Effects of prenatal chlorocholine chloride exposure on pubertal development and reproduction of male offspring in rats

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HIGHLIGHTS

- Chlorocholine chloride (CCC) prenatal exposure delayed the onset of F1 puberty.
- The HPT axis were involved in the abnormal development in F1 offspring.
- CCC might inhibit the expression of PP1γ2 to decrease the sperm motility in F1.

GRAPHICAL ABSTRACT
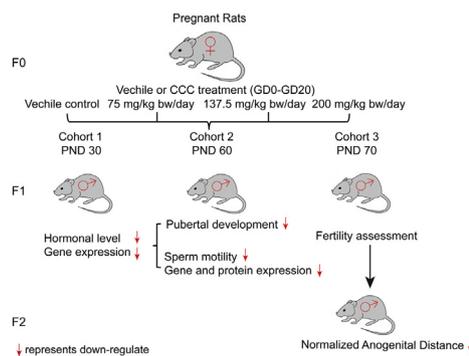
ABSTRACT

Chlorocholine chloride (CCC) promote plant growth as a regulator. Emerging evidence by our group showed that CCC might restrain the puberty onset and impair the reproductive functions in male rats through HPT axis. In this study, we further investigated the effects of prenatal CCC exposure on pubertal development, reproduction of male offspring in rats and explored the underlying mechanisms. The results showed that CCC of 137.5 and 200 mg/kg bw/day delayed the age of preputial separation (PPS), decreased the sperm motility of male offspring. PP1γ2 which is an essential protein in spermatogenesis reduced in 137.5 and 200 mg/kg bw/day groups. Crucial hormones involved in hypothalamic-puititary-testicular (HPT) axis decreased at postnatal day (PND) 30. It was indicated that CCC exposure in pregnancy might disturb the pubertal development, reproductive functions of male offspring through HPT axis and disturb the sperm motility through PP1γ2.

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1. Introduction

The plant growth regulator is widely used in agricultural food production, especially in the production of cereals. Chlorocholine chloride (2-chloro-N, N, N-trimethylethananminium chloride, CCC) is the most commonly used one in European Union (Poulsen et al., 2007; European Food Safety Authority, 2016). It has the ability to shorten and strengthen the stems through reducing the height of the crops accompanied by increasing the stem diameter (Tolbert, 1960; Galea et al., 2015). Food and Agricultural Organization (FAO) and World Health Organization (WHO) established the acceptable daily intake (ADI) of CCC is 0.05 mg/kg bw/day (International Programme on Chemical Safety, 1997). CCC remains its substance in some plants for many years (European Food Safety Authority, 2016). The studies showed that the residue of CCC in pears in UK was 11 mg/kg (Reynolds et al., 2004) and in the grapes from India was 0.5 mg/kg which were much higher than the maximum residue levels (MRLs) 0.05 mg/kg (European Food Safety Authority, 2013). Based on the European Union reports on pesticide residues in food, it was continually found that CCC residues exceeded their MRLs and had the highest frequency of multiple residues in cereals like rye, wheat and oat, and in fruits like pears and tomatoes (European Food Safety Authority, 2013, 2014, 2016). CCC was also detectable in animal products when CCC-treated plants were included in feed rations and offered to the animals (Blinn, 1967; Bier and Ackermann, 1970). It was reported that the concentration of CCC in a chicken sample and a goat meat sample were 15.9 and 2.43 times of its MRL in China (Li et al., 2012).

CCC exerted its action in its intact form without any metabolization in mammlians and it was excreted mainly through urine (EFSA, 2008; Hou et al., 2018). The study using ^{14}C-labeled CCC in rats showed that absorption and elimination practically completed within 24 h and unmetabolized CCC were detected in urine (FAO et al., 1999). The application of CCC resulted in a widespread exposure in human beings. A study indicated that CCC was detectable in 100 urine samples in Sweden (Lindh et al., 2011). In 2019, Registry to Toxic Effects of Chemical Substances (RTECS) of U.S. National Institute for Occupational Safety and Health (NIOSH) classified CCC as a potential endocrine disrupting chemical (EDC) (Registry of Tosic Effects of Chemical Substances, 2019). Emerging evidence suggest that EDCs *in utero* exposure have been associated with adverse reproductive outcomes due to disturbing hormonal regulation (Caserta. et al., 2008).

CCC was reported to disrupt the animal development and reproduction. In our previous *in vitro* studies, it was demonstrated that rat embryo growth and development were suppressed by CCC (Xiagedeer et al., 2016). Even a low dose CCC pregnancy exposure disrupted the embryonic growth and disordered the growth regulators (Xiagedeer et al., 2020). CCC performed the adverse effects on skeletal development (Huang et al., 2016). When pigs were fed with grain included CCC, the mating rate and estrus were reduced (Danielsen et al., 1989). The hens which were applied with 5 p.p.m. CCC had a remarkable depression in production and weight of egg (Gultom et al., 2001). In mice, CCC was found to suppress the functional competence of epididymal spermatozoa and to cause a significantly diminished fertilization (Torner et al., 1999). However, female mice reproduction damage was not observed at an applied level of CCC intake (Sorensen and Danielsen, 2006). It implied that reproduction of male mammal performed more sensitive to CCC than female.

The hypothalamic-puititary-testicular (HPT) axis was compromised of gonadotropin-releasing hormone (GnRH), gonadotropins containing leuteinizing hormone (LH) and follicle-stimulating hormone (FSH) together with sex steroids. It is wide consensus that GnRH which is secreted from the hypothalamus in a periodic pulsatile manner stimulates the synthesis and secretion of LH and FSH (Jin and Yang, 2014). GnRH performs a critical role in puberty onset and regulates the reproduction (Javed et al., 2015). Kisspeptin and its receptor, G protein-coupled receptor-54 (GPR-54), are the upstream regulators to stimulate the pulsatile generation of GnRH and play a key role in the onset of puberty (Seminara et al., 2003; Wahab et al., 2011). The biosynthesis of testosterone (T) is regulated by HPT axis (Hou et al., 2018). Previous studies from our group found that pubertal CCC exposure resulted in the reduction of T in male rats (Hou et al., 2018), delayed the onset of puberty and impaired the reproductive functions through HPT axis (Hou et al., 2020).

In this study, we further investigated the effects of prenatal CCC exposure on pubertal development, reproduction of male offspring in rats and explored the underlying mechanisms. It aimed to provide further scientific data for the safety use and risk assessment of CCC.

2. Materials and methods

2.1. Chemicals

CCC (CAS No. 999-81-5, 99 % pure) was purchased from Beijing Galaxy Tianhong Chemical Company.

2.2. Animals

Adult and healthy Sprague-Dawley rats (SPF) were obtained from Beijing Vital River Laboratory Animal Technology. All rats were housed under controlled conditions of temperature (20∠26 ° C), humidity (40–70 %) and illumination (12/12 h light/dark cycle) with free access to standard rodent chow (20 % protein rodent diet) and autoclaved tap water. All animal procedures were approved by the Animal Welfare & Ethical Inspection Committee of Peking University according to the government guidelines for the Care and Use of Laboratory Animals (Approval No. LA2017024).

2.3. Experimental design

The experimental procedures and treatment methods in this study were described as follows.

After a habituation period of one week, the rats weighting 200–220 g (female) and 300–320 g (male) were mated overnight at a rate of 2:1 to obtain pregnant rats. The day that the sperms were detected was considered as gestational day (GD) 0. On GD0, 24 pregnant rats were randomly assigned into four groups according to body weights. One group of pregnant rats received distilled water as vehicle control and other groups of pregnant rats were treated with CCC at three different doses (75, 137.5, 200 mg/kg bw/day). The ADI of CCC is 0.05 mg/kg bw/day suggested by WHO. Here, the uncertainty factor of 100-fold was used for extrapolation from data of animal to human beings. E.P.A suggested that another 10-fold might be used for developmental toxicity under pesticide exposure (Office of pesticide programs, E.P.A, 2001). The low dose 75 mg/kg bw/day divided by 1000 was 0.075 mg/kg which was very close to the "ADI value" of CCC with an uncertainty factor of 1000. Moreover, the doses which were same as our previous study could have a better comparison of the effects of CCC exposure in different period (Hou et al., 2020). Vehicle or CCC was daily administered to each pregnant rat by oral gavage from GD0 to GD20. Body weight of each pregnant rat was recorded every day. Pregnant females delivered their pups naturally to produce the F1 offspring. The day on which parturition occurred was considered as PND 0 for the pups. The following reproductive indices were determined for each group, gestational length: days from copulation to delivery, gestational index: number of pregnant

females with live pups/number of pregnant females × 100, litter size, survival index: number of live pups on PND4/number of live pups on PND0 × 100, body weight of pups at birth.

F1: After birth, the litters were adjusted to eight pups with four males and four females to ensure standardized nutrition until weaning on PND23. During lactation, body weight of each male pup was recorded on PND4, 7, 14 and 21. Anogenital distance (AGD) of each male pup was measured on PND4, 14 and 21 and normalized by the cube root of body weight. The F1 male offspring were randomly assigned to one of three cohorts after weaning:

Cohort 1: One male from each litter of each group was randomly selected and maintained until PND30. Male offspring were weighted and euthanized on PND30. All rats were sacrificed by exsanguination from the femoral artery after anesthetization with isoflurane. Blood sample was extracted from the angular vein through a capillary siphon and serum was isolated for enzyme linked immunosorbent assay (ELISA) analysis. The hypothalamus was rapidly collected as described (Quennell et al., 2011). Pituitaries and hypothalami were stored at −80 °C for mRNA analysis.

Cohort 2: One male from each litter of each group was randomly selected and maintained until adult (PND60). Body weight of each male was recorded every day. Testis descent (TD) and preputial separation (PPS) were daily monitored until they were present or until PND60. AGD of each male was measured on PND30, 40, 50 and 60 and normalized as mentioned above. Blood sample was extracted from the angular vein through a capillary siphon and serum was isolated for ELISA analysis. Males were sacrificed by exsanguination from the femoral artery after anesthetization with isoflurane on PND60. Pituitaries, hypothalami and left testes were excised and conserved at −80 °C for further analysis. Right testes were fixed for morphological observation. Left epididymides were subsequently removed for sperm analysis. Sperms were collected from right epididymides for western blot analysis.

Cohort 3: One male from each litter of each group was randomly selected and maintained until PND70. Each male was mated overnight with an untreated, sexually mature and virgin female until a copulation occurred or 2 weeks had elapsed. Vaginal smear was examined in the next morning to confirm the presence of sperm as evidences of copulation. Pregnant females delivered their pups naturally to produce the F2 offspring. Reproductive indices including mating index (number of mated females with successful copulation/total number of mated females × 100), fertility index (number of pregnant females/number of mated females with successful copulation × 100), gestational length, gestational index, litter size, survival index. On PND4, body weight and AGD of F2 male pups were recorded. AGD was normalized as mentioned above. All rats were sacrificed by exsanguination from the femoral artery after anesthetization with isoflurane.

2.4. ELISA analysis

Kisspeptin (E-EL-R2530c), GnRH (E-EL-0071c), FSH (E-EL-R0391c), LH (E-EL-R0026c) levels in serum from PND30 and PND60 male offspring were measured with ELISA kits (Elabscience Biotechnology Company, Beijing, China). Serum T (F4421-B) were assayed using ELISA kits (Meimian Industry Co., Ltd, Jiangsu, China). The sensitivities of Kisspeptin, GnRH, FSH, LH and T were 46.88 pg/mL, 9.38 pg/mL, 1.88 ng/mL, 0.94 mIU/mL, 1.255 nmol/L respectively. All procedures followed the manufacturers• instruction.

2.5. Histological analysis

After fixed in Davidson•s fixative (Leagene, DF0004) for 24 h, right testes were processed through graded alcohols and subsequently embedded in paraffin. Sections (5 μm thickness) were cut and stained using hematoxylin and eosin, and then photographed with a light microscope (Nikon E400, Japan) for morphological evaluation.

2.6. Sperm analysis

Sperms in the left epididymides from PND60 male offspring were collected as our previous description (Hou et al., 2018). Epididymal sperm concentration was measured by a hemocytometric counter and sperm motility was determined microscopically. Sperm density was evaluated as the ratio of sperm count to epididymal weight. Sperm morphology was observed by smears to get the percent of abnormal spermatozoa of each male (Dobrzynska and Gajewski, 2020; Hsu et al., 2021).

2.7. Gene expression analysis

The gene transcript expression was examined by real-time polymerase chain reaction (RT-PCR) analysis in hypothalami and pituitaries from PND30 and PND60 male offspring as well as in left testes from PND60 male offspring. In brief, total RNA was extracted from tissues samples using Transzol Up reagent (Transgen Biotech, Beijing, China). The total RNA concentration was quantified and 1 μg of RNA was reverse-transcribed to cDNA using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (Transgen Biotech, Beijing, China) according to the manufacturer•s protocol. Analysis of the transcript levels for gene targets was performed with primes shown in Supplementary Table 1. RT-PCR was conducted using TransStart Top Green qPCR SuperMix kit (Transgen Biotech, Beijing, China). Comparison of the transcript level was made and the relative differences in gene expression were calculated with threshold cycle (C_T) values which were first normalizes to those of actin gene as the endogenous control in the same sample. The relative differences in gene expression compared with control C_T values were calculated using the $2^{-\triangle\triangle C_T}$ method (Livak and Schmittgen, 2001).

2.8. Western blot analysis

After excised, right epididymides from PND60 male offspring were punctured using a needle and kept in 1 mL physiological

Table 1
Sperm quality of male offspring after *in utero* CCC treatment.

Parameters	Dose (mg/kg bw/day)			
	0	75	137.5	200
Sperm concentration (10^6/mL)	22.38 ± 9.84	23.81 ± 4.54	26.00 ± 4.60	28.28 ± 10.14
Sperm density (10^6/g)	68.03 ± 27.78	72.63 ± 12.75	76.32 ± 14.87	87.21 ± 26.69
Sperm motility (%)	21.17 ± 9.43	22.00 ± 1.67	11.67 ± 7.47*	11.67 ± 6.86*
Percent of abnormal spermatozoa (%)	3.95 ± 0.75	4.67 ± 0.66	4.88 ± 1.64	4.28 ± 0.91

Data were presented as mean ± SD, n = 6. *$P < 0.05$ as compared with the control group.

saline prewarmed to 37 °C for 10 min to allow sperms to swim out. The sperm suspension was washed twice in phosphate buffer saline, centrifuged at 1000 g for 10 min at 4 °C and resuspended in 80 μL RIPA buffer with 1% PMSF (Beyotime Biotechnology, China) and sonicated. After sonication, the sperm suspension was centrifuged at 12,000 g for 10 min at 4 °C. Left testicular tissue about 0.05 g was homogenized for 10 s at 4 °C in 400 μL RIPA buffer with 1% PMSF. The homogenate was centrifuged at 12,000 g for 10 min at 4 °C. The sperm and testis supernatant were collected for western blot analysis. The protein quantification was performed by the Enhanced Bicinchoninic Acid (BCA) Protein Assay Kit (Beyotime Biotechnology, China). Aliquots of protein (50 μg of testis and 120 μg of sperm) were analysed by electrophoresis on 10 % SDS-PAGE gel and transferred onto a nitrocellulose membrane. The membrane was incubated for 2 h with 5% skim milk (w/v, Sigma-aldrich, USA) in TBST to block nonspecific binding. The membrane was then incubated with the respective primary antibody: phospho-GSK3α (Ser21) (1: 1000), GSK3α (1: 1000), PP1γ2 (1: 1000), β-actin (1: 2000) or Tubulin (1: 2000) in 5% BSA diluted in TBST overnight at 4 °C. After washed three times in TBST, the membrane was incubated with HRP-conjugated anti-rabbit IgG (1: 5000) as the second antibody in TBST for 2 h at room temperature. After washed three times the immunoreactive bands on the nitrocellulose membrane were detected by chemiluminescence using an ECL-plus kit (GE Health, USA).

2.9. Statistical analysis

The data were presented as mean ± standard deviation (SD) and analysed by SPSS 20.0 (Peking University). A litter was used as a sample unit for all offspring indexes. The data of body weight, AGD and normalized AGD of male offspring were analysed by two-factor repeated measures ANOVA. The data of sperm motility and percent of abnormal spermatozoa were analysed by Pearson chi-square test. The data of survival index were analysed by Fisher•s exact test. For all other data, statistical analysis was performed using the one-way ANOVA followed by homogeneity of variances test. Multiple comparison of Dunnett-t test was conducted when the assumption of equal variance was satisfied, otherwise Tamhane•s T2 test was implemented. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. The effects of CCC on pregnant rats

As shown in Fig. 1, body weights of pregnant rats in the 200 mg/kg bw/day CCC treated group reduced significantly compared with the control group from GD8. However, there were no differences in the other CCC treated groups compared with the control group.

The gestational index, gestational length, litter size, survival index of pups and body weight of pups at birth were detected to evaluate the reproductive performance of pregnant rats after CCC treatments. The results showed that CCC had no influences on the reproductive performance of pregnant rats (Supplementary Table. 2).

3.2. The effects of prenatal exposure CCC on the development of male offspring

There were no significant differences of body weights, AGD and normalized AGD between the CCC-treated groups and the control group of the male offspring (Fig. 2A-F). All male offspring showed TD and PPS. The male offspring in control and 75 mg/kg bw/day CCC treated groups showed complete PPS on PND41, the offspring in 137.5 and 200 mg/kg bw/day CCC treated groups presented

complete PPS on PND44 (Fig. 2K). However, in utero CCC exposure did not affect the age and body weight at TD (Fig. 2G-H), the age and body weight at initial PPS (Fig. 2I-J) and the body weight at complete PPS (Fig. 2L) of male offspring.

3.3. The effects of in utero CCC exposure on sperm quality of male offspring and the expression of spermatogenic proteins

Sperm motility of PND60 male offspring in 137.5 and 200 mg/kg bw/day CCC treated groups was significantly decreased compared with the control group. However, in utero CCC exposure did not affect sperm concentration, density and percent of abnormal spermatozoa of male offspring (Table 1).

Histological analysis of testes in male offspring indicated that CCC treatment didn•t cause any apparent morphological changes. Seminiferous tubules of testes from all male offspring were normal and germ cells in different development stages were regularly arranged (Fig. 3).

The protein expression of PP1γ2 was significantly decreased in sperm (Fig. 4A) and testes (Fig. 4B) from PND60 male offspring exposed to 137.5 and 200 mg/kg bw/day CCC in utero. As shown in Fig. 4C, the gene expression of Ppp1cc which was the coding gene of PP1γ2 consistently reduced in 137.5 and 200 mg/kg bw/day CCC treated groups. However, in utero exposure didn•t affect the protein expression of phospho-GSK3α (Ser21) in sperm (Fig. 4D). All sample replicas were provided in supplementary Fig. 1.

3.4. The effects of in utero CCC exposure on hormonal expression levels of PND30 and PND60 male offspring

In utero exposure to 137.5 and 200 mg/kg bw/day CCC significantly reduced serum levels of kisspeptin (Fig. 5A) and GnRH (Fig. 5B) of PND30 male offspring. Moreover, in utero exposure to 200 mg/kg bw/day CCC also lowered serum level of FSH (Fig. 5C). In utero CCC exposure did not change serum LH level of male offspring (Fig. 5D). However, serum T level of male offspring in all in utero CCC treated groups was significantly decreased (Fig. 5E).

No differences were observed in gene expression levels of Kiss1, Gpr54, Gnrh, Gnrhr and LHβ in the hypothalami and pituitaries samples (Fig. 6A-D, F). The expression of FSHβ was decreased significantly in PND30 male offspring exposed to CCC in utero (Fig. 6E).

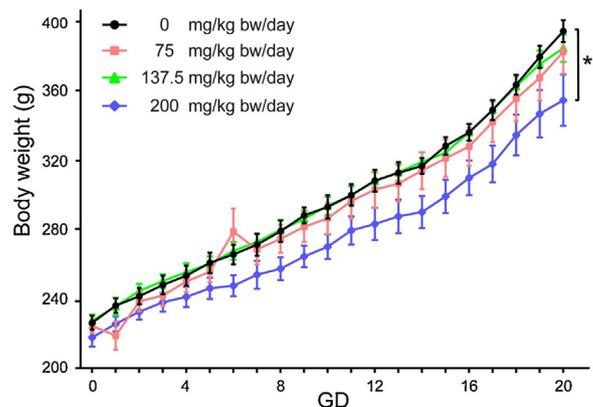At PND60, the secretion of T showed no change, same as the secretion of kisspeptin, GnRH, FSH, LH and the related gene

Fig. 1. Body weights of pregnant rats. Data were presented as mean ± SD, n = 6. *$P <$ 0.05 as compared with the control group. GD, gestational day.
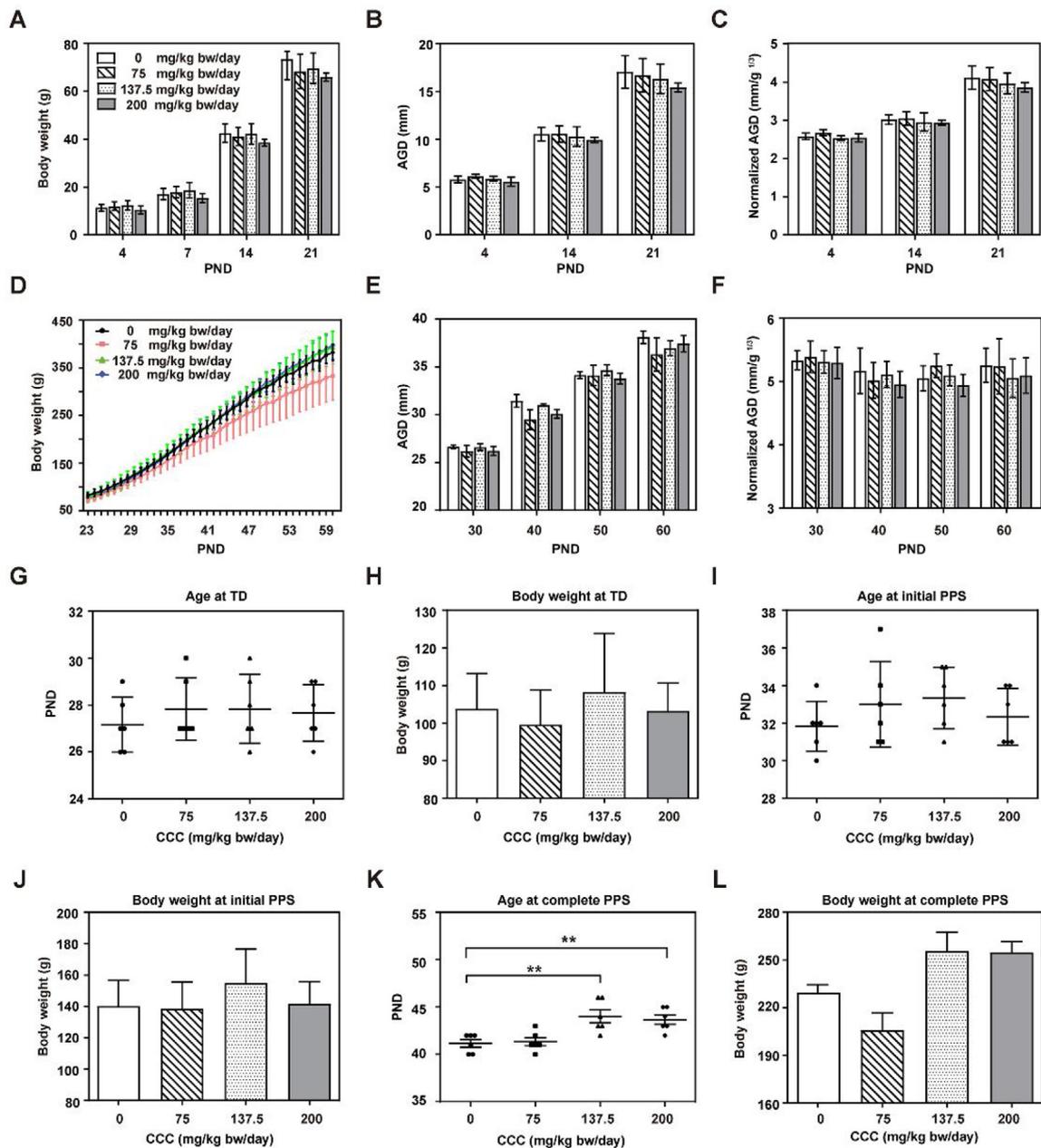
Fig. 2. Body weight and developmental parameters of male offspring. A: Body weight of male offspring before weaning; B: AGD before weaning; C: normalized AGD before weaning; D: Body weight of male offspring after weaning; E: AGD after weaning; F: normalized AGD after weaning; G: Age at TD; H: Body weight at TD; I: Age at initial PPS; J: Body weight at initial PPS; K: Age at complete PPS; L: Body weight at complete PPS; Data were presented as mean ± SD, n = 6. **$P < 0.01$ as compared with the control group. TD, testes descent; PPS, preputial separation; AGD, anogenital distance; PND, postnatal day.

expression remained unchanged after *in utero* CCC exposure (Supplementary Fig. 2–3).

3.5. The effects of prenatal CCC exposure on reproductive performance of male offspring

CCC *in utero* exposure showed no significant effects on reproductive performance of male offspring, including mating index, gestational index, gestational length, litter size and survival index (Supplementary Table 3). There were 2 copulated females not to be successfully pregnant in the 75 and 137.5 mg/kg bw/day groups, respectively.

3.6. The effects of CCC exposure on body weight and AGD of F2 male pups

At PND4 of F2 male pups, body weights (Fig. 7A) and AGD (Fig. 7B) showed no difference among the groups. Whereas, normalized AGD reduced significantly in the F2 male pups from male offspring *in utero* exposed to 137.5 and 200 mg/kg bw/day CCC (Fig. 7C).

4. Disscussion

It is well acknowledged that EDCs have a relationship with adverse pregnancy outcomes like premature birth, abortion, fetal
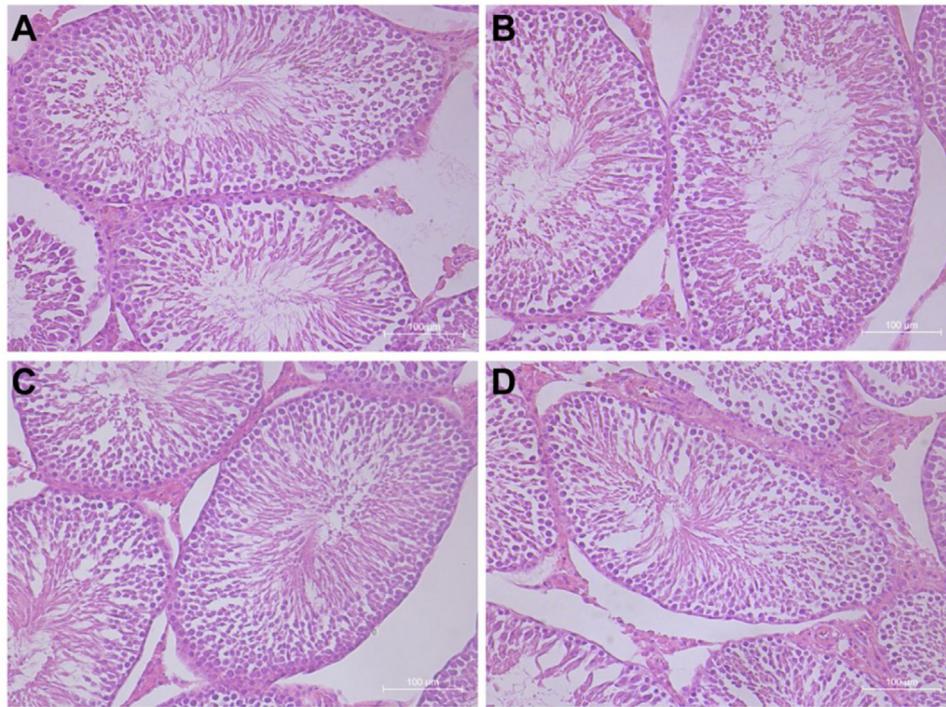
Fig. 3. Testicular histopathology of male offspring after prenatal CCC treatment. A: *In utero* exposure of 0 mg/kg bw/day CCC; B: *In utero* exposure of 75 mg/kg bw/day CCC; C: *In utero* exposure of 137.5 mg/kg bw/day CCC; D: *In utero* exposure of 200 mg/kg bw/day CCC. Representative photomicrographs were captured at magnification × 200.
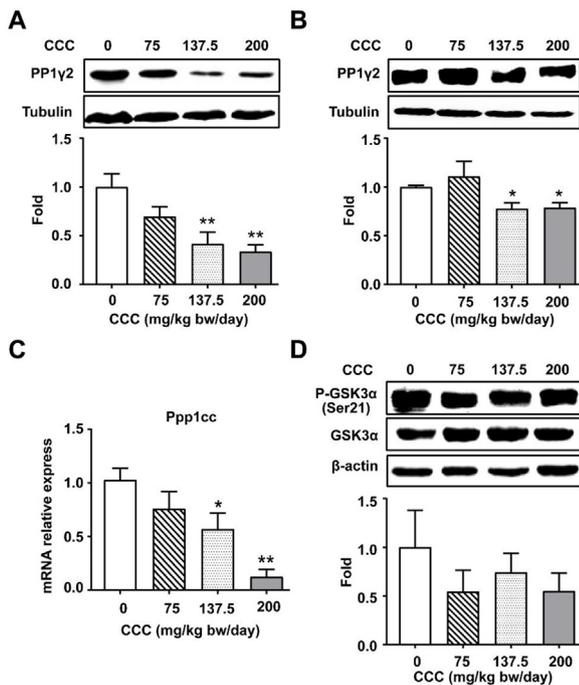

Fig. 4. The expression of spermatogenic proteins in PND60 male offspring. A: the protein expression of PP1γ2 in sperm; B: the protein expression of PP1γ2 in testes; C: the gene expression of Ppp1cc l in testes; D: the protein expression of phospho-GSK3α (Ser21) in sperm; Data were presented as mean ± SD, in A, B, D, n = 3 (randomly selected samples); in C, n = 6. *P <0.05, **P <0.01 as compared with the control group.

growth disorder, low birth weight and so on (Ho et al., 2016). As a suspected EDCs, the prenatal exposure of CCC should be paid more attention. Puberty is a key developmental period which stimulates

the gonadal axis and determines the adequate reproductive capacity (Yardimci et al., 2019). Our previous studies showed that CCC treatments might result in the delay of puberty and impaired the reproductive functions by suppressing the secretion of kisspeptin through HPT axis (Hou et al., 2020) and decreased the T synthesis (Hou et al., 2018). In this research, we focused on the reproductive development of male offspring after prenatal CCC exposure.

It was demonstrated that the reproductive capacity of pregnant rats was not affected by CCC (Supplementary Table. 2). AGD and normalized AGD of male offspring had no apparent differences among all the groups (Fig. 2B-C, E–F). PPS was a representative parameter of puberty onset (Hou et al., 2020). Prenatal CCC exposure delayed average 3 days at complete PPS of male offspring in 137.5 and 200 mg/kg bw/day groups (Fig. 2K). It was indicated that prenatal CCC exposure delayed the puberty onset of offspring.

The results showed that the sperm motility was inhibited compared to the control group (Table 1) in 137.5 and 200 mg/kg bw/day groups. Sperm motility is regulated by phosphoproteins such as type 1 protein phosphatase γ2 (PP1γ2) and glycogen synthase kinase 3α (GSK3α) (Dey et al., 2018; Martin-Hidalgo et al., 2020). PP1γ2 expresses specifically in testis and sperm which is the only one isoform of PP1 expressed in spermatozoa. It is essential for flagellar integrity and the development of flagellar structures (Chakrabarti et al., 2007). PP1γ2 is encoded by Ppp1cc (Sinha et al., 2013). The results showed that both in sperm and in testes, the protein expression of PP1γ2 reduced in 137.5 and 200 mg/kg CCC treated groups (Fig.4A-B). Consistently, CCC caused a reduced Ppp1cc gene expression (Fig. 4C). In addition, the expression of p-GSK3α showed no statistically significant difference between the control group and other groups (Fig. 4D). Therefore, it indicated that prenatal CCC exposure might inhibit the sperm motility by inactivating the expression of PP1γ2 rather than GSK3α.
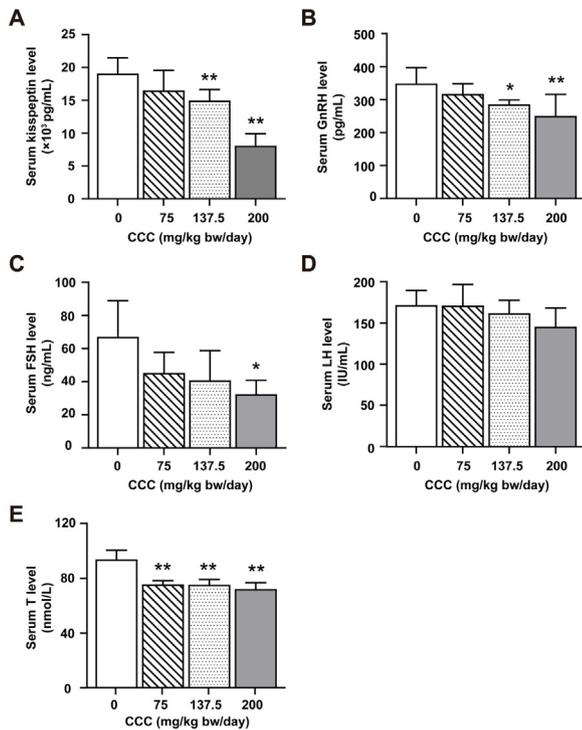
Fig. 5. Hormonal levels of PND30 male offspring. A: Serum kisspeptin level; B: Serum GnRH level; C: Serum FSH level; D: Serum LH level; E: Serum T level. Data were presented as mean ± SD, n = 6. *$P < 0.05$, **$P < 0.01$ as compared with the control group. GnRH, gonadotropin-releasing hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone; T, testosterone.

Fig. 6. Gene expression levels in hypothalami and pituitaries from PND30 male offspring. Genes in hypothalamic: A, Kiss1; B, Gpr54; C, Gnrh. Genes in pituitaries: D, Gnrhr; E, Fshβ; F, Lhβ. Data were presented as mean ± SD, n = 6. *$P < 0.05$, **$P < 0.01$ as compared with the control group.

The HPT axis is essential during the puberty, androgen synthesis, spermatogenesis and reproduction (Dent et al., 2015; Jin and Yang, 2014; O● Shaughnessy, 2014). Kiss1/kisspeptin/GPR-54 system plays an important role in puberty onset and the regulation of reproductive functions through acting upstream of GnRH (Hou et al., 2020). In this study, the serum level of kisspeptin and GnRH reduced at PND30 in 137.5, 200 mg/kg bw/day groups (Fig. 5A-B), which was consistent with the delay of puberty after prenatal CCC exposure. It was indicated that in utero CCC exposure induced the impairment of GnRH secretion might disturb the secretion of kisspeptin, then disturb the pubertal development.

FSH and LH are in response to the GnRH from the hypothalamus which are secreted into the blood stream (Thackary et al., 2010). FSH stimulates Sertoli cell and spermatogonial proliferation, whereas LH/testosterone is mandatory to complete spermatogenesis (Koskenniemi et al., 2017). It has been confirmed that a decreased frequency of pulsatile GnRH favors FSH secretion, meanwhile an increased frequency favors LH secretion (Thompson and Kaiser, 2014). In this study, the serum FSH level (Fig. 5C) and the encoded gene Fshβ expression (Fig. 6E) reduced at PND30 in 137.5 and 200 mg/kg bw/day after prenatal CCC exposure, but the serum LH level (Fig. 5D) and the encoded gene Lhβ (Fig. 6F) remained unchanged. Those results demonstrated that the reduction of FSH secretion might response to the decreased frequency of pulsatile GnRH secretion which was inhibited by prenatal CCC exposure, besides, PND30 was the early age of pubertal development. The secretion of FSH was more sensitive than that of LH at PND 30.

T is an important androgen mainly secreted from Leydig cells under the regulation of HPT axis (Chen et al., 2017a). It plays a vital role in pubertal development and reproductive functions in males (Hou et al., 2020). LH bin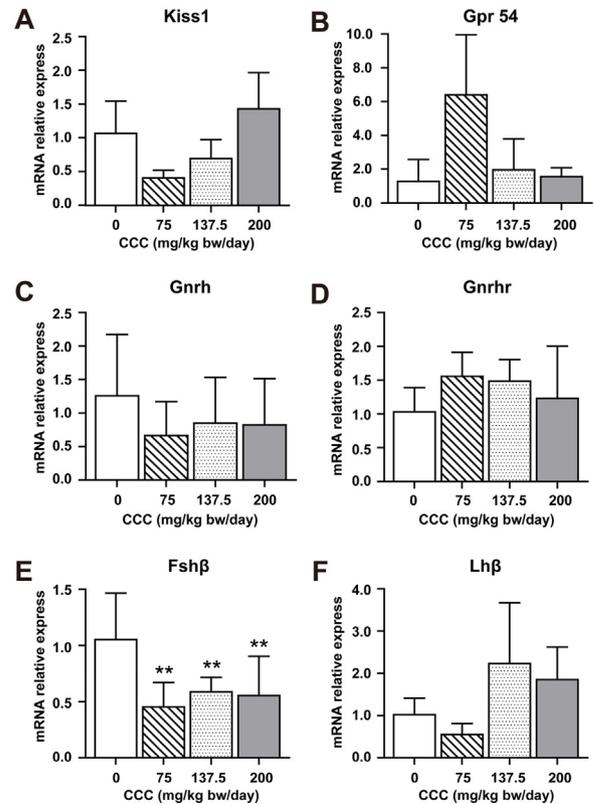ds LH receptor on testicular Leydig cells to active the secretion of T (Hou et al., 2018). Besides, the serum T level was inhibited in all CCC treated groups after prenatal CCC exposure whereas the secretion of LH remained unchanged (Fig. 5D-E). It indicated that CCC reduced the T level which might be not regulated by LH. The capacity of steroidogenesis of the Leydig cells and the number of Leydig cells determined the production of T (Li et al., 2018; Ye et al., 2017). Leydig cells develop sequentially in two cell populations, one is during the fetal period and the other is during the puberty. In puberty, Leydig cell development begins with the appearance of progenitor Leydig cells (PLCs) at PND21 from the commitment of stem Leydig cells (SLCs), then at PND35 SLCs transit through immature Leydig cells (ILCs), and finishes the maturation into adult Leydig Cells (ALCs) at PND49↙56 (Chen et al., 2017b). ILCs start to have the capacity to produce T from androstenedione, but the T is quickly metabolized by androgenmetabolizing enzyme called 5α-reductase 1 (SRD5A). Until ILCs develop into ALCs, the secretion of T is at a stable, dynamic level because of the silence of SRD5A (Ge and Hardy, 1998). It suggested that in utero CCC exposure might inhibit the differential capacity or numbers of fetal Leydig cells and disturbed the process which ILCs transited into ALCs. In this study, at PND60, the secretion of T showed no change, same as the secretion of kisspeptin, GnRH, FSH, LH and the related gene expression remained unchanged after in utero CCC exposure (Supplementary Fig. 2–3). In contrast, in our previous study, CCC pubertal exposure induced the level of kisspeptin, GnRH, FSH and T (Hou et al., 2020). It suggested that CCC pubertal exposure showed a stronger reduction in HPT axis than prenatal exposure.

AGD is an androgen-sensitive marker of reproductive development in human and rodent models which shows more severe
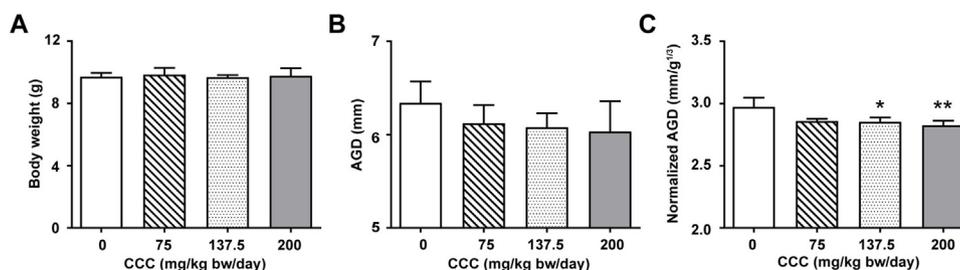
Fig. 7. Body weight and AGD of F2 male pups from male offspring with or without *in utero* CCC treatment. A: Body weight; B: AGD; C: Normalized AGD. Data were presented as mean $\pm$ SD, n = 6. *P <0.05, **P <0.01 as compared with the control group.

when exposure occurs during prenatal or early postnatal development (Nelson et al., 2020). Furthermore, normalized AGD was developed to adjust for the effect of body weight on AGD in animal studies (Gallavan et al., 1999; Thankamony et al., 2016). In this study, prenatal CCC exposure reduced the normalized AGD of F2 male offspring in 137.5 and 200 mg/kg bw/day groups on PND4 (Fig. 7C). The results indicated that CCC treatment might have a biologically significant disturbance in the development of F2 males. However, it was observed that no differences in PND4 AGD or normalized AGD for F1 male offspring in CCC treated groups (Fig. 2B-C). Comparing the PND4 normalized AGD for the F1 and F2 male offspring, the measurements in the treated groups appeared similar, but the higher control of F2 might cause the reduced normalized AGD in treated groups statistically. It still needed more evidence to explore the effects of CCC prenatal exposure on F2 generation.

5. Conclusion

This work focused on the effects of CCC exposure during the critical period of development-prenatal window. Our data suggested that CCC prenatal exposure might inhibit the secretion of crucial hormones in HPT axis resulting in the delay of the puberty onset and down-regulate the expression of PP1γ2 to decrease the sperm motility of F1 male offspring in 137.5 and 200 mg/kg bw/day groups. CCC *in utero* exposure decreased the level of testosterone. Our findings have a new insight into the CCC *in utero* exposure affect the pubertal and developmental outcomes.

CRediT authorship contribution statement

Qianqian Xiao: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Xiaohong Hou:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Validation. **Chenping Kang:** Conceptualization, Methodology, Writing - review & editing. **Bayindala Xiagedeer:** Software, Formal analysis. **Hong Hu:** Software, Formal analysis. **Qinghe Meng:** Validation. **Jianjun Jiang:** Validation. **Weidong Hao:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

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