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 administered 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*.

Key Words

- ▶ glucocorticoid
- ▶ IGF
- ▶ Leydig cells
- ▶ male reproduction
- ▶ testis

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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

retardation (IUGR) (Fortier *et al.* 1993, Chen *et al.* 2014, Greenwood *et al.* 2014). There is evidence that caffeine intake of 300 mg/day in pregnant women is associated with an increased risk of IUGR according to the World Health Organization (WHO) (Guilbert 2003). However, there are some pregnant women whose caffeine intake exceeds this level (>300 mg/day), and may even reach 500 mg/day (CARE Study Group 2008). In addition, PCE has irreversible effects on reproductive parameters and fertility in males (Ramlau-Hansen *et al.* 2008, Dorostghoal *et al.* 2012, Cavalcante *et al.* 2014). IUGR refers to the poor growth of a baby while in the mother's womb during pregnancy. IUGR is believed to put infants at risk for adult reproductive disorders and testicular dysplasia (Francois *et al.* 1997, Moller & Skakkebaek 1997). Despite the known negative effects of PCE and IUGR on testicular development, the underlying mechanism in reference to its intrauterine origin has not been clarified.

Androgens, which are primarily released from Leydig cells (LCs), are synthesized from cholesterol under the catalysis of a plurality of steroid synthetases. Deficient androgen action is important in the origin of male reproductive disorders and in programming male reproductive dysfunctions (Macleod *et al.* 2010). A deficiency in androgens can further induce disease states of the body, such as obesity and insulin resistance (Cohen 1999, Pitteloud *et al.* 2005). Thus, an appropriate level of androgens must be maintained. The role of insulin-like growth factor 1 (IGF1) has been demonstrated in the regulation of testis development, number of LCs and steroidogenesis (Wang *et al.* 2003). The deletion of *Igf1* was shown to result in diminished number of LCs and lower circulating testosterone level in adult males (Wang & Hardy 2004). Our previous studies found that PCE-induced maternal glucocorticoid overexposure in fetal rats and inhibited the expression of *Igf1* in a variety of tissues (Tan *et al.* 2012, Wang *et al.* 2014, Chen *et al.* 2018). Therefore, it is reasonable to speculate that the effect of PCE on testicular development may be influenced by fetal blood glucocorticoid level and local *Igf1* expression.

Altered fetal growth or development can fundamentally change the risk of health disorders in adulthood and perhaps, in future generations (Aiken & Ozanne 2014). This phenomenon is called 'intrauterine programming'. Indeed, early-life events will trigger changes in fetal development trajectory that remain in specific circumstances after birth (Fowden *et al.* 2008). Increasing studies have reported that excessive glucocorticoid exposure is a key initiating factor of intrauterine programming (Moisiadis & Matthews 2014a,b),

and these changes likely involve epigenetic processes (Crudo *et al.* 2013). A study of IUGR in rats found that alterations to the histone code were responsible for reduced *Igf1* expression (Fu *et al.* 2009). Evidence from our laboratory also revealed that histone modification played a role in the permanent reprogramming of the genome in response to PCE (Tan *et al.* 2012). These results suggest that the high glucocorticoid level induced by PCE may program IGF1 expression in the testis through an epigenetic mechanism.

In this study, pregnant rats were treated with different doses of caffeine during middle and late pregnancy as our previous study described (Ao *et al.* 2015, Shangguan *et al.* 2017). By detecting the morphological and functional indexes in male offspring rats, we aimed to confirm the presence of testicular development toxicity and explore its intrauterine programming mechanism by PCE. Moreover, by combining a chronic stress model *in vivo* and cell experiment *in vitro*, we clarified a glucocorticoid-mediated intrauterine programming mechanism of IGF1 and testosterone synthesis. This study elucidates the glucocorticoid-insulin-like growth factor 1 (GC-IGF1) axis-related intrauterine programming mechanism for testicular development toxicity and provides an experimental and theoretical basis for the early prevention and treatment of testicular development toxicity-related diseases.

Materials and methods

Chemicals and reagents

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. 3 β -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 administered 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 (%) = $(\text{PW}_x \text{ bodyweight} - \text{PD}_1 \text{ bodyweight}) \times 100 / \text{PD}_1 \text{ bodyweight}$. 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 ($5-7^{\circ}\text{C}$) 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 $\times$ 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. 10 μ L 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 $\times$. The sperm count was determined with a hemocytometer and expressed as $\times 10^6$ 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 2 μ L of the epididymal fluid were homogenized in 2 mL 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 expressional 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 LCs per unit square of interstitial tissue areas (10⁴ μ m²) 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 4×10^5 cells per well in six-well plates in medium. The cells were treated with various concentrations of caffeine (1, 10 and 100 μ M) or corticosterone (250, 750 and 1250 nM) 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, 2.5 μ M RU486 or 100 ng/mL IGF1 were used alone or combined with 1250 nM 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 nuclease-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 80,000g (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 quantified using a Protein 200 Lab-chip 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

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 $\times$). The testicular morphology was observed by hematoxylin-eosin dyeing (C, 200 $\times$, 400 $\times$), and Ki67 protein expression (red arrows) was detected by immunohistochemistry (D, $n = 3$, 200 $\times$, 400 $\times$). The Leydig cells structure (red arrows represent injured mitochondria) was observed by transmission electron microscopy (E, 5000 $\times$). Mean $\pm$ 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>.

Figure 2

Effects of prenatal caffeine exposure (PCE) on testicular steroidogenesis 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. Serum testosterone concentration and intra-testicular testosterone content were measured by ELISA (A, $n = 8-12$). The mRNA expression of steroidogenic acute regulatory protein (*Star*), cytochrome P450 cholesterol side chain cleavage (*P450scc*) and 3 β -hydroxysteroid dehydrogenase (*Hsd3b*) were detected by RT-qPCR (B, $n = 8-12$). HSD3B protein level was detected by immunohistochemistry (C, $n = 3$, 400 $\times$). Leydig cells (stained with HSD3B, a cytoplasmic steroidogenic enzyme marker) counts were detected by immunofluorescence staining (D, $n = 3$, 200 $\times$). Mean $\pm$ 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>.

($P < 0.01$, 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*) 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. 4A), as well as the HSD3B expression ($P < 0.05$, Fig. 4B and C). Moreover, the number of LCs and the amount of spermatozoon in

Figure 3

Effects of prenatal caffeine exposure (PCE) on testicular morphology in male offspring rats after birth. Pregnant rats in the PCE(H) group were allowed to deliver spontaneously at term. Bodyweight/bodyweight gain rate, testis weight/index and seminiferous tubule diameter/epithelium thickness were recorded and calculated at different time points after birth (A, B, $n = 8-12$) (C, $n = 3$, 400 $\times$). Mean $\pm$ s.d., * $P < 0.05$, ** $P < 0.01$ vs control by the Student's *t*-test. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-18-0684>.

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(H) 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

Figure 4

Effects of prenatal caffeine exposure (PCE) on testicular function in male offspring rats after birth. Pregnant rats in the PCE(H) group were allowed to deliver spontaneously at term. Serum testosterone concentration and intra-testicular testosterone content were measured by radioimmunoassay (A, $n = 8-12$). The mRNA expression of steroidogenic acute regulatory protein (*Star*), cytochrome P450 cholesterol side chain cleavage (*P450scc*) and 3 β -hydroxysteroid dehydrogenase (*Hsd3b*) were detected by RT-qPCR (B, $n = 8-12$). HSD3B protein level was detected by Western blot (C, $n = 3$). Leydig cells (stained with HSD3B, a cytoplasmic steroidogenic enzyme marker) counts were detected by immunofluorescence staining (D, $n = 3$, 400 $\times$). Sperm counts and sperm motility were measured (E, $n = 5$). The rate of pregnancy and bodyweight of F2 generation male rats were recorded (F, $n = 8-12$). Representative photomicrographs of sperm smear collected from epididymal cauda in PCE(H) group were shown (head abnormalities (thin arrows) and tail abnormalities (thick arrows)) (G). The frequency of spermatozoa with abnormal morphology was observed (G, $n = 5$). Mean $\pm$ s.d., * $P < 0.05$, ** $P < 0.01$ vs control by the Student's *t*-test. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-18-0684>.

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

Figure 5

Effects of prenatal caffeine exposure (PCE) on corticosterone concentration and insulin-growth factor 1 (IGF1) expression in male offspring rats before and after birth. 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. And pregnant rats in the PCE(H) group were allowed to deliver spontaneously at term. Serum corticosterone concentration was measured by ELISA (A, F, $n = 8-12$). The mRNA expression of glucocorticoid receptor (*Gr*) and *Igf1* were detected by RT-qPCR (B, C, G, $n = 8-12$). The levels of histone 3 lysine 14 acetylation (H3K14ac) in the *Igf1* promoter were measured by ChIP-PCR (E, I, $n = 3$). IGF1 protein level was detected by immunohistochemistry (D, $n = 3$, 400 $\times$) or Western blot (H, $n = 3$). Mean $\pm$ s.d., * $P < 0.05$, ** $P < 0.01$ vs control by one-way ANOVA or Student's *t*-test. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-18-0684>.

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 ($155 \pm 28 \mu\text{M}$, 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 $100 \mu\text{M}$) and corticosterone (250, 750 and 1250 nM).

No cytotoxicity was observed up to $100 \mu\text{M}$ 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 $2.5 \mu\text{M}$ 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

Figure 6

Effects of chronic stress on testicular insulin-growth factor 1 (IGF1) expression and steroidogenesis in male offspring rats followed prenatal caffeine exposure (PCE). Pregnant rats in the PCE(H) group were allowed to deliver spontaneously at term. Male pups were randomly selected from each dam and subjected to two weeks chronic stress or not from postnatal week 10 to 12. Serum corticosterone concentration was measured by ELISA (A, $n = 8-12$). The mRNA expression of *Igf1*, steroidogenic acute regulatory protein (*Star*), cytochrome P450 cholesterol side chain cleavage (*P450scc*) and 3β -hydroxysteroid dehydrogenase (*Hsd3b*) were detected by RT-qPCR (B, D, $n = 8-12$). The levels of histone 3 lysine 14 acetylation (H3K14ac) in the *Igf1* promoter were measured by ChIP-PCR (C, $n = 3$). Serum testosterone concentration and intra-testicular testosterone content were measured by radioimmunoassay (E, F, $n = 8-12$). Mean $\pm$ s.d., * $P < 0.05$, ** $P < 0.01$ vs control by independent t-test; # $P < 0.05$, ## $P < 0.01$ vs no stress by paired t-test.

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, Nordenvall *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,

Figure 7

Direct effects of caffeine and corticosterone on steroidogenesis in mouse Leydig TM3 cells. The TM3 cells were treated with different concentrations of caffeine and corticosterone for 3 days. Above-mentioned treatment of the cells showed no cytotoxicity. Then, TM3 was treated in the presence of corticosterone (750 nM) with insulin-growth factor 1 (IGF1) (100 ng/mL) or RU486 (2.5 μ M). The mRNA expression of *Igf1*, steroidogenic acute regulatory protein (*Star*), cytochrome P450 cholesterol side-chain cleavage (*P450scc*) and 3 β -hydroxysteroid dehydrogenase (*Hsd3b*) were determined by RT-qPCR (A, B, D, E, G, I, $n = 5$). The testosterone concentration in the cell culture supernatant was detected by radioimmunoassay (C, F, H, $n = 5$). The levels of histone 3 lysine 14 acetylation (H3K14ac) in the *Igf1* promoter were measured by ChIP-PCR (J, $n = 3$). Mean $\pm$ s.d., * $P < 0.05$, ** $P < 0.01$ vs control; # $P < 0.05$, ## $P < 0.01$ vs 750 nM corticosterone by one-way ANOVA or Student's *t*-test.

tissues of developmental inhibition do not always fully recover to normal during catch-up growth (Finkelstein *et al.* 2013). In the present 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.

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

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

Figure 8

The intrauterine programming alteration of GC-IGF1 axis caused by prenatal caffeine exposure is involved in testicular dysplasia and dysfunction in male offspring rats. GC, glucocorticoid; H3K14ac, histone 3 lysine 14 acetylation; HPA, hypothalamic-pituitary-adrenal; IGF1, insulin-like growth factor 1. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-18-0684>.

decreased while the testicular IGF1 expression gradually increased. Intriguingly, when adult offspring (PW12) underwent 2 weeks of chronic stress (ice swimming), 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 Y F Z were contributed to materials, experiments and analysis tools. F L, D J L and S Z were involved in technical assistance, discussion and consulting. All authors reviewed the manuscript.

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