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Pubertal cadmium exposure impairs testicular development and spermatogenesis via disrupting testicular testosterone synthesis in adult mice

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ABSTRACT

Cadmium (Cd) is a well-known testicular toxicant. However, the effects of pubertal Cd exposure on testicular development and spermatogenesis remained to be elucidated. The present study investigated the effects of pubertal Cd exposure on testicular development and spermatogenesis. Male CD-1 mice were intraperitoneally injected with $CdCl_2$ (1 mg/kg) daily from postnatal day 35 (PND35) to PND70. As expected, pubertal Cd exposure significantly decreased the number of spermatozoa in epididymides. In addition, pubertal Cd exposure markedly reduced the weights of testes, epididymides and prostate and seminal vesicle in adult mice. A significant decrease in serum and testicular testosterone (T) was observed in mice exposed to Cd during puberty. Moreover, pubertal Cd exposure markedly reduced mRNA and protein levels of testicular StAR, P450scc, $P450_{17\alpha}$ and 17β-HSD. Taken together, these results suggest that the decreased testicular T synthesis might partially contribute to pubertal Cd-induced impairment on testicular development and spermatogenesis in mice.

1. Introduction

Cadmium (Cd) is one of major occupational and environmental toxicants. Cd is frequently used in electroplating, pigments, paints, welding, and Ni–Cd batteries. Workers in these occupations are exposed to Cd at significantly higher levels than the general public. Volcanic activity, fossil fuel combustion, forest fires, and transportation of contaminated soil particles by wind constitute the major natural activities responsible for introducing Cd to the environment [1]. The general population is exposed to Cd via drinking water, food and cigarette smoking. It has been suggested that Cd is involved in carcinogenesis in multiple organs including lung, kidney, prostate, liver, pancreas, and testes [2]. Cd is also a potent teratogen in rodent animals [3]. When administered to mice during gestation, Cd-induced malformations of the neural tube, craniofacial region, limbs, trunk, viscera, and axial skeleton in fetuses [4–7].

Within the last decades, numerous studies have demonstrated that environmental and occupational Cd exposures are associated with male infertility and the poor semen quality in humans. According to several epidemiological investigations, a significant inverse correlation was observed between blood Cd level and semen quality [8–10]. Even a low level of Cd accumulation in semen might contribute to male infertility by reducing sperm quality [11]. A recent study showed that the level of Cd in seminal plasma in general population was associated with semen quality and oxidative DNA damage in human spermatozoa [12].

Cd was also a testicular toxicant in rodent animals [13]. At high doses, Cd-induced testicular damage included interstitial edema, hemorrhage and necrosis, accompanied by damage to seminiferous tubules affecting sperm cells and their precursors [14,15]. In addition, treatment with cadmium chloride ($CdCl_2$) caused a significant decrease in sperm concentration, motility, weight of testes and epididymis, and increase in dead and abnormal sperm [16]. Androgens, primarily testosterone (T), are essential for normal spermatogenesis [17]. Although Cd-mediated testicular toxicity was recognized decades ago [18,19], previous studies mainly focused on Cd-induced changes in testicular histopathology. Few studies have investigated the effects of post-pubertal Cd exposure on testicular T synthesis. According to several earlier studies, the level of serum T was significantly decreased in Cd-treated adult rodent animals [20,21]. Two in vitro studies also observed a decreased T secretion in Cd-treated primary rat Leydig cells [22,23]. However, little is known about the effects of pubertal Cd exposure on testicular T synthesis.

In the present study, we investigated the effects of pubertal Cd exposure on testicular development and spermatogenesis in mice. We found that pubertal Cd exposure impaired testicular development and spermatogenesis. In addition, pubertal Cd exposure

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Table 1
Primers the number of cycles and annealing temperature for RT-PCR.

Name	Sequence	Denaturation (°C)	Annealing (°C)	Extension (°C)	Number of cycles (n)	Size (bp)
GAPDH	Forward: 5′-GAGGGGCCATCCACAGTCTTC-3′ Reverse: 5′-CATCACCATCTTCCAGGAGCG-3′	94	56	72	35	340
StAR	Forward: 5′-TGTCAAGGAGATCAAGGTCCTG-3′ Reverse: 5′-CGATAGGACCTGGTTGATGAT-3′	94	57	72	41	310
P450scc	Forward: 5′-AGGTGTAGCTCAGGACTTCA-3′ Reverse: 5′-AGGAGGCTATAAAGGACACC-3′	94	56	72	38	370
$P450_{17\alpha}$	Forward: 5′-CCAGGACCCAAGTGTGTTCT-3′ Reverse: 5′-CCTGATACGAAGCACTTCTCG-3′	94	56	72	38	250
17β-HSD	Forward: 5′-ATTTTACCAGAGAAGACATCT-3′ Reverse: 5′-GGGGTCAGCACCTGAATAATG-3′	94	52	72	40	367

disrupted testicular T synthesis via downregulating the expression of testicular steroidogenic acute regulatory (StAR) protein and several T synthetic enzyme genes. The decreased testicular T synthesis might, at least partially, contribute to Cd-induced impairment on testicular development and spermatogenesis in mice.

2. Materials and methods

2.1. Chemicals

Cadmium chloride ($CdCl_2$) was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were from Sigma or as indicated in the specified methods.

2.2. Animals and treatments

Male CD-1 mice (4-week old, 18–22 g) were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories, Inc. The animals were allowed free access to food and water at all times and were maintained on a 12-h light/dark cycle in a controlled temperature (20–25 °C) and humidity (50 ± 5%) environment. After a week of quarantine and acclimation, 36 male mice were randomly divided into two groups. In Cd group, mice were intraperitoneally (i.p.) injected with $CdCl_2$ (1 mg/kg, 0.1 mg/ml dissolved in sterile normal saline) daily from PND35 to PND70. The control mice were administered with the same volume of normal saline (NS). Our preliminary results showed that testicular hemorrhage, edema and necrosis were observed in Cd-treated mice at doses of 2 or 4 mg/kg/day (data not shown) but not at 1 mg/kg/day. In order to investigate the low dose effects of Cd on testicular development in more detail, we dosed the mice in the current study with the sub-necrotic dose of 1 mg/kg/day. All mice were anesthetized with sodium pentobarbital and killed at PND70. Blood sera were collected. Cauda epididymides from 12 mice each group were removed for analysis of sperm quality. Testes were excised, dissected, weighted, and then divided in two parts: left one was kept at −80 °C for subsequent measurements. The other part of the testes was immersed in modified Davidson's fluid (mDF) for 12–24 h for histology and apoptosis analysis [24]. Sera and testes from 12 mice were randomly chosen for measurement of T by RIA. Six testes from six different mice were applied to RT-PCR or immunoblotting analysis. The determination of Cd in biological samples (serum, testis, kidney and liver) was performed by graphite furnace atomic absorption spectrometry (GFAAS). All procedures on animals followed the guidelines for humane treatment set by the association of laboratory animal sciences and the center for laboratory animal sciences at Anhui medical university.

Table 2
Cd level in serum, liver, kidney and testis in mice at PND70.

	NS	Cd
Serum Cd (μg/l)	0.76 ± 0.158	32.19 ± 3.189**
Cd in liver (μg/g)	0.04 ± 0.017	30.09 ± 3.596**
Cd in kidney (μg/g)	0.13 ± 0.035	41.25 ± 5.480**
Cd in testis (μg/g)	0.07 ± 0.021	1.70 ± 0.256**
The relative amount of Cd in liver against Cd in serum	0.055 ± 0.0217	0.961 ± 0.1117**
The relative amount of Cd in kidney against Cd in serum	0.171 ± 0.0456	1.281 ± 0.1702**
The relative amount of Cd in testis against Cd in serum	0.097 ± 0.0281	0.053 ± 0.0080

Data were expressed as means ± SEM. $n = 12$.
** $P < 0.01$ as compared with NS group. NS, normal saline.

2.3. Sperm analysis

The cauda epididymides were removed and immediately immersed into the F12 medium supplemented with 0.1% bovine serum albumin (BSA). Spermatozoa were released by mincing the epididymides in the collection medium. The sperm suspensions were incubated at 37 °C. After incubation, sperm suspension was analyzed for the number of spermatozoa according to WHO laboratory manual 4th edition [25].

2.4. Histology and immunohistochemistry

Two cross-sections from each testis were embedded in paraffin using standard procedures performed by Pathological Lab at Anhui Medical University. Paraffin-embedded tissues were serially sectioned. At least two nonserial sections were stained with hematoxylin and eosin (H&E) using standard procedures for morphological analyses. Leydig cells were identified by staining for 3β-hydroxysteroid dehydrogenase (3β-HSD). Sections of 5 μm were mounted onto coated slides, dewaxed, and rehydrated. Antigen retrieval was performed by pressure cooking slides for 5 min in 0.01 M citrate buffer (pH 6.0). Slides were incubated for 30 min in 0.3% (v/v) hydrogen peroxide in methanol to block endogenous peroxidase activity and then washed in Tris-buffered saline [TBS; 0.05 M Tris, 0.85% (w/v) NaCl (pH 7.4)]. Nonspecific binding sites were blocked with an appropriate normal serum diluted 1:5 in TBS containing 5% (w/v) BSA before the addition of rabbit polyclonal antibody against mouse 3β-HSD (Santa Cruz, USA) and overnight incubation at 4 °C. After washing in TBS, slides were incubated for 30 min with the appropriate secondary antibody conjugated to biotin goat anti-rabbit (Santa Cruz, USA), diluted 1:500 in the blocking mixture. This was followed by 30 min incubation with horseradish peroxidase-labeled avidin–biotin complex (Dako). Immunostaining was developed by application of diaminobenzidine (liquid DAB^+; Dako), and slides were counterstained with hematoxylin, dehydrated, and mounted using Pertex mounting medium (Cell Path, Hemel Hempstead, UK). The number of 3β-HSD-positive cells was counted in 12 randomly selected fields from each slide at a magnification of 400×.

2.5. Radioimmunoassay (RIA)

Serum was separated by centrifugation and stored at −80 °C until assay for T. T in serum was measured using ^{125}I-based RIA kits from Beijing north institute of biological technology (Beijing, China). For measuring testicular T in mice, testes

Table 3
Body weight, reproductive organs weight and sperm count in mice at PND70.

	NS	Cd
Body weight (g)		
PND35	21.8 ± 0.57	22.0 ± 0.65
PND70	35.1 ± 0.76	31.1 ± 0.51**
Absolute weight of testes (mg)	239.7 ± 7.18	203.0 ± 9.06**
Absolute weight of epididymides (mg)	90.2 ± 5.45	71.0 ± 5.15*
Absolute weight of prostates and seminal vesicles (mg)	329.6 ± 22.01	229.4 ± 20.17**
Relative weight of testes (mg/g body weight)	6.8 ± 0.20	6.5 ± 0.29
Relative weight of epididymides (mg/g body weight)	2.6 ± 0.16	2.3 ± 0.17
Relative weight of prostates and seminal vesicles (mg/g body weight)	9.4 ± 0.63	7.4 ± 0.65*
Sperm count ($\times 10^6$)	15.6 ± 0.81	10.0 ± 0.85**

Data were expressed as means ± SEM. $n = 12$; NS, normal saline.
* $P < 0.05$ as compared with NS group.
** $P < 0.01$ as compared with NS group.

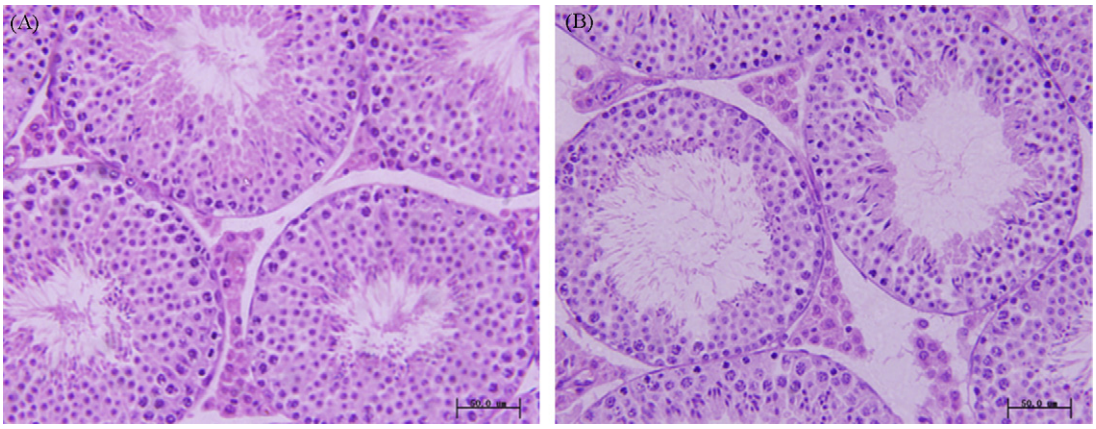

Fig. 1. Effects of pubertal Cd exposure on testicular histology. Male mice were injected with $CdCl_2$ (1 mg/kg, i.p.) daily from PND35 to PND70. Testes were collected at PND70. Testicular cross-sections from control (A) and Cd-treated (B) mice were stained with H&E. Magnification: 400×. NS, normal saline.

were homogenized in 0.5 ml PBS (pH 7.4). T was extracted from homogenate using diethyl ether. After extraction, the organic phase was evaporated into dryness in a fume hood, the steroids were solubilized in an aliquot of PBS, and measured using ^{125}I-based RIA kits (Beijing, China) following the manufacturer's protocols for serum samples. The concentration of testicular T was expressed as ng/mg protein.

2.6. Semiquantitative RT-PCR

Total cellular RNA was extracted using TRI-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. RNase-free DNase (Promega, Madison, WI) was used to remove genomic DNA. The integrity and concentration of RNA was determined by measuring absorbance at 260 nm followed by electrophoresis on agarose gels. For the synthesis of cDNA, 1.0 μg of total RNA from each sample was reverse transcribed in a 40 μl reaction volume containing 0.5 μg oligo$(dT)_{15}$ (Promega, Madison, WI), 1 mM of each dNTP, 15 units AMV RT (Promega), and 1 U/μl recombinant RNasin RNase inhibitor (Promega) in 5 mM $MgCl_2$, 10 mM Tris·HCl (pH 8.0), 50 mM KCl, and 0.1% Triton X-100. The mixture was heated to 70 °C for 10 min, maintained at 42 °C for 30 min, and then heated to 95 °C for 5 min to terminate the reaction. Reaction mixtures (25 μl) for PCR were assembled using 2 μl cDNA template, 0.625 units Hot Start Polymerase (Promega), 200 μM of each dNTP, 1.5 mM $MgCl_2$, and 1.0 μM of each primer in 1× Flexi buffer. The primers were synthesized by Sangon Biological Technology (Shanghai, China), according to sequence designs previously described. The primer pairs, the number of cycles and annealing temperature are shown in Table 1. The amplified PCR products were subjected to electrophoresis at 75 V through 1.5% agarose gels (Sigma, St. Louis, MO) for 45 min. The pBR322 DNA digested with Alu I was used for molecular markers (MBI Fermentas). Agarose gels were stained with 0.5 mg/ml ethidium bromide (Sigma, St. Louis, MO) TBE buffer.

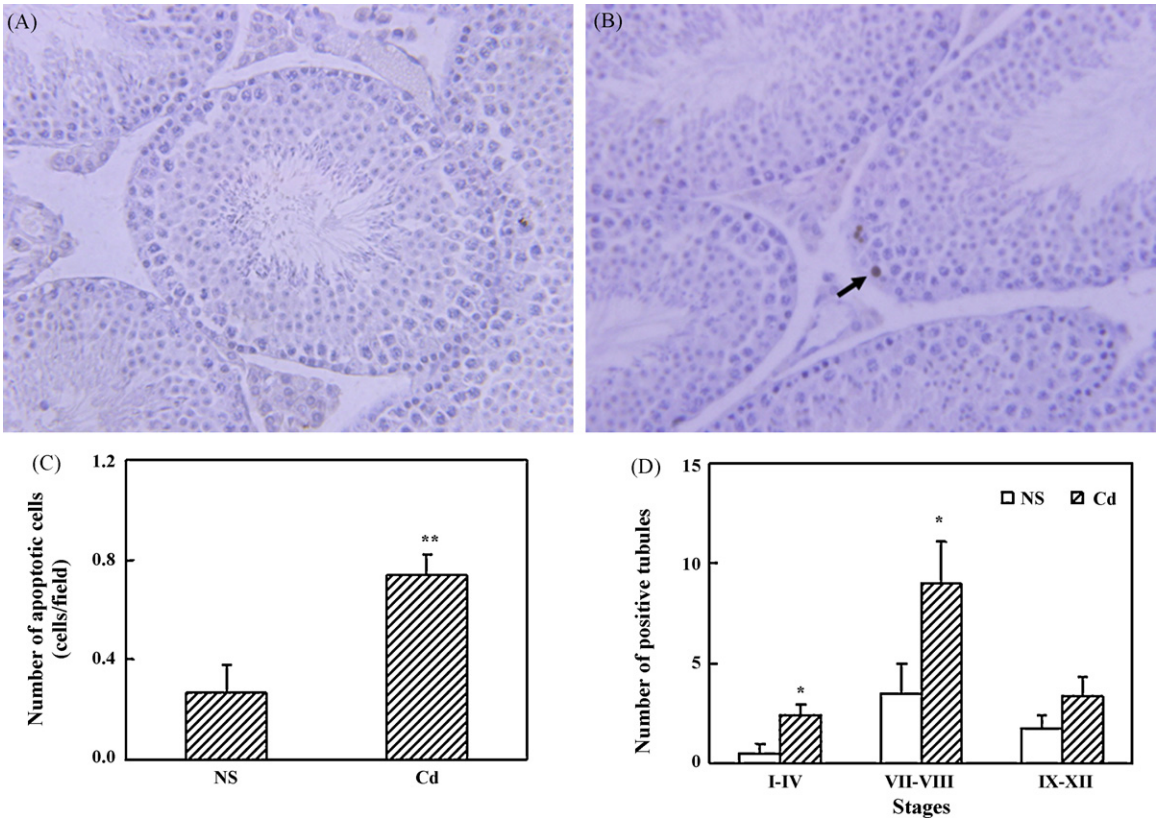

Fig. 2. Effects of pubertal Cd exposure on apoptosis in testes. Male mice were injected with $CdCl_2$ (1 mg/kg, i.p.) daily from PND35 to PND70. Testes were collected at PND70. Apoptosis was analyzed with TUNEL staining of testicular sections from (A) control and (B) Cd-treated mice. Arrows show TUNEL-positive germ cells. (C) $TUNEL^+$ cells in testes were counted in 12 randomly selected fields from each slide at 400× magnification. (D) The number of TUNEL-positive tubules from stages I–VI, VII–VIII, and IX–XII was counted in each slide at a magnification of 400×. Data were expressed as means ± SEM of 12 samples from 12 different mice. $^*P < 0.05$, $^{**}P < 0.01$ as compared with controls. NS, normal saline.

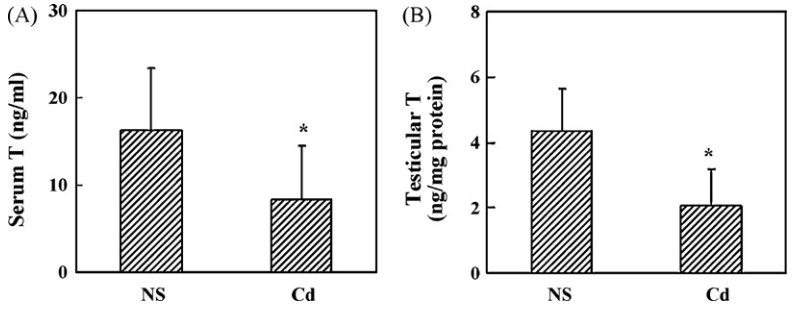

Fig. 3. Effects of pubertal Cd exposure on serum and testicular T. Male mice were injected with $CdCl_2$ (1 mg/kg, i.p.) daily from PND35 to PND70. Sera and testes were collected at PND70. The level of T in (A) sera and (B) testes was measured by RIA. Data were expressed as means ± SEM of 12 samples. $^*P < 0.05$ as compared with controls. NS, normal saline.

2.7. Immunoblotting

Testes were homogenized in lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsulphate (SDS), and 1 mM phenylmethylsulfonyl fluoride (PMSF). Samples were then centrifuged at 15,000 × *g* for 15 min. Supernatants from each sample were added to a gel loading buffer (100 mM Tris, pH 6.8, 20% glycerol, 200 mM DTT, 4% SDS, 0.03% bromophenol blue) and boiled for 5 min. Proteins (50 μg/sample) in loading buffer were subjected to electrophoresis in 10% SDS-polyacrylamide gel for 3 h. The gel was transferred electrophoretically onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA, USA) and blocked in 5% nonfat powdered milk in Dulbecco's PBS (DPBS) overnight at 4 °C. The membranes were then incubated for 2 h with rabbit polyclonal antibody against StAR, $P450_{17\alpha}$, 17β-HSD and P450scc (Santa Cruz Biotechnology, USA) (1:1000 dilutions) or β-actin (Beijing Biosynthesis Biotechnology, Beijing, China) (1:2000 dilutions) at room temperature. After washes in DPBS containing 0.05% Tween-20 four times for 10 min each, the membranes were incubated with goat anti-rabbit IgG antibody for 2 h. The membranes were then washed for four times in DPBS containing 0.05% Tween-20 for 10 min each, followed by signal development using an enhanced chemiluminescence (ECL) detection kit from Pierce (Pierce Biotechnology, Rockford, IL).

2.8. Terminal dUTP nick-end labeling (TUNEL) staining

For the detection of apoptosis in testes, paraffin-embedded sections were stained with the TUNEL technique using an in situ apoptosis detection kit (Promega) according to the manufacturer's protocols. All sections were counterstained with hematoxylin. The number of TUNEL-positive germ cells was counted in 12 randomly selected fields from each slide at a magnification of 400×. And the number of TUNEL-positive tubules from stages I–VI, VII–VIII, and IX–XII was counted in each slide at a magnification of 400×.

2.9. Measurement of Cd

The determination of Cd in biological samples (serum, testis, kidney and liver) was performed by graphite furnace atomic absorption spectrometry (GFAAS) using the stabilized-temperature-platform-furnace coupled with a D_2-lamp background correction system. The external quality-control program did not show any time trend in the accuracy of the cadmium measurements. Detection limits were based on three times the relative standard deviation of 20 consecutive measurements of the blank solution (1% HNO_3) according to IUPAC, being 0.10 μg/l Cd. Precision of the method was measured by coefficients of variation (CV). Mean CV for measurement of Cd in biological samples was 3.5% for within-day determinations and 3.9% for day-to-day determinations. For serum samples, initially studied a 1 + 4 (v/v) dilution with 1% HNO_3. For other biological samples (testis, kidney, and liver), samples were decomposed in duplicate according to the following procedure: 200 mg of samples were accurately weighed in a digestion tube, a 3 ml freshly prepared mixture of concentrated HNO_3–H_2O_2 was added to each tube, and the solutions were kept at room temperature for 12 h, the clear transparent digests were obtained. After this period, the mixture was boiled nearly to dryness, and the residue quantitatively transferred to a 10 ml volumetric flask with 1.0% HNO_3. The resulting solution was analyzed by GFAAS by taking 10 μl of the digest under the aforementioned condition.

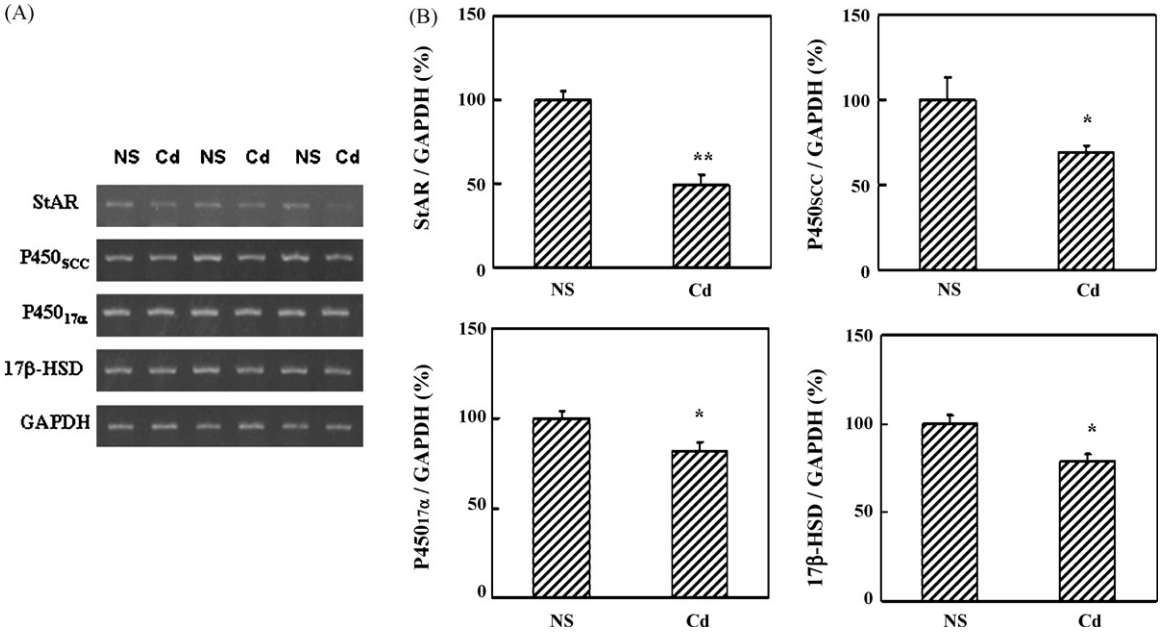

Fig. 4. Effects of pubertal Cd exposure on mRNA levels of testicular StAR and T biosynthetic enzymes. Male mice were injected with $CdCl_2$ (1 mg/kg) daily from PND35 to PND70. Testes were collected at PND70. (A) StAR, P450scc, $P450_{17\alpha}$ and 17β-HSD mRNA was measured using RT-PCR. (B) Quantitative analysis for the level of StAR, P450scc, $P450_{17\alpha}$ and 17β-HSD mRNA from six individual RNA samples was performed. StAR, P450scc, $P450_{17\alpha}$ and 17β-HSD mRNA were normalized to GAPDH mRNA level in the same samples. StAR, P450scc, $P450_{17\alpha}$ and 17β-HSD mRNA level of the control was assigned as 100%. All data were expressed as means ± SEM of six samples from six different mice. $^*P < 0.05$, $^{**}P < 0.01$ as compared with controls. NS, normal saline.

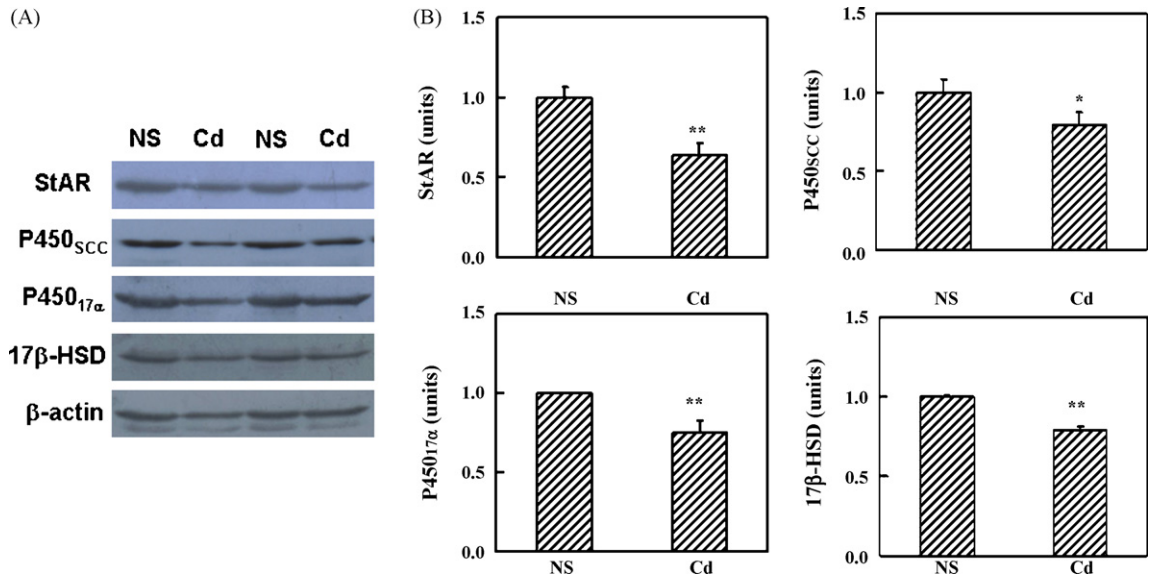

Fig. 5. Effects of pubertal Cd exposure on protein level of testicular StAR and T biosynthetic enzymes. Male mice were injected with $CdCl_2$ (1 mg/kg) daily from PND35 to PND70. (A) The protein level of StAR, $P450_{17\alpha}$, 17β-HSD and P450scc was measured using immunoblotting. (B) Quantitative analysis of scanning densitometry was performed. StAR, P450scc, $P450_{17\alpha}$ and 17β-HSD were normalized to β-actin level in the same samples. The densitometry unite of the control was assigned as 1. All data were expressed as means ± SEM of six samples from six different mice. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with controls. NS, normal saline.

2.10. Statistical analysis

For RT-PCR, StAR, $P450_{17\alpha}$, 17β-HSD and P450scc mRNA were normalized to GAPDH mRNA level in the same samples. StAR, $P450_{17\alpha}$, 17β-HSD and P450scc mRNA level of the control was assigned as 100%. For Western blot studies StAR, $P450_{17\alpha}$, 17β-HSD and P450scc were normalized to β-actin level in the same samples. The densitometry unite of the control was assigned as 1. All quantified data were expressed as means ± SEM at each point. Student's *t*-test was used to determine differences between the Cd-treated mice and controls.

3. Results

3.1. Level of Cd in sera, livers, kidneys and testes

The level of Cd in sera, livers, kidneys and testes at adulthood (PND70) was measured by GFAAS. As shown in Table 2, the absolute amounts of Cd in sera, livers, kidneys and testes were significantly increased in Cd-treated mice as compared with controls. In addition, pubertal exposure to Cd also obviously increased the relative contents of Cd in mouse livers, kidneys against sera. However, no significant difference on the relative contents of Cd in testes was observed between Cd-treated mice and controls.

3.2. Weights of male reproductive organs and sperm quality

No animals died during Cd administration period. The effects of pubertal Cd exposure on body weight and the absolute and relative weights of testes, epididymides, prostates and seminal vesicles on PND70 are presented in Table 3. Results showed that pubertal Cd exposure significantly reduced body weight and the absolute weights of testes, epididymides, and prostates and seminal vesicles on PND70. In addition, the relative weight of prostate and seminal vesicle at PND70 was markedly decreased in mice exposed to Cd during puberty. The effects of pubertal Cd exposure on sperm count in cauda epididymides were analyzed. As shown in Table 3, pubertal Cd exposure significantly reduced the number of sperm in cauda epididymides.

3.3. Histology and apoptosis in testes

The effects of pubertal Cd exposure on testicular histology are shown in Fig. 1. Testicular morphology in control mice was normal (Fig. 1A). Although no interstitial edema, hemorrhage and necrosis were observed in testes of Cd-treated mice, pubertal Cd exposure markedly increased the inside diameter of seminiferous tubules and disturbed the array of spermatogenic cells in testicular sections of adult mice (Fig. 1B). The effects of pubertal Cd exposure on germ cell apoptosis in testes were determined using TUNEL assay. As shown in Fig. 2, pubertal Cd exposure significantly increased the number of apoptotic germ cells in testes. Furthermore, the increase in apoptotic germ cells appeared to be stage-specific, with the highest incidence of TUNEL-positive tubules presented in stages VII–VIII and slight increase in labeled germ cells shown in stages I–VI.

3.4. Serum and testicular T

The effects of pubertal Cd exposure on serum and testicular T are presented in Fig. 3. Results showed that pubertal Cd exposure significantly reduced the level of serum T (Fig. 3A). In addition, pubertal Cd exposure significantly reduced the level of testicular T in adult mice (Fig. 3B).

3.5. Testicular mRNA level of StAR and T synthetic enzymes

The effects of pubertal Cd exposure on mRNA level of testicular StAR and T synthetic enzymes are determined using RT-PCR. As shown in Fig. 4, pubertal Cd exposure significantly decreased mRNA level of testicular StAR. In addition, pubertal Cd exposure significantly decreased mRNA level of testicular P450scc, $P450_{17\alpha}$ and 17β-HSD in adult mice.

3.6. Testicular protein level of StAR and T synthetic enzymes

The effects of pubertal Cd exposure on protein level of testicular StAR and T synthetic enzymes are presented in Fig. 5. Results showed that pubertal Cd exposure significantly reduced protein level of testicular StAR. In addition, pubertal Cd exposure significantly reduced protein level of testicular P450scc, $P450_{17\alpha}$ and 17β-HSD in adult mice.

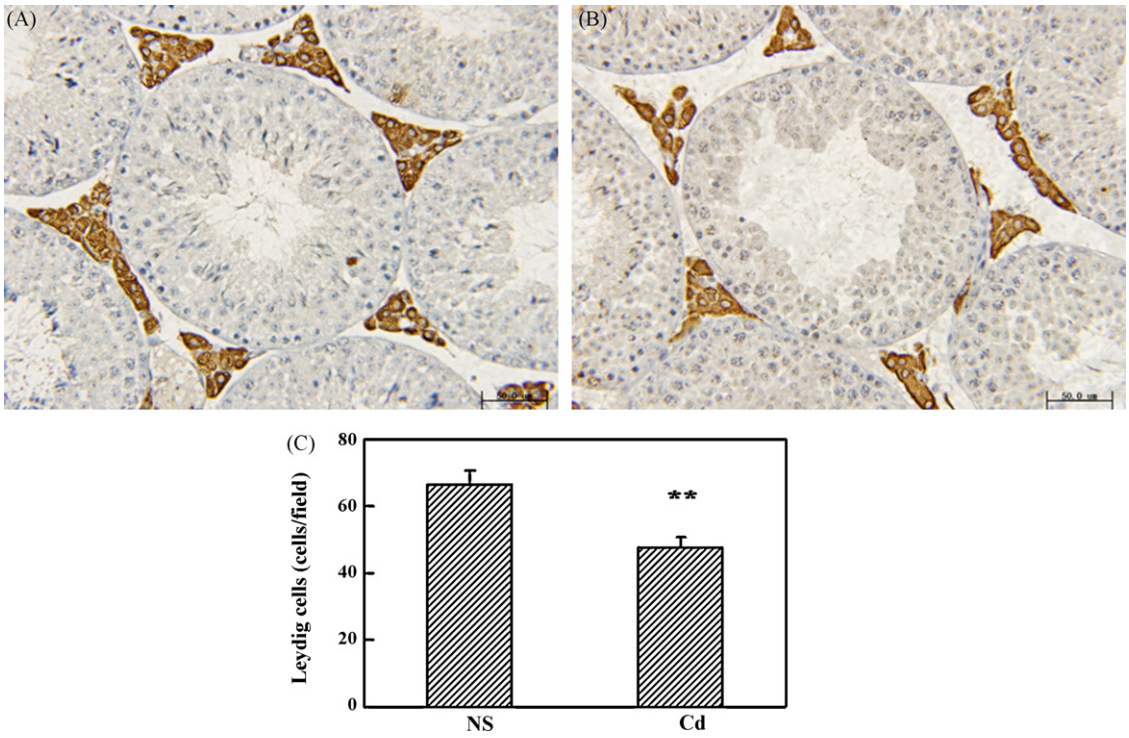

Fig. 6. Effects of pubertal Cd exposure on the number of Leydig cells in testes. Male mice were injected with $CdCl_2$ (1 mg/kg, i.p.) daily from PND35 to PND70. Testes were collected at PND70. Leydig cells in testes of (A) control and (B) Cd-treated mice were immunolocalized by staining with a polyclonal antibody specific for 3β-HSD. (C) The numbers of Leydig cells in testes were counted in 12 randomly selected fields from each slide at 400× magnification. Data were expressed as means ± SEM of 12 samples from 12 different mice. $^{**}P < 0.01$ as compared with controls. NS, normal saline.

3.7. Number of Leydig cells in testes

Leydig cells in testes were identified by staining for 3β-HSD. As shown in Fig. 6, the number of Leydig cells in testes was significantly decreased in mice exposed to Cd during puberty.

4. Discussion

In the present study, we investigated the effects of pubertal Cd exposure on testicular development and spermatogenesis in mice. We found that pubertal Cd exposure significantly reduced the absolute weights of testes and epididymides, and the absolute and relative weights of prostates and seminal vesicles in adult mice. In addition, pubertal Cd exposure markedly decreased the layers of spermatogenic cells, increased the inside diameter of seminiferous tubules, and disturbed the array of spermatogenic cells in testes of adult mice. The number of spermatozoa in cauda epididymides was significantly decreased in mice exposed to Cd during puberty. These observations are different from the interstitial edema, hemorrhage and necrosis that were observed in the testes of pubertal mice dosed at 2 or 4 mg/kg/day in the preliminary dose ranging phase of this study (data not reported), or from adult rats and mice administered higher doses of $CdCl_2$ in the published literature [14,15]. The changes seen in the testes and epididymides and the decreased weight of the accessory sex organs in the current study using lower dose levels of $CdCl_2$, are consistent with the changes caused by decreased levels of T.

Androgens, primarily T, which act through the somatic cells to regulate germ cell differentiation, are a prerequisite for normal spermatogenesis [17]. A Sertoli cell-selective knockout of the androgen receptor resulted in spermatogenic arrest in meiosis and infertility with defective spermatogenesis and hypotestosteronemia [26,27]. Several studies showed that post-pubertal Cd exposure significantly decreased the level of serum T [20,21,28,29]. However, the effects of pubertal Cd exposure on testicular T synthesis remained obscure. According to an earlier study, follicle stimulating hormone (FSH) levels decreased in Cd-treated post-pubertal rats, but was not altered in Cd-treated pubertal rats, suggesting that pubertal and post-pubertal Cd exposure differentially disrupts the hypothalamic-pituitary-testicular axis function in the rat [28]. To investigate the effects of pubertal Cd exposure on testicular T synthesis in mice, the present study measured the level of serum and testicular T at 24 h after the last administration of Cd. We found that the level of serum T was significantly decreased in mice exposed to Cd during puberty. In addition, pubertal Cd exposure markedly reduced the level of testicular T in adult mice.

StAR is an essential and limiting factor in testicular T synthesis, responsible for the transport of cholesterol into mitochondria [30]. A recent study showed that administration of Cd (10 μmol/kg) to adult male Sprague–Dawley rats significantly reduced protein level of StAR in Leydig cells [31]. In the present study, we investigated the effects of pubertal Cd exposure on the expression of testicular StAR in adult mice. As expected, pubertal Cd exposure markedly reduced protein expression of testicular StAR in adult mice. In addition, mRNA level of testicular StAR was significantly decreased in mice exposed to Cd during puberty. These results suggest that StAR might be important in Cd-induced decrease in testicular T synthesis.

T synthetic enzymes in Leydig cells include cytochrome P450 cholesterol side-chain cleavage (P450scc) enzyme, cytochrome P450 17α-hydroxysteroid dehydrogenase ($P450_{17\alpha}$) and 17β-hydroxysteroid dehydrogenase (17β-HSD). P450scc converted cholesterol to pregnenolone that is catalyzed by $P450_{17\alpha}$ to produce 17-hydroxyprogesterone and androstenedione, with the latter then being converted to T by 17β-HSD [32]. Several studies showed that administration of Cd to adult rodents significantly decreased testicular 17β-HSD activities [21,33–35]. In the present study, we investigated the effects of pubertal Cd exposure on the expres-

sion of testicular P450scc, $P450_{17\alpha}$ and 17β-HSD in adult mice. We found that protein level of testicular P450scc, $P450_{17\alpha}$ and 17β-HSD was significantly decreased in mice exposed to Cd during puberty. In addition, pubertal Cd exposure obviously reduced mRNA level of testicular P450scc, $P450_{17\alpha}$ and 17β-HSD in adult mice. Thus, the decreased testicular T synthesis in mice exposed to Cd during puberty might partially be attributed to downregulation of T synthetic enzymes in testes.

Aside from the expression of testicular StAR and T synthetic enzymes, we searched for other possible factors involved in the decreased T production in mice exposed to Cd during puberty. Recently, several studies showed that abnormal Leydig cell aggregation in the testis of rats exposed to di-(n-butyl) phthalate (DBP) or di-(2-ethylhexyl) phthalate (DEBP), two endocrine disruptors, was associated with the decreased level of testicular T [36–38]. In the present study, we analyzed the number of Leydig cells in testes of mice exposed to Cd during puberty. Our results showed that the number of Leydig cells in testes was significantly decreased in mice exposed to Cd during puberty. Indeed, an in vitro study demonstrated that Cd was directly toxic to primary Leydig cells [23]. Therefore, the present study does not exclude that pubertal Cd exposure reduces testicular T synthesis through directly injuring Leydig cells and reducing the number of Leydig cells in mouse testes.

Some endocrine disruptors have been demonstrated to induce germ cell apoptosis in testes [39–43]. According to a recent study, mono-(2-ethylhexyl) phthalate (MEHP), a well-known endocrine disruptor, significantly reduced the number of germ cells by increasing apoptosis without change in T production in human fetal testes [44]. Indeed, several studies showed that the number of apoptotic germ cells was increased in testes of Cd-treated adult rats [45–47]. In the present study, we investigated the effects of pubertal Cd exposure on apoptosis in testes of adult mice. Our results found that pubertal Cd exposure markedly increased the number of apoptotic cells in testes of adult mice, indicating that pubertal Cd exposure might also impair testicular development and spermatogenesis through proapoptotic mechanism. It had been shown that reduced levels of intratesticular T resulted in a cell and stage-specific apoptosis of round spermatids and spermatocytes in stages VII and VIII tubules [48]. The present results showed that the increase in apoptotic germ cells is stage-specific, with the highest incidence of TUNEL-positive germ cells in stages VII–VIII tubules. These results suggest that the decrease in testicular T level might contribute, at least partially, to Cd-evoked apoptosis in germ cells.

Our results also showed that the body weight of the Cd-treated mice was 12% lower than the controls at the end of the dosing period. For mice, this is a significant lowering of body weight gain. Earlier studies showed that even a 10% lowering of body weight gain in mice is sufficient to cause significant decreases in the weights of male reproductive organs of the mouse and that the mouse is more sensitive to this effect than the rat [49,50]. In addition, another study also demonstrated that body weight reduction in rats can result in decreased accessory sex gland weights, decreased T levels and apoptosis of spermatocytes and spermatids in stage VII/VIII tubules [51]. These results suggested that Cd-induced the body weight loss might be, at least partially, attributed to cause decreased weights of testes, epididymides and accessory sex organs as well as lowering of T levels.

In summary, the present study investigated the effects of pubertal Cd exposure on testicular development and spermatogenesis in mice. We found that pubertal Cd exposure impaired testicular development and spermatogenesis. In addition, pubertal Cd exposure markedly disrupted T synthesis via downregulating the expression of testicular StAR and T synthetic enzymes in adult mice. The decreased testicular T synthesis might, at least partially, contribute to Cd-induced impairment on testicular development and spermatogenesis in mice.

Conflict of interest

There are no conflicts of interest.

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