THEMATIC RESEARCH

The GC-IGF1 axis-mediated testicular dysplasia caused by prenatal caffeine exposure

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Abstract

Prenatal caffeine exposure (PCE) can induce testicular developmental toxicity. Here, we aimed to explore the underlying mechanism of this process in reference to its intrauterine origin. Pregnant rats were intragastrically administrated caffeine (30 and 120 mg/kg/day) from gestational days 9 to 20. The results showed that the male fetuses exposed to high dose of caffeine (120 mg/kg/day) had a decreased bodyweight and inhibited testosterone synthetic function. Meanwhile, their serum corticosterone concentration was elevated and their testicular insulin-like growth factor 1 (Igf1) expression was decreased. Moreover, the histone 3 lysine 14 acetylation (H3K14ac) level in the Igf1 promoter region was reduced. Low-dose (30 mg/kg/day) caffeine exposure, however, increased steroidogenic enzymes expression in male fetuses. After birth, the serum corticosterone concentration gradually decreased in the PCE (120 mg/kg/day) offspring rats, whereas the expression and H3K14ac level of Igf1 gradually increased, with obvious catch-up growth and testicular development compensation. Intriguingly, when we subjected the offspring to 2 weeks of chronic stress to elevate the serum corticosterone concentration, the expression of Igf1 and testosterone synthesis were inhibited again in the PCE (120 mg/kg/day) group, accompanied by a decrease in the H3K14ac level in the Igf1 promoter region. In vitro, corticosterone (rather than caffeine) was proved to inhibit testosterone production in Leydig cells by altering the H3K14ac level and the expression of Igf1. These observations suggested that PCE-induced testicular developmental toxicity is related to the negative regulation of corticosterone on H3K14ac levels and the expression of Igf1.

Introduction

Caffeine, which is a common component of foods, beverages and pharmaceuticals, is widely ingested by people, even pregnant women (Fredholm et al. 1999, Kluger 2004). Approximately 75% of women consume caffeine during pregnancy in the United States (Signorello & McLaughlin 2004). Previous studies have indicated that prenatal caffeine exposure (PCE) resulted in adverse birth outcomes, including intrauterine growth
Caffeine (CAS#58-08-2) was purchased from Sigma-Aldrich Co., Ltd. Isoflurane was purchased from Baxter Healthcare Co. (Deerfield, IL, USA). The ELISA kits for rat corticosterone and testosterone were obtained from R&D Systems, Inc. Rat radioimmunoassay kit (lot number: 20160620) was purchased from Beijing North Institute of Biological Technology (Beijing, China). Primary antibodies such as goat anti-rabbit immunoglobulin G (IgG) (ab172730) and anti-Ki67 (ab15580) were purchased from Abcam. 3β-Hydroxysteroid dehydrogenase (HSD3B) antibody (sc-30820) and IGF1 antibody (sc-9013) were purchased from Santa Cruz Biotechnology, Inc. β-Actin (AC004) was purchased from ABclonal Technology (Wuhan, China). The antibodies for histone 3 lysine 9 acetylation (H3K9ac) (A7255) and histone 3 lysine 14 acetylation (H3K14ac) (A7254) were also purchased from ABclonal Technology. Specific antibody information is provided in Supplementary Table 1 (see section on
supplementary data given at the end of this article). Chromatin immunoprecipitation (ChIP) assay kits were purchased from Millipore Co., Ltd. Mifepristone (RU486) (ODR-4395) and proteinase K (20 mg/kg) (ST533) were purchased from Kori Biotech Co., Ltd (Wuhan, China). IGF1 (791-MG) was purchased from Abcam. Reverse transcription and real-time quantitative polymerase chain reaction (RT-qPCR) kits were purchased from Takara Biotechnology Co., Ltd. The SYBR green dye was purchased from Applied Biosystems (Thermo Fisher Scientific). All of the primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

Animals and treatment

The animal experiments were performed in the Center for Animal Experiments of Wuhan University, which has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Wuhan University School of Medicine ( Permit No. 201709). All animal experimental procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Chinese Animal Welfare Committee. To reduce bias in the animal experiments, the rats were housed and treated by a technician, and different co-authors were in charge of testis and blood sample harvesting and data analysis, respectively. The experimental procedures and treatment methods in this study were described as follows (Supplementary Fig. 1).

The Wistar rats raised in this study were bred and mated as previously described (Xu et al. 2012b). The rat dams were randomly distributed into three groups: a control group, a low-dose (30 mg/kg/day) caffeine exposure (PCE(L)) group and a high-dose (120 mg/kg/day) caffeine exposure (PCE(H)) group. From gestational days (GDs) 9 to 20, the pregnant rats were intragastrically administrated caffeine or same volume of saline once per day. According to the dose conversion between human and rat (human:rat 1:6.17 by body surface area comparisons) (Reagan-Shaw et al. 2008), the dosage of the PCE(L) group corresponds to the daily human (60 kg) consumption of 292 mg caffeine and can be achieved in daily consumption.

On GD20, 36 rat dams from the control, PCE(L) and PCE(H) groups (n=12 per group) were anesthetized with 3% isoflurane and killed to extract the male fetuses. The other 24 rat dams from the control and PCE(H) groups (n=12 per group) were allowed to normally deliver for male offspring. The fetal bodyweight was recorded. IUGR was diagnosed when the bodyweight of an animal was two standard deviations less than the mean bodyweight of the control group (Engelbregt et al. 2001). Then, the male fetuses were immediately decapitated to collect blood samples and testes, and the blood samples of the different male fetuses from each litter were pooled as one sample. Fetal testes from each littermate were pooled together and immediately frozen in liquid nitrogen, followed by storage at −80°C for subsequent analyses, and partial fetal right testis was randomly selected and fixed for morphological observation.

Beginning at postnatal day (PD) 1, the pups were weighed weekly until postnatal week (PW) 12. The bodyweight gain rate was calculated as follows (Xu et al. 2012a): bodyweight gain rate (%) = (PW<sub>n</sub> bodyweight – PD<sub>1</sub> bodyweight) × 100/PD<sub>1</sub> bodyweight. At PW10, one male offspring rat was randomly selected from each litter (the chronic stress group, n=12), and was then exposed to an ice-water (5–7°C) swim for 5 min once per day for 2 weeks. At PW12, another male offspring rat was randomly selected to mate with normal females (to avoid inbreeding). The pregnant rats were kept until normal delivery, and the birthweight of the F2 generation was recorded. The animals were anesthetized by isoflurane and euthanized at PW2, PW6 and PW12, respectively. Blood samples were collected, and the serum was isolated. The testes were immediately frozen in liquid nitrogen, followed by storage at −80°C for subsequent analyses, and partial right testis was randomly selected and fixed for morphological observation.

Hormonal level measurements

The concentrations of serum corticosterone and fetal serum testosterone were measured by ELISA kits, following the manufacturer protocol. Fetal intra-testicular testosterone content was measured using previously described methods (Mylchreest et al. 2002), and the assay had a 40 pg/tissue limit of detection. Adult serum testosterone and intra-testicular testosterone content were measured using radioimmunoassay kits following the manufacturer protocol.

Histological and ultra-microstructure measurements

As described by Park et al. (2015), immediately after removal, one testis from each animal was processed for sectioning. Serial sections of 5 μm thickness were
taken from the mid portion of each testis and stained with hematoxylin-eosin (HE). All histomorphometric evaluations were performed by the same trained, calibrated, and blinded examiner using an image analysis system (Olympus) coupled to an Olympus AH-2 light microscope (Olympus). Four serial sections were traced for fetal right testis per animal (n=3), and then diameter and area of fetal testis were measured to obtain a mean value per animal at 100× magnification.

The fetal testis was dehydrated through a graded series of ethanol and embedded in Epon 812. Ultrathin sections (~50nm) were cut with LKB-V ultramicrotome (Bromma, Sweden), dually stained with uranyl acetate and lead citrate, and examined with a Hitachi H600 transmission electron microscope (TEM) (Hitachi). Digital images were acquired directly by a computer (Dell).

**Sperm count, motility and morphology examinations**

Samples were obtained from the right caudal epididymides of the rats at PW12 for the sperm count and motility analysis as previously described (Toure et al. 2007). The caudal epididymides were cut into pieces in phosphate-buffered saline (PBS, pH 7.4, 37°C). The sperm released into the PBS were then incubated for 15–30 min at 37°C in 5% CO₂ to allow sperm diffusion. 10μL of sperm suspension was placed on a preheated (37°C) slide, covered with a cover slip and immediately examined using a light microscope at 100×. The sperm count was determined with a hemocytometer and expressed as ×10⁶ cells/mL. Another part of the suspension was drawn by capillary action into a prewarmed (37°C) chamber slide for the quantitative assessment of motility using a Hamilton-Thorne motility analyzer (Hamilton-Thorne Biosciences, Beverly, MA, USA).

Sperm samples were obtained from the cauda of the left epididymis for morphology examinations. Samples of 2μL of the epididymal fluid were homogenized in 2mL of bidistilled water. One drop of the solution was smeared onto a glass slide and air-dried. The smears were stained with HE. For the morphological evaluation, 200 spermatozoa were randomly analyzed, and the percentage of abnormal spermatozoa was obtained. Morphological abnormalities consisting of the head and tail of the spermatozoa were classified according to the modified descriptions and were adapted for the experimental model used.

**Immunohistochemistry and immunofluorescence measurements**

Testes were fixed overnight in 4% paraformaldehyde fixative and embedded in paraffin. For immunological histological chemistry (IHC) analysis, the sections were incubated overnight at 4°C with the following antibodies: anti-Ki67 (1:1000), anti-IGF1 (1:500) and anti-HSD3B (1:500). Immunohistochemical analysis was performed using a DAB staining kit to determine the expression levels of Ki67, HSD3B and IGF1 proteins in the testes. Immunostaining for the negative control was performed on parallel sections, in which the primary antibody was replaced with non-immune rabbit IgG. The intensity of staining was determined by measuring the mean optical density in five random fields for each section. For immunofluorescence (IF) analysis, primary antibody was diluted as optimized (HSD3B 1:100) and was incubated overnight at 4°C. After rewarming for 15 min, the corresponding fluorescent secondary antibody (1:400) were added, then incubated at room temperature for 1 h in the dark. Nuclear counterstain (DAPI; Sigma-Aldrich) was diluted 1:500 in Tris-buffered saline (TBS) and incubated for 10 min. As described previously (Motohashi et al. 2016), the number of GCs per unit square of interstitial tissue areas (10⁴μm²) were calculated by examining 25 randomly selected sites in each group to avoid a sampling bias. All images were captured using an Olympus AH-2 light microscope (Olympus). Analysis of the stained images was performed using Olympus software.

**Leydig cell culture**

Mouse Leydig TM3 cells were grown and maintained in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The TM3 cells liberated during digestion were collected and plated at a density of 4 × 10⁵ cells per well in six-well plates in medium. The cells were treated with various concentrations of caffeine (1, 10 and 100μM) or corticosterone (250, 750 and 1250nM) for 3 days, and then harvested for further analysis. To confirm that the effect of corticosterone on TM3 cells was mediated by increased glucocorticoid receptor (GR) and decreased IGF1, 2.5μM RU486 or 100ng/mL IGF1 were used alone or combined with 1250nM corticosterone.
Total RNA extraction, reverse transcription and RT-qPCR

Total RNA was isolated from testicular tissue and TM-3 cells using TRIzol Reagent following the manufacturer’s protocol. After isolation, the quality of the RNA samples was assessed using a NanoDrop spectrophotometer and 2–3% agarose gel (Rivera-Torres 2015). For each RNA sample, 1 µg of RNA was used for cDNA synthesis. First, genomic DNA contamination was removed by incubating the RNA with dsDNase at 37°C for 2 min. After heat inactivation, we proceeded to cDNA synthesis by incubating the RNA with 1 µL of oligo(dT)18 primer (100 pM), 0.5 mM of dNTP Mix, 4 µL of 5× RT Buffer, 1 µL of Maxima H Minus Enzyme Mix and 3 µL of nuclelease-free water at 50°C for 30 min followed by 5 min at 85°C. The controls for each reaction were the RNA sample without reverse transcriptase (RNA-RT) and no RNA with reverse transcriptase (no RNA+RT). RT-qPCR was performed using the ABI StepOne RT-PCR thermal cycler (Thermo Fisher Scientific) in a 10 µL reaction mixture. The reaction mixture contained 5.6 µL of oligo mix (0.5× Power SYBR Green PCR Master Mix and 50–100 nM of forward and reverse primers) and 4.4 µL of diluted cDNA (1–10 ng). The cycling conditions were as follows: 2 min at 50°C, followed by 10 min at 95°C for polymerase activation and then 40 cycles of 95°C for 15 s and 60°C for 1 min for primer annealing and extension. To quantify the gene transcripts more precisely, the mRNA level of the housekeeping gene glyceraldehyde 3-phosphatedehydrogenase (Gapdh) was measured and used as a quantitative control. The optimal primer was determined by a separate set of experiments to ensure that both the target gene and Gapdh were amplified with equal efficiency. Each sample was normalized against the Gapdh mRNA content. The sequences for each set of primers are shown in Supplementary Table 2.

Western blot analysis

Specimens were homogenized in 50 mM Tris–HCl, 150 mM KCl (pH 7.4), 1% Triton X-100 and 0.25 mM phenylmethylsulfonyl fluoride and centrifuged at 80,000 g (30 min, 4°C). The pellet was lysed with lysis buffer (10 mM Tris–HCl, 1% sodium dodecyl sulfate (SDS), 1.0 mM ethylene diamine tetraacetic acid, 10% glycerol and 5% 2-mercaptoethanol) The protein concentrations in lysates (4 µL) were determined using a Protein 200 Labchip kit (Agilent Technologies Inc.) and run on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc.). An equal amount of protein (10 µg) from each lysate was resolved on 10% SDS polyacrylamide gels under denaturing conditions and then transferred to Immuno-Blot PVDFs (Bio-Rad). After 1 h of blocking by immersion in 5% non-fat dried milk in TBS with 0.1% (v/v) Tween 20 (TBST), Western blot analyses were performed using antibodies to IGF1 (diluted 1:500), HSD3B (diluted 1:500) and β-actin (diluted 1:4000) and incubated overnight at 4°C in an orbital shaker. After washing three times with TBST, the membranes were incubated with a 1:2000 dilution of secondary antibodies for 1 h. Finally, the membranes were detected using the ECL Plus Western Blotting Detection System (Applied Biosystems). To verify the relative amounts of protein in each lane, the level of β-actin was determined as an internal control.

Chromatin immunoprecipitation-polymerase chain reaction (ChIP-PCR)

The homogenate of testicular tissues was fixed with 1% formaldehyde for 15 min at 37°C to cross-link DNA and its associated proteins. Glycine (0.125 M final concentration) was added to terminate the reaction for 8 min. The lysates were then sonicated to shear the DNA to a size of 200–800 bp. After sonication, the samples were collected by centrifugation and diluted with dilution buffer. After mixing, 10 µL of the supernatant was saved as input DNA for normalization of chromatin input. The remainder was divided into 200 µL per Eppendorf tube and incubated overnight at 4°C on nutator/rocker with specific antibody for H3K9ac (1:50 dilution), H3K14ac (1:50 dilution) or IgG (1:50 dilution) and BSA-treated Protein G beads to reduce nonspecific background binding. The immunoprecipitated DNA–protein complex was collected by centrifugation and washed sequentially with low-salt, high-salt, LiCl immune complex and Tris-EDTA washing buffer. Prepared elution buffer (1% SDS, 0.1 M NaHCO3) was used to elute the DNA–protein complex. Samples were incubated overnight at 65°C with 200 µg/mL proteinase K, and subsequently were purified using a DNA purification kit, following the manufacturer protocol. Purified DNA was dissolved in 100 µL of elution buffer finally.

Statistical analysis

SPSS 13.0 (SPSS Science Inc.) and Prism (GraphPad Software) were used for data analysis. The IUGR rate and bodyweight gain rate were calculated and then transformed by arcsine square-root prior to t-test evaluations (Engelbregt et al. 2001, Luo et al. 2014). A paired t-test was used to compare the mean values of the groups without chronic stress.
and after chronic stress. Student’s *t*-test and one-way ANOVA followed by a *post hoc* Dunnett *t*-test or a *post hoc* Bonferroni *t*-test were performed as appropriate. Statistical significance was defined as *P*<0.05.

**Results**

**PCE inhibited testis development in male fetal rats**

The results showed that the PCE(H) group had a reduced bodyweight (89.3% of the control) and increased IUGR rate (*P*<0.01, Fig. 1A), but it did not differ between the control and PCE(L) groups (Fig. 1A). Meanwhile, the fetal testicular maximum area and maximum diameter of the PCE(L) and PCE(H) groups were decreased (*P*<0.01, Fig. 1B). No remarkable disorganized cell arrangement or morphological changes were observed by HE staining (Fig. 1C). However, the immunohistochemical analyses showed that testicular Ki67 protein expression in the PCE(H) group was decreased (*P*<0.05, Fig. 1D). In addition, the TEM images showed some mitochondrial vacuolation in the testicular LCs of the PCE(H) group, whereas the PCE(L) group had no such changes (Fig. 1E). These findings suggested that PCE can induce testicular dysplasia in male fetal rats.

**PCE changed testosterone synthetic function in male fetal rats**

As shown in Fig. 2, the serum testosterone concentration and intra-testicular testosterone content in the PCE(H) group were markedly lower than their respective controls.

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**Figure 1**

Effects of prenatal caffeine exposure (PCE) on testicular morphology in male fetal rats. Pregnant rats were intragastrically administered with low-dose (30 mg/kg/day) and high-dose (120 mg/kg/day) caffeine once per day from gestational day (GD) 9 to 20, which were assigned to PCE(L) and PCE(H) groups, respectively. At GD20, the fetal rats were taken out. The bodyweight, intrauterine growth retardation (IUGR) rate, testicular diameter and area of male fetal rats were recorded (A, *n* = 12) (B, *n* = 3, 100×). The testicular morphology was observed by hematoxylin-eosin dying (C, 200×, 400×), and Ki67 protein expression (red arrows) was detected by immunohistochemistry (D, *n* = 3, 200×, 400×). The Leydig cells structure (red arrows represent injured mitochondria) was observed by transmission electron microscopy (E, 5000×). Mean ± s.d., *P*<0.05. **P**< 0.01 vs control by one-way ANOVA. A full colour version of this figure is available at https://doi.org/10.1530/JOE-18-0684.
The GC-IGF1 axis-mediated testicular dysplasia

L Pei, Q Zhang et al.

The GC-IGF1 axis-mediated testicular dysplasia (P<0.01, Fig. 1A), as well as the mRNA and protein expressions of HSD3B (P<0.05, P<0.01, Fig. 1B and C). Although the serum testosterone concentration remained unchanged and the intra-testicular testosterone content decreased (P<0.05, Fig. 1A) in the PCE(L) group, the mRNA expression of steroidogenic acute regulatory protein (Star) and cytochrome P450 cholesterol side chain cleavage (P450scc) and 3β-hydroxysteroid dehydrogenase (Hsd3b) increased (P<0.05, Fig. 1B). The number of LCs (HSD3B+) (P<0.05, Fig. 1D) decreased obviously in both groups. The above results indicated that PCE could alter the testosterone synthetic function in male fetal rats.

PCE-induced postnatal catch-up growth in male offspring rats

After birth, we recorded the bodyweight and observed the testicular morphology at different time points (PW2, PW6 and PW12) in the PCE(H) groups. The male offspring rats exhibited a lower bodyweight from PW0-1, which gradually increased and was close to the control level at PW12, whereas the corresponding body weight gain rates were significantly higher (P<0.01, Fig. 3A). Meanwhile, the testicular weight, testis index and seminiferous tubule diameter increased gradually in the PCE(H) group (P<0.05, P<0.01, Fig. 3B and C). These results suggested that the IUGR offspring rats in the PCE(H) group exhibited catch-up growth after birth.

PCE-induced testicular dysfunctions in male offspring rats

We also observed postnatal alterations to testicular function in the male offspring rats. Compared to the control group, the serum testosterone concentration in the PCE(H) group decreased at PW2 and PW12, whereas there was no change at PW6 (P<0.01, Fig. 4A). Meanwhile, the intra-testicular testosterone content continued to be lower than that of the control groups (P<0.05, P<0.01, Fig. 4B and C). Moreover, the number of LCs and the amount of spermatozoon in
the PCE(H) group were lower at PW12 (P<0.05, P<0.01, Fig. 4D and E). However, there was no difference in sperm motility between the control and PCE(H) groups (Fig. 4E). Male offspring rats were mated with normal female rats at PW12 to produce F2 generation. The pregnancy rate of the normal female rats remained unchanged, whereas naturally born F2-generation male rats had a decreased birth weight (87.1% of the control) (P<0.05, Fig. 4F). In addition, a significantly higher frequency of morphologically abnormal spermatozoa was noted in the PCE group (P<0.05, Fig. 4G). Collectively, these results indicated that PCE induced low steroidogenesis and abnormal spermatogenesis in the male offspring rats.

**PCE-induced changes of serum corticosterone concentration and testicular IGF1 expression before and after birth**

We further investigated the serum corticosterone concentration and the expression of testicular IGF1 at different time points (GD20, PW2, PW6 and PW12). In utero, the serum corticosterone concentration and testicular Gr expression were higher (P<0.05, P<0.01, Fig. 5A and B) but the IGF1 expression was lower in response to PCE(H) (P<0.05, Fig. 5C and D). The H3K14ac level in the Igf1 promoter region was also decreased (P<0.01, Fig. 5E). However, these indexes did not change
in the PCE(L) group (Fig. 5A, B, C, D and E). After birth, the serum corticosterone concentration showed a decreasing trend over time and was particularly significantly reduced at PW12 ($P<0.05$, $P<0.01$, Fig. 5F). Conversely, testicular IGF1 expression, whether at mRNA or protein levels, increased gradually from PW2-12 ($P<0.01$, Fig. 5G and H). We then found that the H3K14ac level of Igf1 was reduced at PW2, but displayed no noticeable changes after that ($P<0.05$, Fig. 5I). Taken together, these findings suggested that high serum glucocorticoid level can negatively regulate testicular IGF1 expression.

### Effects of chronic stress on serum corticosterone level, testicular IGF1 expression and steroidogenesis

To gain further insight into the negative regulation of serum corticosterone on testicular IGF1 expression and steroidogenesis, we treated male offspring rats with 2 weeks of chronic stress to increase their serum corticosterone level. We then examined the testicular IGF1 expression and steroidogenesis-related indices with or without stress. With chronic stress, both the control and PCE(H) groups showed elevated serum corticosterone concentrations...
and reduced testicular Igf1 expression ($P<0.05$, $P<0.01$, Fig. 6A and B). Moreover, the level of H3K14ac in the Igf1 promoter was downregulated by chronic stress ($P<0.05$, $P<0.01$, Fig. 6C). The expressions of steroid synthetase Star, P450scC, and Hsd3b decreased, as did the serum testosterone and intra-testicular testosterone levels ($P<0.05$, $P<0.01$, Fig. 6D, E and F). These data indicated that a high glucocorticoid level could negatively alter the H3K14ac and expression levels of IGF1, which further inhibited the testicular steroidogenesis.

Corticosterone (rather than caffeine) inhibited IGF1 expression and steroidogenesis in TM3 cells

We employed TM3 cells, which are a type of LCs, to investigate the effects of caffeine and corticosterone on steroidogenesis. Based on the fetal serum caffeine concentration (155±28 μM, Wang et al. 2014) and the aforementioned fetal serum corticosterone concentration (Fig. 5A), we treated the TM3 cells with different concentrations of caffeine (1, 10 and 100 μM) and corticosterone (250, 750 and 1250 nM).

No cytotoxicity was observed up to 100 μM caffeine or 1250 nM corticosterone treatment for 3 days (data not shown). Under the caffeine treatment, there was no obvious change in the expression of Igf1 and Hsd3b (Fig. 7A and B), but the expression of Star and P450scC increased ($P<0.01$, Fig. 7B), as well as the testosterone concentration in the cell culture supernatant ($P<0.05$, $P<0.01$, Fig. 7C), conversely, these indicators were inhibited following corticosterone treatment ($P<0.05$, $P<0.01$, Fig. 7D, E and F), as was the level of H3K14ac in the Igf1 promoter region ($P<0.01$, Fig. 7J). These results indicated that caffeine enhanced steroidogenesis, whereas corticosterone inhibited steroidogenesis in the TM3 cells. To further confirm the effect of corticosterone and IGF1 in steroidogenesis, we administered 2.5 μM RU486 (a GR inhibitor) or 100 ng/mL IGF1 to treat the TM3 cells. The expressions of steroid synthetase and testosterone production were significantly reversed ($P<0.05$, $P<0.01$, Fig. 7G and H). Moreover, the expression and H3K14ac level of Igf1 were also upregulated after RU486 treatment ($P<0.01$, Fig. 7I and J). These results indicated that corticosterone (rather than caffeine) downregulated the
H3K14ac level and expression of IGF1 via GR, which further inhibited steroidogenesis in the LCs.

Discussion

Altered IGF1 expression by PCE may contribute to testicular dysplasia and dysfunction

Testicular development has been depreciated because of maternal undernutrition, changes in lifestyle and exposure to xenobiotics during pregnancy (Dupont et al. 2012). The fetal androgen (testosterone) level is known to be a major determinant of male reproductive disorders (Welsh et al. 2008, 2010) and can be affected by maternal caffeine consumption (Ramlau-Hansen et al. 2008, Dorostghoal et al. 2012, Cavalcante et al. 2014). Previous studies have suggested that IGF1 could influence LC development and testosterone-related steroid synthase expression. In Igf1-knockout mice, the differentiation of LCs and the expression of steroid synthetase were inhibited, as was the level of circulating testosterone (Wang & Hardy 2004, Hu et al. 2010). However, the data still appear to be strikingly absent concerning whether PCE could reduce IGF1 expression and thus influence testicular development during the intrauterine period. Therefore, we detected the expression of local IGF1 in testis tissue and found that it was lower in the PCE(H) group at GD20. The fetal testis size and the expression of Ki67 were decreased, accompanied by obvious mitochondrial damage in LCs. Meanwhile, the PCE(H) group exhibited fewer LCs and lower testosterone production. These results suggest that IGF1 is likely involved in PCE-induced fetal testicular dysplasia.

Studies have shown that low birth weight is one of the risk factors of reproductive disorders in males (Main et al. 2006, Nordin et al. 2014). In the present study, the PCE(H) group displayed a reduced bodyweight and increased IUGR rate at GD20. After the intrauterine period of growth inhibition, body growth often does not merely return to a normal rate; in fact, it exceeds the normal growth rate, resulting in catch-up growth (Hediger et al. 1998). An increasing number of studies have confirmed that individuals with IUGR will undergo catch-up growth after birth, and this growth is mainly associated with elevated IGF1 expression (Kajantie 2003, Jensen et al. 2015). Despite a greater proliferative capacity than normal,
The GC-IGF1 axis-mediated testicular dysplasia
L Pei, Q Zhang et al.

In this study, the weight results suggested that IUGR offspring exhibited a typical catch-up growth pattern. Under this physical condition, we further observed the testicular IGF1 expression and development at different time points (PW2, PW6 and PW12). Our results showed that the testicular weight, testis index and seminiferous tubule diameter of the PCE(H) group increased gradually from PW2-12 and were close to or even higher than those of the control group at PW12. Meanwhile, the testicular IGF1 expression increased relatively with time. Although there were fewer LCs and less steroidogenesis at PW12 than those of their respective controls, there was still a growing trend with HSD3B expression increasing from 46% (PW2) of the control to 79% (PW12) of the control and the intra-testicular testosterone content increasing from 43% (PW2) to 85% (PW12). Taken together, PCE can induce a series of compensatory effects on testicular development in IUGR offspring after birth, which is associated with increased testicular IGF1 expression.

Glucocorticoid is secreted by the adrenal gland and is a regulatory factor of IGF1 expression (Fernandez-Cancio et al. 2008). Previously, we have confirmed that PCE could inhibit the development of the fetal hypothalamic–pituitary–adrenal (HPA) axis, which may be associated with fetal overexposure to maternal glucocorticoid (Xu et al. 2012b). This would further lead to low basal activity and an increased sensitivity of the HPA axis in the offspring rats and directly influences the secretion of corticosterone and the susceptibility to multiple diseases (Xu et al. 2012a, 2018). Hence, serum corticosterone level gradually decreased after birth but can be upregulated by chronic stress. Studies have shown that there is a negative correlation between the cortisol in human cord blood and the IGF1 signaling pathway (Cianfarani et al. 1998). In the present study, we discovered a similar phenomenon. Specifically, in utero, the fetal serum corticosterone level increased while the testicular IGF1 expression decreased in the PCE(H) group. However, after birth, the serum corticosterone level increased but was still lower than that of the control group.

GC-IGF1 axis programming is involved in testicular dysplasia and dysfunction

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The GC-IGF1 axis-mediated testicular dysplasia

L Pei, Q Zhang et al.

The GC-IGF1 axis-mediated testicular dysplasia

The serum corticosterone concentration was elevated and testicular IGF1 expression and testosterone synthesis were markedly inhibited. This negative correlation between the serum corticosterone concentration and testicular IGF1 expression suggested the existence of a ‘GC–IGF1 axis’ in the testicular development of male PCE offspring. This axis is not only involved in the inhibition of testicular development in utero, but it also mediates postnatal catch-up growth and partial testicular development compensation.

A high level of corticosterone (rather than caffeine) mediated the epigenetic programming mechanism of the GC-IGF1 axis

Both corticosterone and caffeine have easy access to the fetus through the placenta because of their lipophilicity. Our previous study also demonstrated that high concentrations of corticosterone and caffeine co-existed in the fetal blood following PCE (Wang et al. 2014). Which of these mediated the testicular dysplasia induced by PCE in male offspring rats? Studies have shown that corticosterone can inhibit the expression of IGF1 (Fernandez-Cancio et al. 2008). The actions of glucocorticoid are predominantly mediated by an intracellular receptor, GR (Ramamoorthy & Cidlowski 2013). GR is widely distributed in different cell types, including LCs (Silva et al. 2010). In vivo, our results showed that PCE(H) could increase GR expression and decrease IGF1 expression in fetal testes. In vitro, we further confirmed that caffeine and corticosterone have opposite roles for IGF1 expression and steroidogenesis. Specifically, corticosterone inhibited it but caffeine excited it. Thus, it is conceivable that the increased steroid synthetase expression in vivo in the PCE(L) group should be attributed to the direct effect of caffeine. RU486, a GR inhibitor, can significantly reverse the downregulation of IGF1 expression and steroidogenesis by corticosterone. When we treated the TM3 cells with exogenous IGF1, the inhibitory effect on steroidogenesis caused by corticosterone could also be rescued. All these findings indicated that PCE(H)-induced high corticosterone level (rather than caffeine) inhibited testicular IGF1 expression in the fetus via the GR, which reduced steroid synthetase expression and testosterone production.

Intrauterine overexposure to glucocorticoid can alter fetal developmental programming to adapt to the environment (Harris & Seckl 2011). The changes in intrauterine programming caused by high glucocorticoid level during pregnancy are likely associated with the epigenetic regulation of important genes (Drake et al. 2011, Crudo et al. 2013). H3K14ac, which is an epigenetic hallmark of transcriptionally active chromatin, participates in regulating the pluripotency and reprogramming capacity of cells (Jayani et al. 2010, Zhang et al. 2016). Bachagol et al. found that the increase in Igf1 expression was related to an increase in its H3K14ac level in the liver (Bachagol et al. 2018). In the present study, we found that the H3K14ac level of the Igf1 promoter decreased at GD20 and PW2 under high corticosterone conditions, but was close to the normal at PW6 and PW12. However, when chronic stress induced an elevated serum corticosterone concentration, the H3K14ac level of the Igf1 promoter region and IGF1 expression decreased. Moreover, we further confirmed in vitro that corticosterone reduced the H3K14ac level in the Igf1 promoter, which was reversed by the GR inhibitor RU486. These results provide evidence that corticosterone negatively regulates H3K14ac and the expression levels of IGF1 via GR.
Conclusion

In this study, we confirmed that PCE could lead to testicular dysplasia and dysfunction before and after birth in male offspring rats. The intrauterine programming mechanism behind this relationship is as follows: the high level of glucocorticoid caused by PCE decreases the H3K14ac level and expression of IGF1 through GR, which further contributes to testicular dysplasia and dysfunction. Moreover, the corticosterone-dependent regulation on the H3K14ac level of the Igf1 promoter and its expression were involved in testicular development after birth (Fig. 8). Therefore, we first proposed a ‘GC–IGF1 axis’ programming mechanism for PCE-induced testicular dysplasia. This in-depth molecular mechanism requires further study in the future. This work will provide an experimental and theoretical basis for the intrauterine origin and therapeutic targets of testicular developmental toxicity-related diseases.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-18-0684.

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

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Author contribution statement
H W conceived and designed the experiments. L G P and Q Z did experimental work and paper writing. C Y, M L and S Z were involved in technical assistance, discussion and consulting. All authors reviewed the manuscript.

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