAR, P450scc, 3β-HSD, P450c21 and P450c11) and CORT production showed decrease in the PCE fetuses. These results suggested that PCE could induce adrenal dysplasia in male fetal rats.

“Intrauterine programming” is a normal event during pregnancy that is necessary for

natural developmental changes in endogenous hormones and growth factors, while "intrauterine programming alteration" refers to the changes of morphology and function caused by damage *in utero* that can be sustained after birth [26]. Abnormal intrauterine programming of IUGR fetuses will increase the risk of some diseases and mortality after birth, such as hypertension, cardiovascular disease and metabolic disease [27, 28]. Our previous study showed that PCE could inhibit adrenal steroidogenesis in the offspring [9, 17]. The present study further demonstrated that this inhibition showed a programming effect and could continue to after birth. In this study, we found that the IUGR offspring caused by PCE had continuous decrease expression of StAR, P450scc and 3β-HSD (but not P450c21 and P450c11) at PD1~PD168, suggesting that the inhibitory effect of PCE on fetal adrenal steroidogenesis could be extended to adulthood, which is characterized by an intrauterine low-functional programming of adrenal steroidogenesis in the offspring rats and may be related to the epigenetic modification of steroidogenic factor 1 [17].

GC-IGF1 axis programming by PCE probably participated in adrenal development abnormality before and after birth

IGF1 signaling pathway is the core of the endocrine regulation system and modulates adrenal cell proliferation, differentiation and metabolism before and after birth [29]. *In utero* blood IGF1 mainly comes from the liver and adrenal could express IGF1 after birth by autocrine or paracrine mechanisms [29]. "Catch–up growth" was used to describe the phenomenon when growth retardation fetuses induced by pathological factors displayed growth acceleration after removal of the growth-inhibiting condition [30], stimulated mainly by increased circulation IGF1, and it was closely related with the high risk of developing metabolic disease [31]. Previous studies have shown that the IGF1 signaling pathway plays an important role in regulating

expression of P450c21 and P450c11 [32]. In the present study, we found that PCE could inhibit fetal adrenal IGF1 signaling pathway expression, accompanied by an impaired adrenal structural and functional development, similar to the above description. Furthermore, when adrenal IGF1 pathway expression was increased in adult PCE male offspring, adrenal cross-sectional area and cortex Ki67 expression were close to the control level (87.5% and 78.6% of the control respectively), meanwhile, adrenal steroidal synthetases P450c21 and P450c11 were increased and CORT production was close to the control level. These results suggested that the changes of adrenal IGF1 signaling pathway by PCE participated in adrenal development abnormality before and after birth.

Many studies have shown that GC could inhibit IGF1 expression in multiple tissues and cells [22, 33]. Fowden reported that changes of blood GC concentrations in fetus induced by adverse intrauterine conditions might modify blood IGF1 level [34]. Human and animals studies revealed that there was a negative relationship between serum GC and IGF1 levels [35, 36]. Recently, combined with our previous reports that prenatal caffeine, nicotine and ethanol exposure could cause fetal rats over-exposure to maternal GC and increase the susceptibility to adult MS-related NAFLD in IUGR offspring, we proposed a "GC-IGF1 axis programming" hypothesis in the liver of IUGR offspring [12, 37, 38]. In the present study, we also found that PCE male offspring *in utero* showed a high serum GC level but a lower serum IGF1 level; after birth, serum CORT level increased but serum IGF1 level reduced from PD1 to PD35, and then serum CORT level gradually decreased but serum IGF1 level gradually increased from PD60 to PD 168. Meanwhile, there was good opposite changes between serum CORT levels and adrenal IGF1 signaling pathway expression before or after birth. Moreover, there were also consistent changes among IGF1 signaling pathway

expression, steroidal synthetases (P450c21 and P450c11) expression and CORT production of adrenals before and after birth. This negative correlation between serum CORT level and serum IGF1/adrenal IGF1 signaling pathway expression also suggested the existence of a "GC-IGF1 axis programming" in the adrenals of IUGR offspring by PCE, which could evoke a "catch–up growth" of adrenal development after birth, and will increase the susceptibility to MS and related NAFLD probably by excessive GC synthesis.

GC-activation system may mediate the intrauterine programming of adrenal GC-IGF1 axis

A large number of investigations have showed that, 11β-HSDs expression changes could modify the local GC concentration [18, 39]. Due to an approximately 10-fold higher ligand affinity of MR to GC than that of GR, low levels of GC would bind with MR firstly and only a high level of GCs could bind to GR after the MR was completely occupied [18]. GC/CR collaborated with C/EBPs could regulate downstream gene expression, such as C/EBPα could inhibit the IGF1R expression [40] and blood IGF1 level decreased in C/EBPβ knockout mice [41]. In the present study, when the PCE male fetuses were over-exposure to maternal GC, adrenal 11β-HSD1 expression increased and 11β-HSD2 decreased, resulted in 11β-HSD1/11β-HSD2 expression ratio increased, meanwhile, fetal adrenal MR and GR expression, C/EBPα expression and C/EBPα/C/EBPβ expression ratio were significantly increased, along with suppressed IGF1 signaling pathway expression. After birth, in the case of lower serum CORT (from PD60 to PD168) in PCE male offspring, adrenal GC-activation system was inhibited, presenting mainly by 11β-HSD1/11β-HSD2 expression ratio reduced, only MR expression increased in CR, and C/EBPα to C/EBPβ expression ratio decreased, at the same time adrenal IGF1 signaling pathway expression increased.

These results suggested that the GC-activation system might be involved in the "GC-IGF1 axis programming" in the adrenal of male IUGR offspring induced by PCE, and the changes of adrenal IGF1 signaling pathway and related organ development abnormality was an adaptive alteration induced by the GC-IGF1 axis programming by PCE.

Materials and methods

Materials

Caffeine (CAS No. C0750) was obtained from Sigma-Aldrich Co., Ltd. (St Louis, MO, USA). Isoflurane was purchased from Baxter Healthcare Co. (Deerfield, IL, USA). Trizol reagent were purchased from Invitrogen Co. (Carlsbad, CA, USA). IGF1 assay kit was provided by Mind Bioengineering Co., Ltd. (Shanghai, China). Corticosterone (CORT) assay kit was from Sangon Biotech Co., Ltd. (Shanghai, China). Reverse transcription and real-time reverse-transcription PCR (RT-PCR) kits were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Other chemicals and agents were of analytical grade.

Animals and treatments

Animal experiments were performed in the Center for Animal Experiment of Wuhan University (Wuhan, China), which has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). All animal experiment procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Chinese Animal Welfare Committee. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Wuhan University School of Medicine (Permit Number: 14016).

Wistar rats (with weights of 200~240 g for females and 260~300 g for males) were obtained from the Experimental Center of Hubei Medical Scientific Academy (No. 2009-0004, Hubei, China). Details of animal feeding, mating and caffeine (30, 120 mg/kg.d) administration were described as before [11]. Feed intake and weight gain of PC-treated group during pregnancy were similar to control. As shown in Fig. 7, on gestational day (GD) 20, partial pregnant rats were euthanized under isoflurane anesthesia. Pregnant rats with litter sizes of 8 to 14 were considered qualified. IUGR was diagnosed when the bodyweight of an animal was two standard deviations less than the mean bodyweight of the control group [42]. Fetal blood samples were collected and serum was isolated. Fetal adrenals from each littermate were separated, pooled together and immediately frozen in liquid nitrogen, followed by storage at -80℃ for subsequent analyses. Partial fetal adrenals were randomly selected (one per litter) and fixed for histological and transmission electron microscopic (TEM) observation.

As shown in Fig. 7, the rest of pregnant rats were kept until normal delivery. The number of pregnant rats in each group was assigned to 8 (the litter size of each pregnant rat was comprised from 8 to 14 at birth). The weights of offspring were recorded continuously. The bodyweight growth rate was calculated as before [11]. Briefly, the offspring were divided into five batches according to the time of maturity. The offspring were divided into five batches according to the time of maturity, including postnatal day (PD) 1, PD7, PD35, PD 100 and PD168, respectively. In order to obtain enough samples, the average of 4, 3 and 2 littermates from each dam were decapitated at PD1, PD7 and PD35 respectively, and these serum and adrenal samples of littermates were pooled to count as one independent sample for subsequent detection. Specially, the offspring in the fourth and fifth batches were fasted overnight from 8 p.m, anesthetized with isoflurane and decapitated the next morning at 8-10 a.m.

in a room separate from the other animals, and the serum and adrenals samples were collected. Partial adrenals in the PD168 were randomly selected and fixed for histological observation.

$$\text{Bodyweight gain rate (\%)} = \frac{\text{Bodyweight of PW x} - \text{Bodyweight of PW 1}}{\text{Bodyweight of PW 1}} \times 100$$

Analysis for blood samples

The CORT and IGF1 concentrations were measured by biochemical assay kits following the manufacturer's protocol. The intra-assay and inter-assay coefficients of variation for CORT and IGF1 were 5.0% and 7.2%, 5.6% and 9.1%, respectively.

Histological, ultrastructural and immunohistochemical examination

The adrenal gland was fixed in 4% paraformaldehyde solution overnight and processed with the paraffin sectioning technique. Sections of 5 μm thickness were stained with hematoxylin and eosin (HE) dyes, observed and photographed with an Olympus AH-2 light microscope (Olympus, Tokyo, Japan). Five HE sections of each group and five random fields of each section were scored under the microscope. On these sections, the cross-sectional areas of cortex were determined by planimetry; cross-sectional areas of the adrenocortical zones were estimated using the Photo Imaging System (Nikon H550S, Japan). The adrenal gland observation by TEM was achieved by standard procedures and observed with a Hitachi H600 transmission electron microscope (Hitachi, Co., Tokyo, Japan).

The immunohistochemical procedures were performed using a streptavidin-peroxidase (SP)-conjugated method according to the manufacturer's instructions. Paraffin-embedded tissues were cut into 5 μm thickness serial sections, and stained for Ki67 with a goat monoclonal antibody (1:200 dilution; sc-7846, Sant Cruz Biotechnology). At least five random fields from each section were examined.

The signal was visualized using light microscopy, imaged, and the positively stained areas were analyzed using the Photo Imaging System (Nikon H550S, Japan). The number of Ki67-stained nuclei from each image was counted with the Photo Imaging System.

Total RNA extraction, reverse transcription (RT), and real-time quantitative RT-PCR

Detailed protocols for total adrenal RNA extraction and RT were previously described [43]. The concentration and purity of the isolated RNA were determined by a spectrophotometer and adjusted to 1 μg/μl. Total RNA was stored in DEPC-H_2O at -80℃. For real-time quantitative RT-PCR analysis, single-strand cDNA was prepared from 2 μg of total RNA according to the protocol of the Exscript RT reagent kit. The sequences of each primers were designed using Primer Premier 5.0 as shown in Table 1. Relative standard curves were constructed for the following target genes using the corresponding RT-PCR products isolated using the DNA extraction kit with different concentrations ranging from 10 pg to 10,000 pg per reaction. PCR was performed in 96-well optical reaction plates using the ABI Step One RT-PCR thermal cycler (ABI Stepone, NY, USA) in a 20 μl reaction mixture. To quantify the gene transcripts more precisely, the mRNA level of the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) was measured and used as a quantitative control. Each sample was normalized against GAPDH mRNA content. The PCR cycling conditions used were as follows: pre-denaturation, 95℃ for 30 s; denaturation, 95℃ for 5 s; the annealing conditions for each gene are listed in Table 1; and elongation, 72℃ for 30 s (the elongation step was performed for the 3β-HSD reactions).

Statistical analysis

SPSS 13 (SPSS Science Inc., Chicago, Illinois) and Prism (GraphPad Software, La

Jolla, CA, USA) were used for statistical analysis. All presented measurement data except IUGR rates were expressed as the Mean ± S.E.M. Mean bodyweights per litter were calculated and the litter mean bodyweights were used for statistically analyses. For IUGR rate, it was calculated first for each per litter and then was transformed by arcsine square root before using t-test [44]. IUGR rate is presented as the group means of the litter proportions. The *t*-test and chi-squared test were used as appropriate. Statistical significance was defined as $P<0.05$.

Conclusions

In conclusion, PCE induced adrenal developmental abnormality in the offspring rats, which may be related to a "two-programming mechanisms" (Fig. 8). Intrauterine lower-functional programming of adrenal steroidogenesis is "the first programming", which result in fetal adrenal dysplasia; on the other hand, GC-activation system-mediated GC-IGF1 axis programming is "the second programming", which is involved in prenatal and postnatal adrenal structural and functional abnormality. This study may provide some experimental basis for addressing the susceptibility to MS in IUGR offspring induced by PCE.

Conflict of interest statement

All authors have no conflicts of interest.

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Figure legends

Fig. 1. Effects of prenatal caffeine exposure (30 and 120 mg/kg.d) on bodyweight, serum corticosterone (CORT) concentrations, adrenal steroidogenic enzymes mRNA expression and corticosterone (CORT) content in male fetal rats. A: Bodyweight; B: Intrauterine growth retardation (IUGR) rate; C: Serum CORT concentration; D: Adrenal steroidogenic acute regulatory (StAR) expression; E: Adrenal cholesterol side chain cleavage enzyme (P450scc) expression; F: Adrenal 3β-hydroxysteroid dehydrogenase (3β-HSD) expression; G: Adrenal steroid 21-hydroxylase (P450c21) expression; H: Adrenal steroid 11β-hydroxylase (P450c11) expression; I: Adrenal CORT content. Mean ± S.E.M., n = 12 for bodyweight and IUGR rate, n = 3 for serum phenotype (serum were randomly merged because of meagre fetal blood), and n=6 for adrenals (six pairs of from two littermates were pooled for homogenization into one sample). Mean ± S.E.M., $^{*}P<0.05$, $^{**}P<0.01$ *vs*. control.

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Fig. 7. The schematic illustration of animal treatment. All the animals were killed at gestational day (GD) 21, postnatal day (PD) 1, PD7, PD35, PD100 and PD168, respectively.

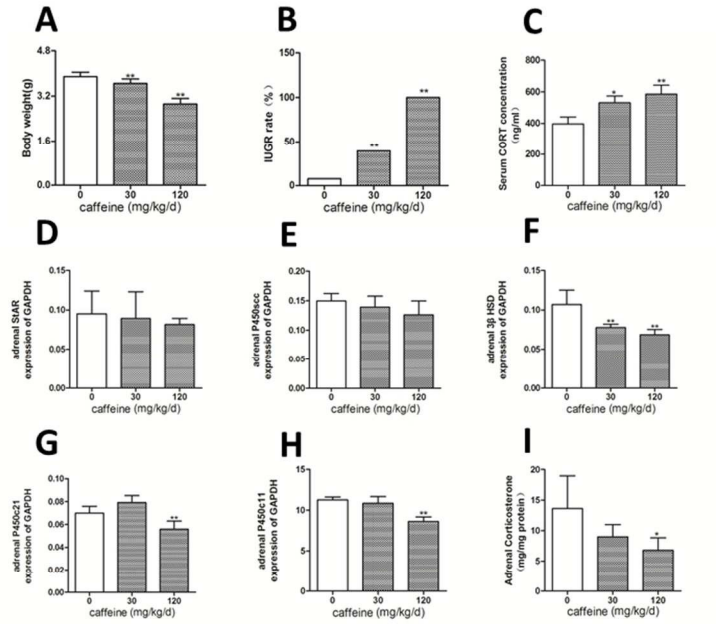
Fig. 8. Two-programming mediated prenatal caffeine exposure-induced adrenal developmental abnormality in male offspring rats. 11β-HSDs, 11β-hydroxysteroid dehydrogenases; CR, corticoid receptor; CEBP, CCAAT enhancer binding proteins; IGF1, insulin-like growth factors 1; GC-IGF1 axis: glucocorticoid and insulin-like growth factor 1 axis.

Table 1

Oligonucleotide primers and PCR conditions of rat in quantitative real-time PCR.

Genes	Forward primer	Reverse primer	Product (bp)	Annealing
StAR	GGGAGATGCCTGAGCAAAGC	GCTGGCGAACTCTATCTGGGT	188	65℃, 30s
P450scc	GCTGCCTGGGATGTGATTTTC	GATGTTGGCCTGGATGTTCTTG	156	63℃, 30s
P450c21	AGGAGCTGAAGAGGCACAAG	GAGGTAGCTGCATTCGGTTC	306	63℃, 30s
P450c11	CCCCTTTGTGGATGTGGTAG	CACGCTCTCAGGTTTCAGGT	190	61℃, 30s
3βHSD	TCTACTGCAGCACAGTTGAC	ATACCCTTATTTTTGAGGGC	271	58℃, 30s
IGF1	GACCAAGGGGCTTTTACTTCAAC	TTTGTAGGCTTCAGCGGAGCAC	148	60℃, 30s
IGF1R	GTCCTTCGGGATGGTCTA	TGGCCTTGGGATACTACAC	195	62℃, 30s
Akt1	ATGAGCGACGTGGCTATTGTGAAG	GAGGCCGTCAGCCACAGTCTGGATG	156	60℃, 30s
11β-HSD1	GAAGAAGCATGGAGGTCAAC	GCAATCAGAGGTTGGGTCAT	133	63℃, 30s
11β-HSD2	TGGCCAACTTGCCTAGAGAG	TTCAGGAATTGCCCATGC	76	63℃, 30s
MR	TGCATGATCTCGTGAGTGA	GAGGCCGTCAGCCACAGTCTGGATG	156	63℃, 30s
GR	CACCCATGACCCTGTCAGTC	AAAGCCTCCCTCTGCTAACC	156	63℃, 30s
C/EBPα	CGCAAGAGCCGAGATAAAGC	CCTAGAGATCCAGCGACCCT	270	60℃, 30s
C/EBPβ	ACTTCTACTACGAGCCCGACT	CGTAGTCGGACGGCTTCTTG	246	60℃, 30s
GAPDH	GCAAGTTCAATGGCACAG	AAGTTCTTCCTGGCCGGTAT	140	63℃, 30s

StAR: steroidogenic acute regulatory protein; P450scc: cytochrome P450 cholesterol side chain cleavage; 3β-HSD: 3β-hydroxysteroid dehydrogenase; P450c21: steroid 21-hydroxylase; P450c11: steroid 11β-hydroxylase; IGF1: insulin-like growth factor 1; IGF1R: IGF1 receptor; AKT1: protein kinase B; 11β-HSD1: 11β-hydroxysteroid dehydrogenase type 1; 11β-HSD2: 11β-hydroxysteroid dehydrogenase type 2; MR: mineralocorticoid receptor; GR: glucocorticoid receptor; C/EBPα: CCAAT enhancer binding proteins α; C/EBPβ: CCAAT enhancer binding proteins β; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.



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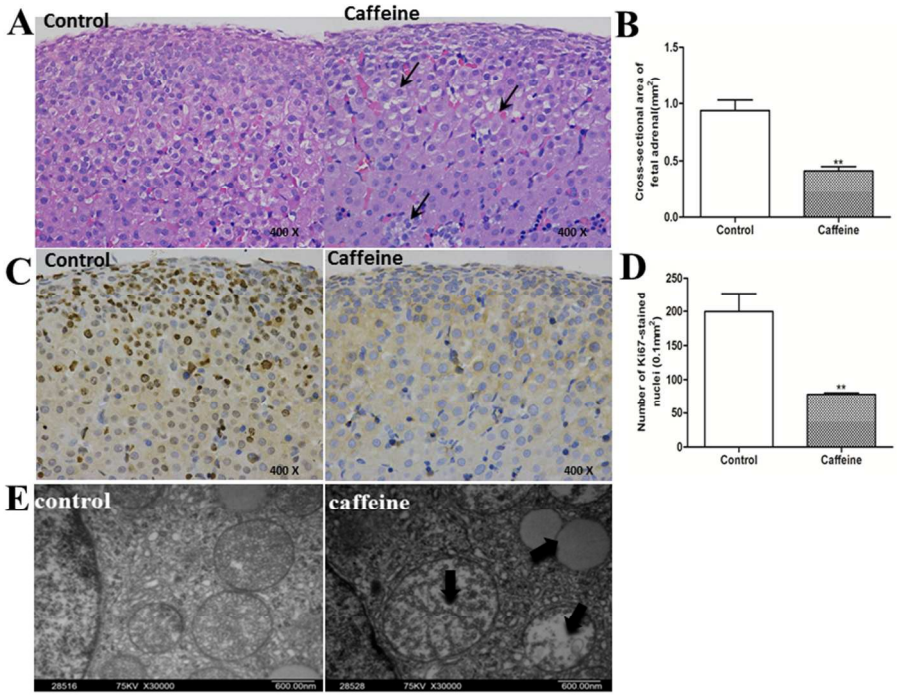

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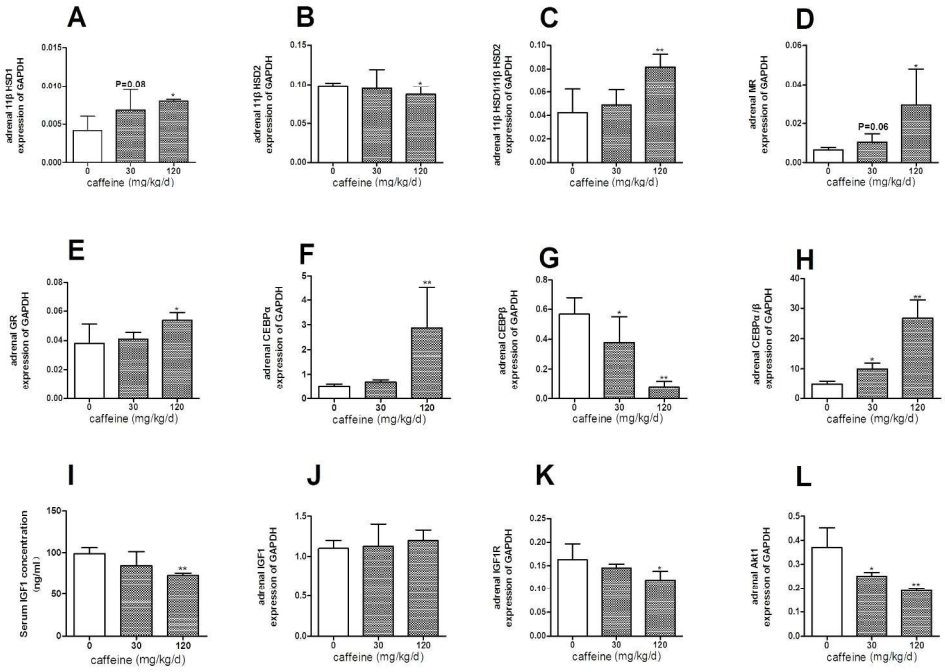

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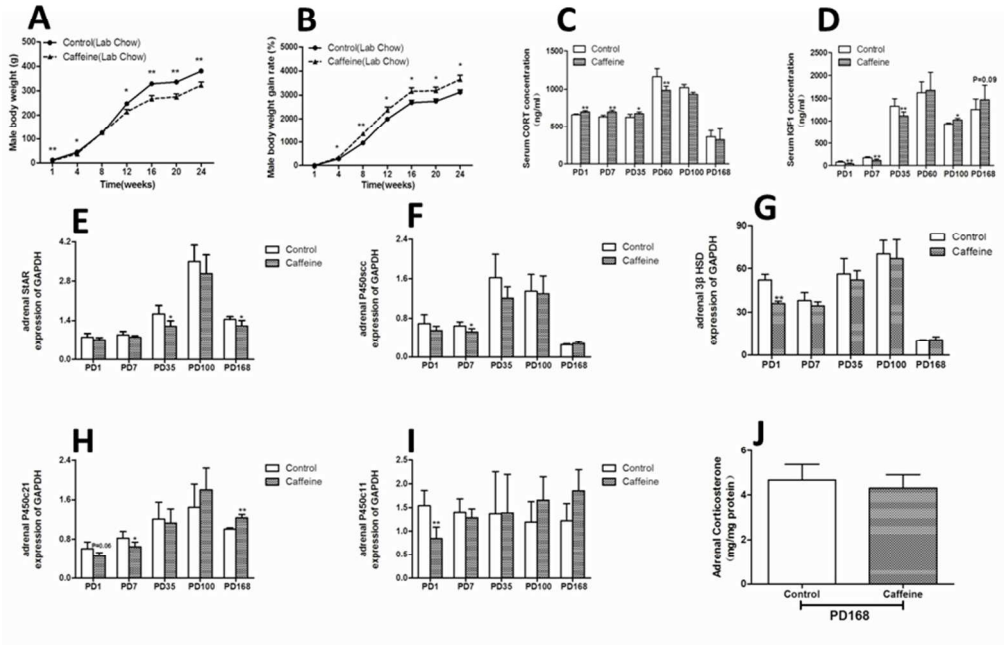

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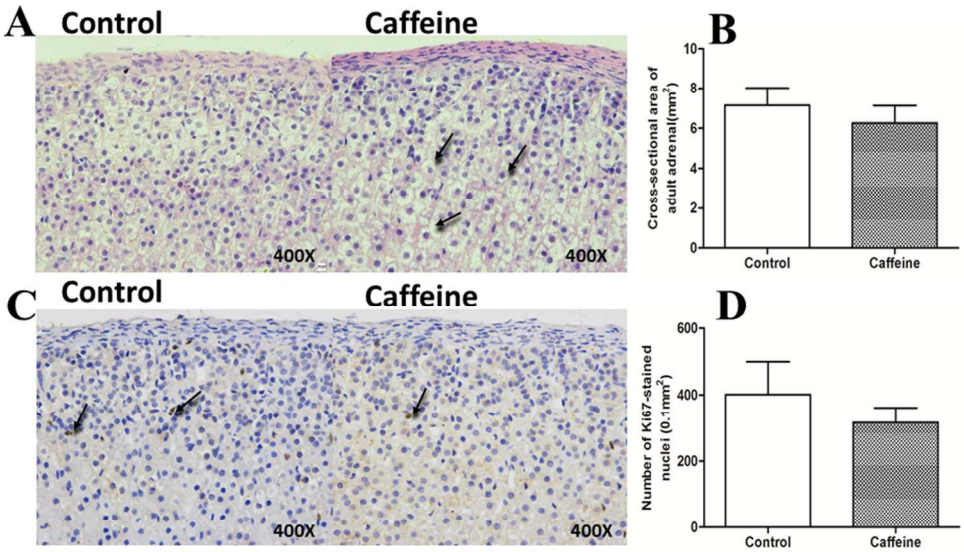

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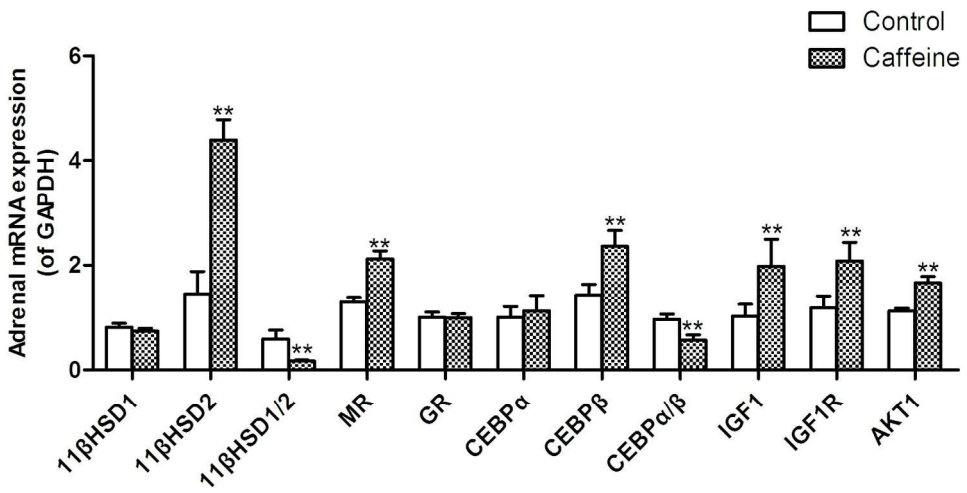

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**P<0.01 vs. control.
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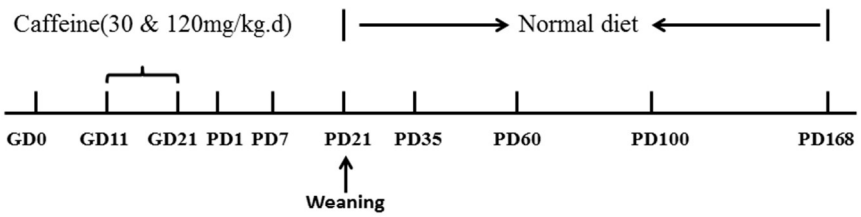

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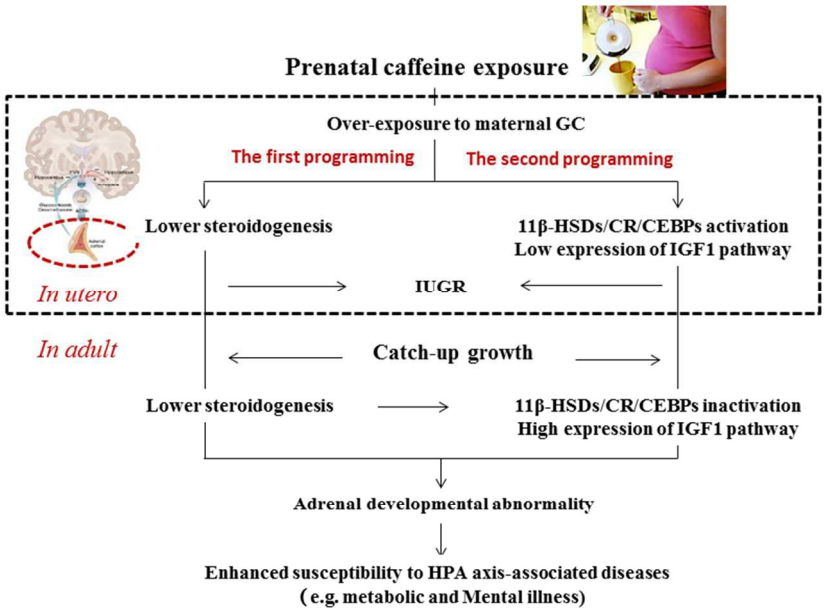

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