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Maternal Cypermethrin Exposure during the Perinatal Period Impairs Testicular Development in C57BL Male Offspring

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Abstract

Numerous studies have demonstrated that endocrine-disrupting compounds (EDC) are a possible cause of male reproductive organ malfunction and malformation. Cypermethrin (CYP) is a widely used synthetic pyrethroid and a potential EDC. This study aimed to examine the effects of perinatal exposure to low-dose CYP on the development and function of the offspring testes. Pregnant mice were intragastrically administered 0.12 to 12 mg/kg/day CYP from embryonic day 0.5 (E0.5) to weaning (PD21.5, postnatal day 21.5). Maternal exposure to 0.12, 1.2, and 12 mg/kg/day CYP affected the body and organ weight of the offspring. Exposure of CYP led to a dose-dependent decrease in the male-to-female sex ratio. A histopathological analysis revealed a thinner seminiferous epithelium layer at PD21.5, interstitial hyperplasia at PD45.5, and germ cell vacuolization at PD90.5 in the 12 mg/kg/day CYP group. The TUNEL assay results revealed increased germ cell apoptosis in the 12 mg/kg/day CYP group. The serum testosterone (T) level decreased, whereas the estradiol level increased with age in the 1.2 and 12 mg/kg/day CYP groups. The RT-PCR analysis demonstrated decreased expression of T production-related, mitosis-related, and meiosis-related genes in the 1.2 and 12 mg/kg/day CYP groups. The *in vitro* experimental results demonstrated reduced expression of steroidogenesis genes and decreased T levels. It is concluded that perinatal exposure to low-dose CYP affects testes development and function in adults.

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Introduction

Compounds that can mimic and block natural hormones and cause adverse health effects in humans and wildlife are referred to as endocrine-disrupting compounds (EDCs) [1,2]. Studies have demonstrated that a wide range of EDCs can lead to serious problems, such as infertility [3–7].

Cypermethrin (CYP), a type II synthetic pyrethroid insecticide, replaces traditional organochlorine and organophosphate pesticides and has been widely used [8]. Different studies had indicated that CYP treatment decreases the layers of spermatogenic cells, increases the inside diameter of seminiferous tubules, decreases Star expression in adolescent mice [9], disturbs the array of spermatogenic cells [10], reduces sperm count and motility in male mice [7,11–14], decreases serum testosterone (T) levels, and increases serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels [15]. It has been demonstrated that CYP exerts anti-androgen effects in androgen receptor reporter gene assays [16,17] and can induce ER transactivity [3]. Most studies have used higher doses of CYP, ranging from 39.66 mg/kg/day [7] to 485 mg/kg/day [12] and even toxicological doses, and most studies have focused on postnatal exposure [9,10,12,15,18]. However, there are no reports describing the effects of lower dosage or environmental exposure levels during fetal exposure on the growth and development of testes. Various studies have investigated the effects of EDCs on the growth and development of the fetus, which is sensitive to hormonal fluctuations [19–22]. Impaired reproductive development has been demonstrated in the sons of female gardeners or farmers where pesticides have been used [23–28]. This study aimed to assess CYP exposure during the perinatal period to determine its effect on fetal development and its long-term impact on male reproduction in C57BL mice.

Materials and Methods

In vivo experimental design, treatment, and sample collection

It has been reported that the LD50 of CYP in mice is 250.0 mg/kg when administered orally in corn oil [15]. Some toxicological studies have used a dosage equal to 1/5LD50 or higher [12,15]. It has been reported that the environmental residue of CYP in surface water varies in different countries from 0.022 to 5.6 ppb [29–32], whereas the CYP residue in human milk can be as high as 945.1 to 1443.8 ppb [33]. Based on these studies, we chose CYP exposure doses of 0.12, 1.2, and 12.0 mg/kg/day for the *in vivo* experiment. Gestating C57BL/6 mice were treated with different doses of CYP through intragastric administration from embryonic day 0.5 (E0.5, day of plug) to weaning (postnatal day 21.5, PD21.5). The gestating control group was treated with vehicle (corn oil; n = 8–10 female mice/group). After birth, sex determination was conducted through *sry* amplification [34] (Fig.

Table 1. Antibodies information.

Antibody	Dilution	Cat No.	Source
β-Actin	1:500[1]	sc-47778	mouse monoclonal IgG
Star	1:500[1]	sc-25806	rabbit polyclonal IgG
3β-HSD	1:200[2] or 1:500[1]	sc-30820	goat polyclonal IgG
Cyp17a1	1:500[1]	sc-66850	rabbit polyclonal IgG
17β-HSD3	1:500[1]	sc-66415	goat polyclonal IgG
Pcna	1:200[2] or 1:500[1]	sc-7907	rabbit polyclonal IgG
AR	1:500[1]	sc-816	rabbit polyclonal IgG
ERα	1:500[1]	sc-542	rabbit polyclonal IgG

[1]represents dilution used in Western blot assay,
[2]represents dilution used in immunohistochemical assay.
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Table 2. The sequences of primers, annealing temperature and amplified products of PCR.

Gene name	Primer	Annealing (°C)	Product size (bp)
L19	F: 5′-gaaatcgccaatgccaact-3′	56	406
	R: 5′-tgagactcgcaggtctaaga-3′		
Star	F: 5′-agatgtgggcaaggtgtttc-3′	56	387
	R: 5′-gagcagccaagtgagtttagt-3′		
Cyp11a1	F: 5′-gctgcctgggatgtgattt-3′	53.5	548
	R: 5′-cggaagtgggtggtatttt-3′		
3β-HSD	F: 5′-aatctgaaaggtacccagaa-3′	51.5	360
	R: 5′-tcatcatagctttggtgagg-3′		
Cyp17a1	F: 5′-ccaggacccaagtgtgttct-3′	56	250
	R: 5′-cctgatacgaagcacttctcg-3′		
17β-HSD3	F: 5′-attttaccagagaagacatct-3′	52	367
	R: 5′-ggggtcagcacctgaataatg-3′		
Cyp19a1	F: 5′-gcttctcatcgcagagtatccg-3′	60	266
	R: 5′-caagggtaaattcattgggctg-3′		
AR	F: 5′-ctgggaagggtctaccac-3′	55	128
	R: 5′-ggtgctatgttagcggcctc-3′		
ERα	F: 5′-cgtgtgcaatgactatgcctc-3′	55	199
	R: 5′-tttcatcatgcccacttcgtaa-3′		
Pcna	F: 5′-caacttggaatcccagaac-3′	53	294
	R: 5′-agacagtggagtggctttt-3′		
Nanos3	F: 5′-tgcaggcaaaaagctgacc-3′	60	101
	R: 5′-cttcctgccacttttggaac-3′		
Cyclin D2	F: 5′-cgatgattgcaactggaagc-3′	54	168
	R: 5′-ttcagcagcagagcttcgat-3′		
Stra8	F: 5′-gtttcctgcgtgttccacaag-3′	54	150
	R: 5′-cacccgaggctcaagcttc-3′		
Cyclin A1	F: 5′-gatgtgtatgaagtcgacacc-3′	54	91
	R: 5′-gtggggtcaaccagcattgg-3′		
Sry	F: 5′-tcttaaactctgaagaagagac-3′	55	404
	R: 5′-gtcttgcctgtatgtgatgg-3′		
Insl3	R: 5′- ggagccgaagtcgagactg-3′	55	186
	R: 5′- gccatctagtccacccctc-3′		

F, forward; R, reverse.
doi:10.1371/journal.pone.0096781.t002

Table 3. Offspring body and organs weight.

Group	Body weight (g)			Liver weight (g)		
	PD21.5	PD45.5	PD90.5	PD21.5	PD45.5	PD90.5
Con	13.65±0.97[a]	32.74±1.30	38.21±2.57[a]	0.67±0.06[a]	2.07±0.11	2.11±0.20
0.12 mg/kg/day	11.09±0.29[b]	33.95±0.76	39.76±0.81[b]	0.43±0.12[b]	2.10±0.21	2.22±0.18
1.2 mg/kg/day	9.31±0.97[b]	34.42±1.30	41.10±1.13[b]	0.38±0.05[b]	2.02±0.06	2.14±0.09
12 mg/kg/day	10.35±0.72[b]	34.82±0.63	44.17±0.73[b]	0.36±0.04[b]	2.08±0.09	2.37±0.07
Group	**Testis weight (g)**			**Epididymis weight (g)**		
	PD21.5	**PD45.5**	**PD90.5**	**PD21.5**	**PD45.5**	**PD90.5**
Con	0.08±0.00[a]	0.17±0.04[a]	0.29±0.01	0.01±0.00	0.06±0.00	0.09±0.00
0.12 mg/kg/day	0.06±0.01[b]	0.20±0.02[b]	0.28±0.03	0.01±0.00	0.07±0.01	0.09±0.01
1.2 mg/kg/day	0.04±0.01[b]	0.22±0.01[b]	0.27±0.02	0.01±0.00	0.06±0.00	0.09±0.00
12 mg/kg/day	0.04±0.01[b]	0.23±0.01[b]	0.28±0.01	0.01±0.00	0.07±0.00	0.10±0.00

Con, control group. Different superscripts ([a], [b]) depict significant differences among body or organs weight ($P<0.05$).

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S1), and the male offspring were subjected to further analyses. The body weights of the male offspring were monitored every five days. The male mice were anaesthetized with pentobarbital sodium (40 mg/kg) and sacrificed randomly by cardiac puncture at three time points: PD21.5 (weaning day, n = 3–10), PD45.5 (juvenile phase, n = 5–10), and PD90.5 (maturation phase, n = 5–11). Serum samples were stored at −20°C until hormone measurement. The testes were dissected and separated into two parts. One part was fixed with 4% paraformaldehyde (PFA) or Bouin's solution (Sigma, USA) for testicular immunohistochemical, apoptosis, and pathological analyses. The other part was snap-frozen in liquid nitrogen and stored at −80°C until use. The testes, epididymises, and livers were weighed.

Sex determination of offspring

The expression of the *Sry* gene [35] was detected through the amplification of tail DNA. The extracted DNA was denatured at 95°C for 5 min and amplified through 30 cycles of PCR using the following conditions: 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. The PCR product was subjected to agarose gel electrophoresis and was visualized by ethidium bromide staining. *L19* was amplified as an internal control.

Ethics statements

The Ethics Committee for Animal Experimentation of the China Agricultural University approved all of the animal experiments (Register No. SKLAB-2011-01-03).

CYP detection

The CYP levels of the prepared dosages and serum were measured by ELISA following the manufacturer's protocol (R&D Systems, USA). Briefly, after proper dilution, the standards and samples were added to a 96-well plate (coated with CYP-coupling antigen). The antibody-enzyme conjugate was then added, and the plate was incubated for 30 min at 37°C and washed. The color-reagents A and B were then added to each of the wells and the plate was incubated for 10 min at 37°C. The stop solution was then added, and the absorbance value at 420 nm was read. The standard curve was generated according to the standard values to calculate the concentration of each of the samples.

Morphological and histological analyses

The testes were embedded in paraffin, and sections (5-μm thick) were deparaffinized using xylene and then stained with hematoxylin and eosin (HE) through standard procedures for histopathological examination. The mounted slides were examined with a light microscope (IX71, Olympus, Japan), and photos were taken with a Nikon DXM1200F camera (Nikon, Japan). The quantification of germ cell numbers and germ cell vacuolation was conducted by counting 5–20 related slides.

Immunohistochemical analyses

The immunohistochemical analyses were performed as previously described [36]. The slides were stained with the following antibodies for chromatic visualization: goat anti-3β-HSD, rabbit anti-CYP17A1 (further information on the primary antibodies is

Table 4. Number of offspring and sex ratio of male to female.

Group	Number of offspring			Male to female ratio
	Male	Female	Total	
Con	31	30	61	1.03:1
0.12 mg/kg/day	13	15	28	0.87:1
1.2 mg/kg/day	29	38	67	0.76:1
12 mg/kg/day	22	32	54	0.69:1

doi:10.1371/journal.pone.0096781.t004

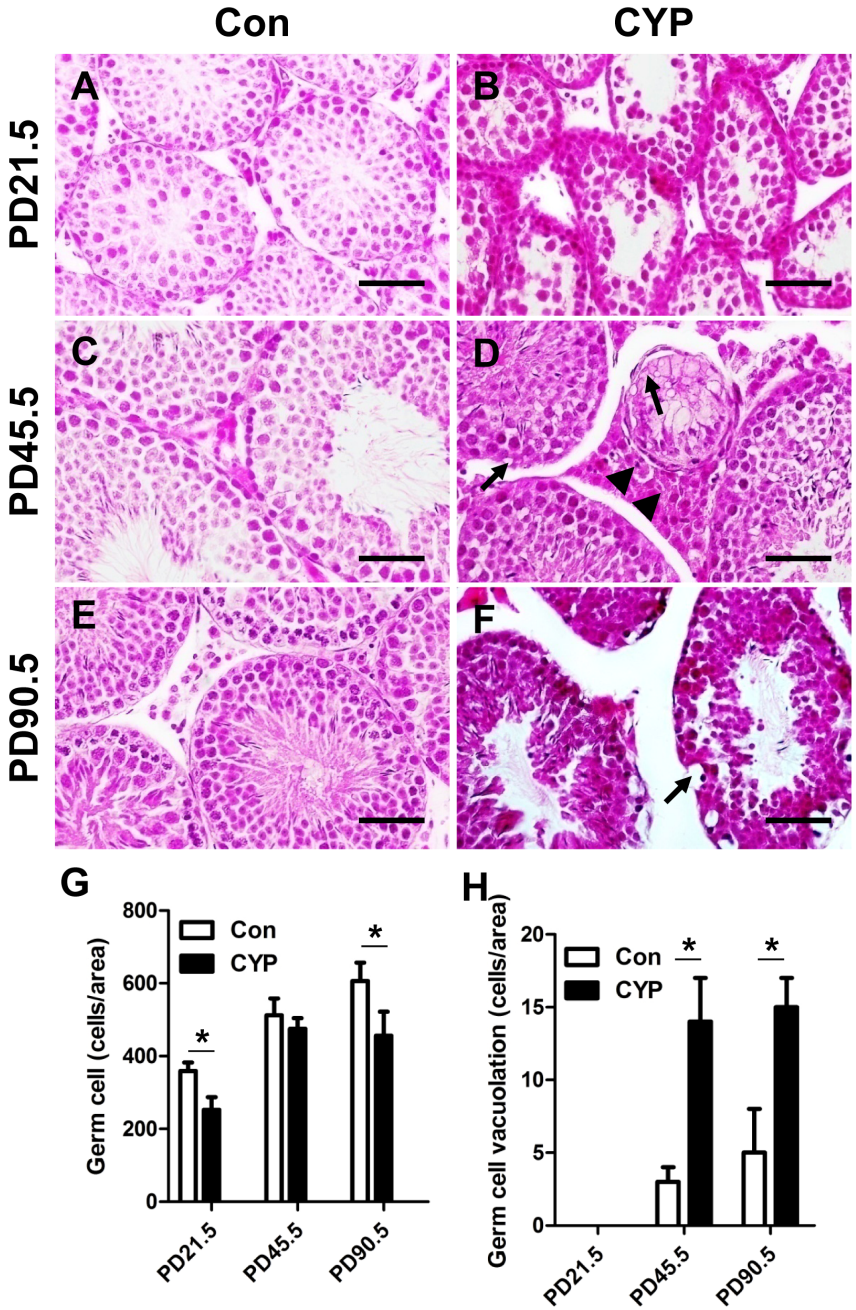

Figure 1. Effects of maternal perinatal CYP-exposure on the testicular histology of offspring. (A) Testicular histology of the control group offspring at PD21.5. (B) Testicular histology of the 12 mg/kg/day CYP-treated group offspring at PD21.5: the seminiferous epithelium layer was thinner than that of the control. (C) Testicular histology of the control group offspring at PD45.5: normal spermatogenesis was observed. (D) Testicular histology of the 12 mg/kg/day CYP-treated group offspring at PD45.5. Some of the germ cells displayed vacuolation (arrow) and hyperplasia of interstitial cells (arrowhead). (E) Testicular histology of the control group offspring at PD90.5. The lumen of the seminiferous tubules was filled with spermatozoa. (F) Testicular histology of the 12 mg/kg/day CYP-treated group offspring at PD90.5. More severe vacuolation of germ cells and destruction of the seminiferous epithelium were observed. All of the images were taken at 400× magnification. Scale bars, 40 μm. (G) Quantification of germ cells in 5–20 related slides of A–F. (H) Quantification of germ cell vacuolation in 5–20 related slides of A–F. The data represent the mean ± SEM. *$P<0.05$.
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presented in Table 1, purchased from Santa Cruz, USA), biotinylated rabbit-anti-goat, goat-anti-rabbit IgG (all at 1:200 dilution, Vector Laboratories, USA), and streptavidin-conjugated HRP (1:200 dilution, Jackson ImmunoResearch, USA). The specific binding was visualized using DAB (1:20 dilution, Leica, Germany). The sections were counterstained with hematoxylin and mounted for further microscopic analyses. The numbers of positive cells in the testes at PD21.5 and the testes at PD45.5 and PD90.5 were counted in five randomly and ten randomly selected fields, respectively. A total of 5-20 related slides were used to quantify the number of positive cells.

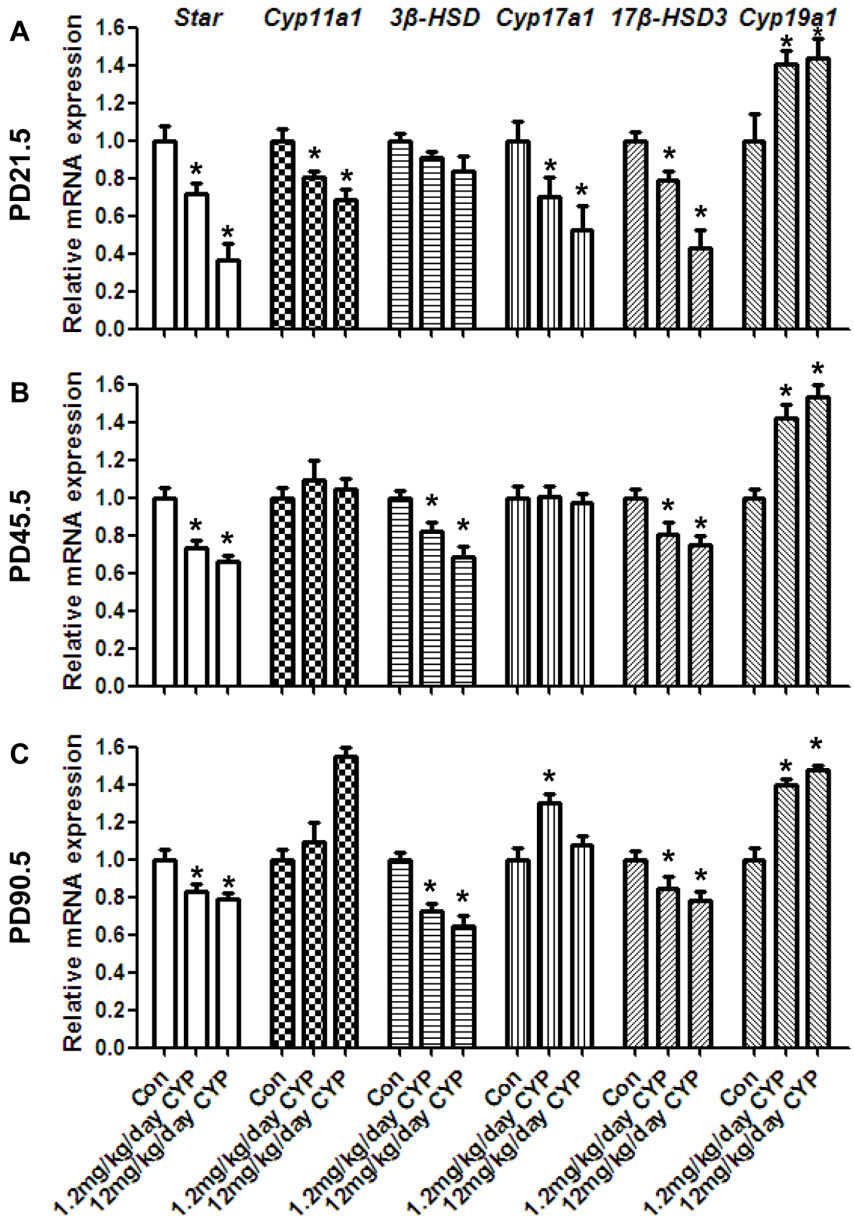

Figure 2. Effects of maternal perinatal CYP-exposure on testicular steroidogenesis-related genes in offspring. (A) The mRNA levels of *Star*, *Cyp11a1*, *3β-HSD*, *Cyp17a1*, *17β-HSD3*, and *Cyp19a1* in the testes at PD21.5 using RT-PCR. *Star*, *Cyp17a1*, and *17β-HSD3* were significantly downregulated in the CYP exposure groups. *Cyp11a1* displayed a downward trend, but this trend was not significant. However, *Cyp19a1* was upregulatedand, and there was no change in *3β-HSD*. (B) mRNA levels of steroidogenesis-related genes at PD45.5. *Star*, *3β-HSD*, and *17β-HSD3* were significantly downregulated in the CYP exposure groups. *Cyp19a1* was upregulated, and there were no changes in the *Cyp11a1* and *Cyp17a1* expression levels. (C) mRNA levels of steroidogenesis-related genes at PD90.5. *Star*, *3β-HSD*, and *17β-HSD3* were downregulated significantly in the CYP exposure groups, and *Cyp19a1* was upregulated significantly. The data represent the mean ± SEM. *$P<0.05$.
doi:10.1371/journal.pone.0096781.g002

Terminal dUTP nick-end labeling staining

The paraffin-embedded slides were stained with the terminal dUTP nick-end labeling (TUNEL) technique to detect the apoptosis of germ cells. An *in situ* cell death detection kit (AP, Roche, Switzerland) was applied according to the manufacturer's protocols. The number of TUNEL-positive cells was counted through immunohistochemical analyses. The quantification of the positive cell number was conducted by counting 5-20 related slides.

RNA extraction and semi-quantitative RT-PCR

The total RNA from the testes and mLTC-1 cells (CRL-2065, ATCC, USA) was isolated using the TRIzol reagent (Invitrogen, USA). In addition, pre-PCR was performed to synthesize the cDNA for the semi-quantitative RT-PCR analyses. One microgram of total RNA was incubated with 10 U of avian myeloblastosis virus reverse transcriptase (Promega, USA), dNTP mix, and oligo-dT primers at 42°C for 1 h. The cDNAs were then denatured at 95°C for 5 min and amplified through 20–36 cycles of PCR using the following conditions: 95°C for 30 sec, 51.5°C to 61°C for 30 sec, and 72°C for 30 sec. An aliquot of the RT-PCR

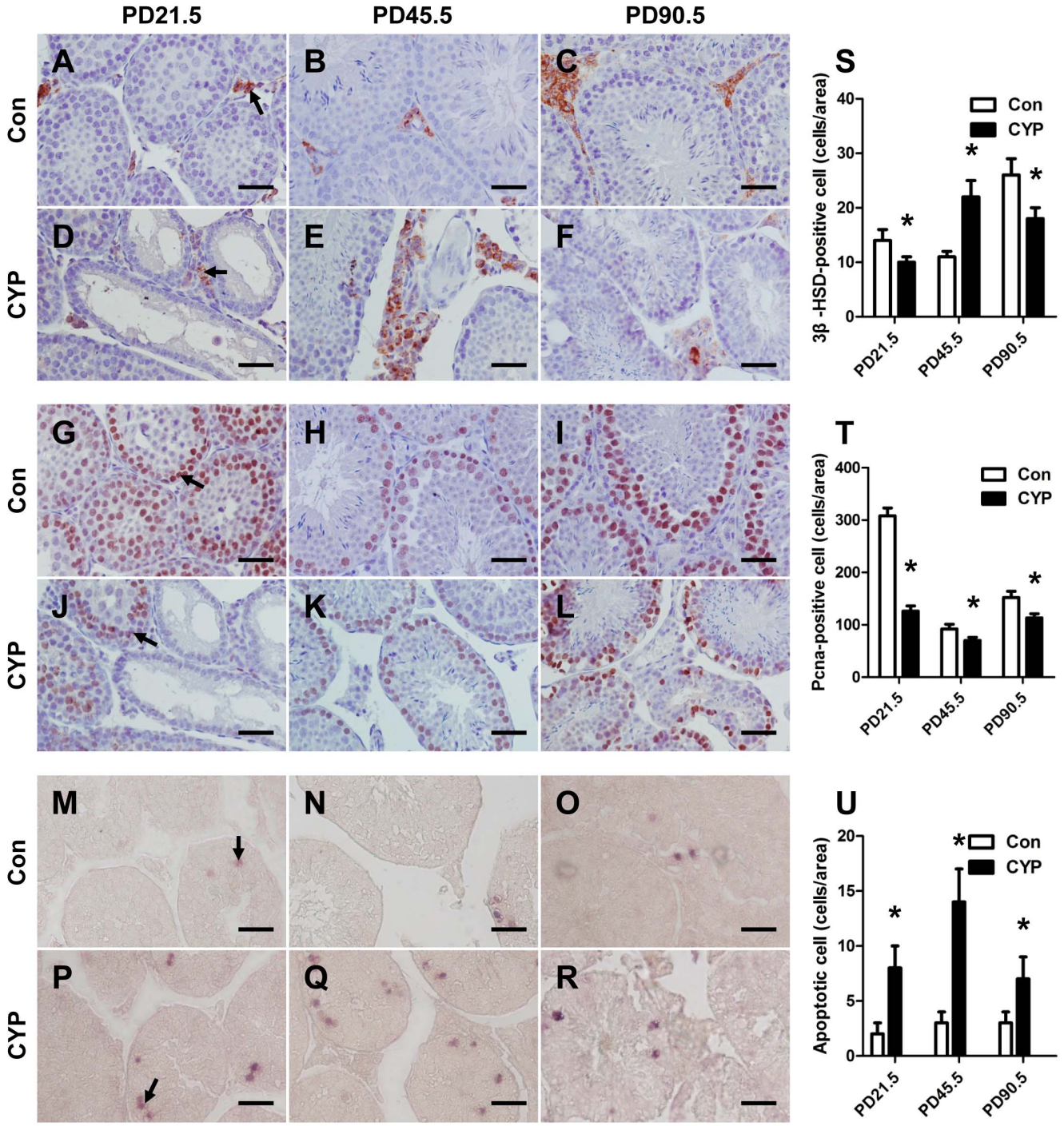

Figure 3. 3β-HSD, Pcna immunohistochemistry and TUNEL assay of testes. Slides of (A, B, C) control and (D, E, F) CYP-treated offspring at PD21.5, PD45.5, and PD90.5, respectively, were immunolocalized with antibody for 3β-HSD. Slides of (G, H, I) control and (J, K, L) CYP-treated offspring at PD21.5, PD45.5, and PD90.5, respectively, were immunolocalized with antibody for Pcna. Slides of (M, N, O) control and (P, Q, R) CYP-treated offspring at PD21.5, PD45.5, and PD90.5, respectively were stained using the TUNEL method. All of the images were taken at 400× magnification. Scale bars, 40 μm. (S) Quantification of 3β-HSD-positive Leydig cells in 5–20 related slides of A–F. (T) Quantification of Pcna-positive germ cells in 5–20 related slides of G–L. (U) Quantification of apoptotic germ cells in 5–20 related slides of M–R. The data represent the mean ± SEM. *$P<0.05$.
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product was subjected to agarose gel electrophoresis and visualized by ethidium bromide staining. The density of the gel bands was quantified using the ImageJ software, version 1.34 (NIH). L19 was amplified as an internal control of the total amount of RNA used. The primer sets are given in Table 2.

Western blot analyses

The Western blots were performed as described previously [36]. Briefly, the total protein from the cells was extracted using RIPA buffer. Aliquots of protein were electrophoresed in SDS-PAGE and transferred to a PVDF membrane. The membrane was

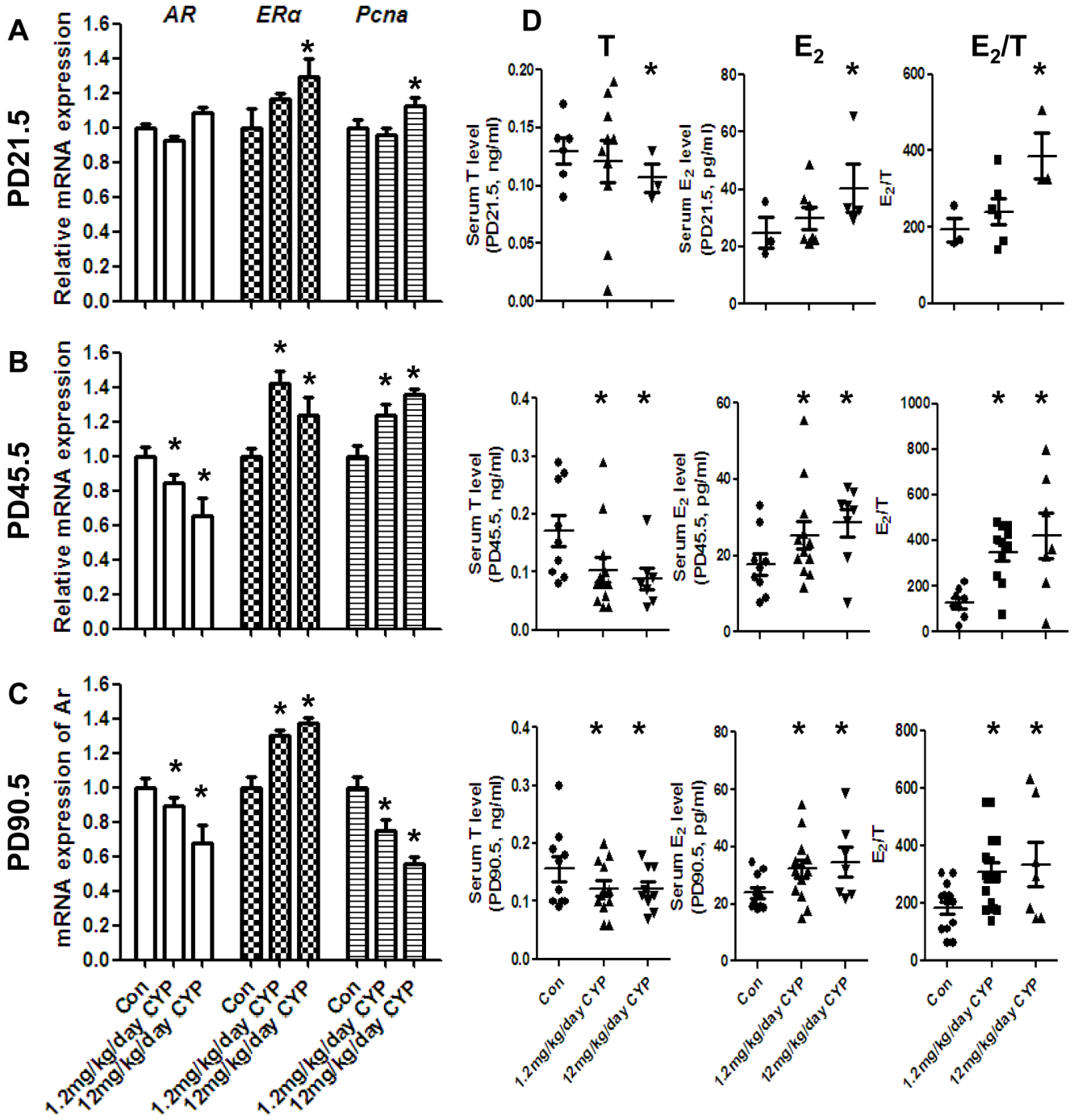

Figure 4. Effects of maternal CYP-exposure on the AR, ERα, Pcna, and E2/T levels of offspring. (A) The mRNA level of *AR* was not affected at PD21.5. The *ERα* level was upregulated significantly in the 12 mg/kg/day-exposure group, and there was an increasing trend in the other exposure group. *Pcna* expression was not altered. (B) *AR* expression was downregulated significantly in the exposure groups, and *ERα* and *Pcna* were upregulated, which may explain the hyperplasia of interstitial cells. (C) *AR* and *Pcna* were downregulated significantly, and *ERα* was upregulated, as observed at PD21.5 and PD45.5. (D) Serum T and E_2 levels and the E_2/T ratio at the three time points. The T level was lower, and the E_2 level was higher in the CYP exposure groups at PD45.5 and PD90.5. The data represent the mean ± SEM. *$P<0.05$.
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incubated with mouse anti-β-Actin, rabbit anti-Star, goat anti-3β-HSD, rabbit anti-CYP17A1, goat anti-17β-HSD3, rabbit anti-Pcna, rabbit anti-AR, and rabbit anti-ERα (further information on the primary antibodies are shown in Table 1, purchased from Santa Cruz, USA). After washing with TBST buffer, the membrane was then incubated with HRP-labeled goat-anti-mouse IgG, goat-anti-rabbit, and rabbit-anti-goat IgG (all at 1:2000 dilution, Jackson ImmunoResearch, USA). The final exposure was obtained using enzymatic chemiluminescence (GE Healthcare, USA). The film was then scanned, and the band density was quantified using the ImageJ software, version 1.34 (NIH).

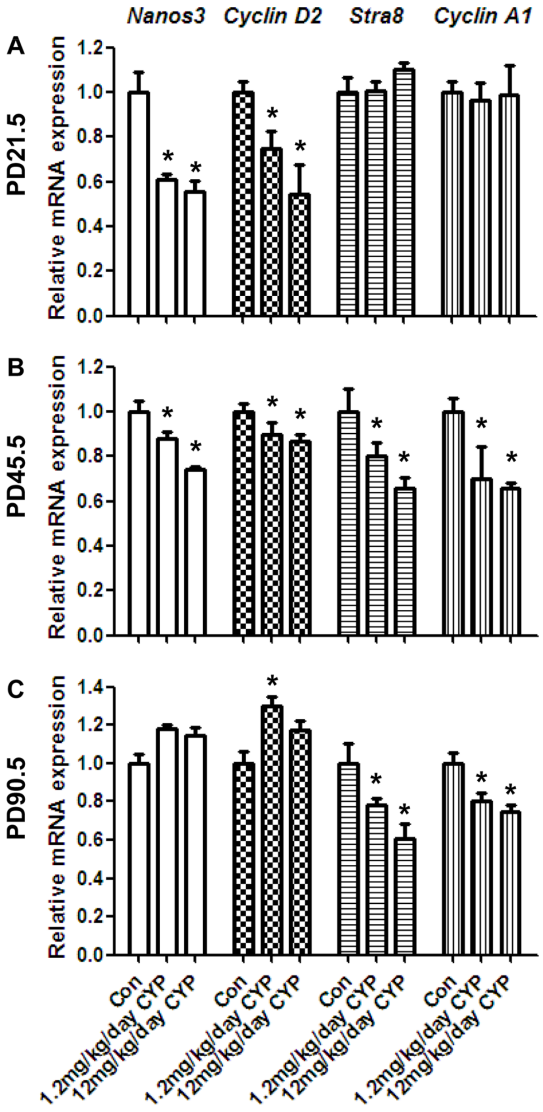

Figure 5. Effects of maternal perinatal CYP-exposure on mitosis and meiosis markers. (A, B, C) mRNA levels of the mitosis markers *Nanos3* and *Cyclin D2* and the meiosis markers *Stra8* and *Cyclin A1* at PD21.5, PD45.5, and PD90.5. The mitosis markers in the exposure groups were downregulated at PD21.5. The mitosis markers were also downregulated at PD45.5. At PD90.5, the mitosis markers were increased, but not significantly, and the meiosis markers were downregulated, as was observed at PD45.5. The data represent the mean ± SEM. *$P<0.05$.
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Cell culture and treatment

It has been reported that the pyrethroid metabolite concentration in human urine ranges from 0.318 to 189 ppb [37–41] and that the CYP residue in human serum can be as high as 0.3 ppm [42]. We chose to use a CYP concentration of 1×10^{-7}–1×10^{-5} M (0.42–42 ppb) in the medium. Leydig mLTC-1 cells were cultured in RPMI1640 containing 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under a 5% CO_2 atmosphere. Serum starvation was performed for 24 h before treatment, and the cells were then treated with three doses of CYP (10^{-7} M, 10^{-6} M, and 10^{-5} M) at 70% confluence for 24 h. DMSO (1/10^{7}) was used as the vehicle control, and LH (5 IU/ml) was used as a positive control. The total RNA and protein were extracted for further analyses.

Measurement of hormone levels

The T and E_2 levels in the serum or culture medium were measured by radioimmunoassay after diethyl ether extraction following the manufacturer's protocol, as described previously [36].

Statistical analyses

The Data were analyzed for statistical significance using SPSS 12.0.1 (SPSS, USA). The data for all of the groups were first tested for normality through the Shapiro-Wilk test. If normally distributed, they were then compared using one-way ANOVA to determine the differences between the treated groups and the vehicle group. Pearson's correlation analysis was performed to determine the dose-response relationship. P values less than 0.05 were set as statistically significant. All of the values are presented as the means ± SEM (standard error of mean). All of the graphs were generated with GraphPad Prism 5.0 (GraphPad, USA).

Results

The serum CYP residues of mothers and F1 male mice were determined using the ELISA method (Fig. S2). The concentrations ranged from 5.04 to 169.84 ppb in the mothers and from 0.57 to 7.63 ppb in the F1 male mice. We speculated that CYP can be transmitted from the mother to the offspring through blood and (or) milk. There was a nonsignificant difference between the treated groups and the control group in overall body weight (BW) (PD0.5–PD90.5) with no observed abnormalities in the liver histology among the groups. At PD21.5, the exposure of pregnant mice to 0.12, 1.2, and 12 mg/kg/day CYP significantly decreased the BW, testis weight (TW), and liver weight (LW) in the male offspring. At PD45.5, the TWs of the two treated groups were increased compared with that of the control group. At P90.5, the BWs of the treated groups were increased. There were nonsignificant differences in the epididymis weight among all of the groups (Table 3). Interestingly, the male-to-female ratio decreased in a dose-dependent manner (Table 4). In addition, lethal embryos were observed at E14.5 of pregnancy, and there were more sites of lethal embryos in the CYP group than in the control group (Fig. S3).

The histological analysis indicated that the seminiferous epithelium of the CYP-treated testes appeared thinner and that the germ cell number was decreased compared with the vehicle-treated group at PD21.5 and PD90.5 (Figs. 1A/B, and E–G). The number of Leydig cells was increased at PD45.5 in the CYP group (Figs. 1C/D). The number of germ cell vacuolations in the CYP group was markedly higher at PD45.5 and PD90.5 than in the control group (Figs. 1C–F and H).

We then examined the steroidogenesis of all of the groups. The expressions levels of these genes were increased with increased age (Fig. S4). The analysis of the different treatment groups revealed that *Star* expression was decreased significantly at the three time points in the CYP groups, and the same results were observed in the analysis of the expression of *3β-HSD* and *17β-HSD3* (Fig. 2). The expression of *Cyp11a1* and *Cyp17a1* was decreased at PD21.5, whereas *Cyp19a1* was upregulated at the three time points in the CYP groups. The immunohistological results indicated that the number of 3β-HSD-positive cells in the CYP group was lower than that observed in the vehicle group at PD21.5 and PD90.5, but the number was higher at PD45.5 (Figs. 3A–F, 3S). Th serum T and estradiol (E_2) levels (Fig. 4D) verified the RT-PCR results,

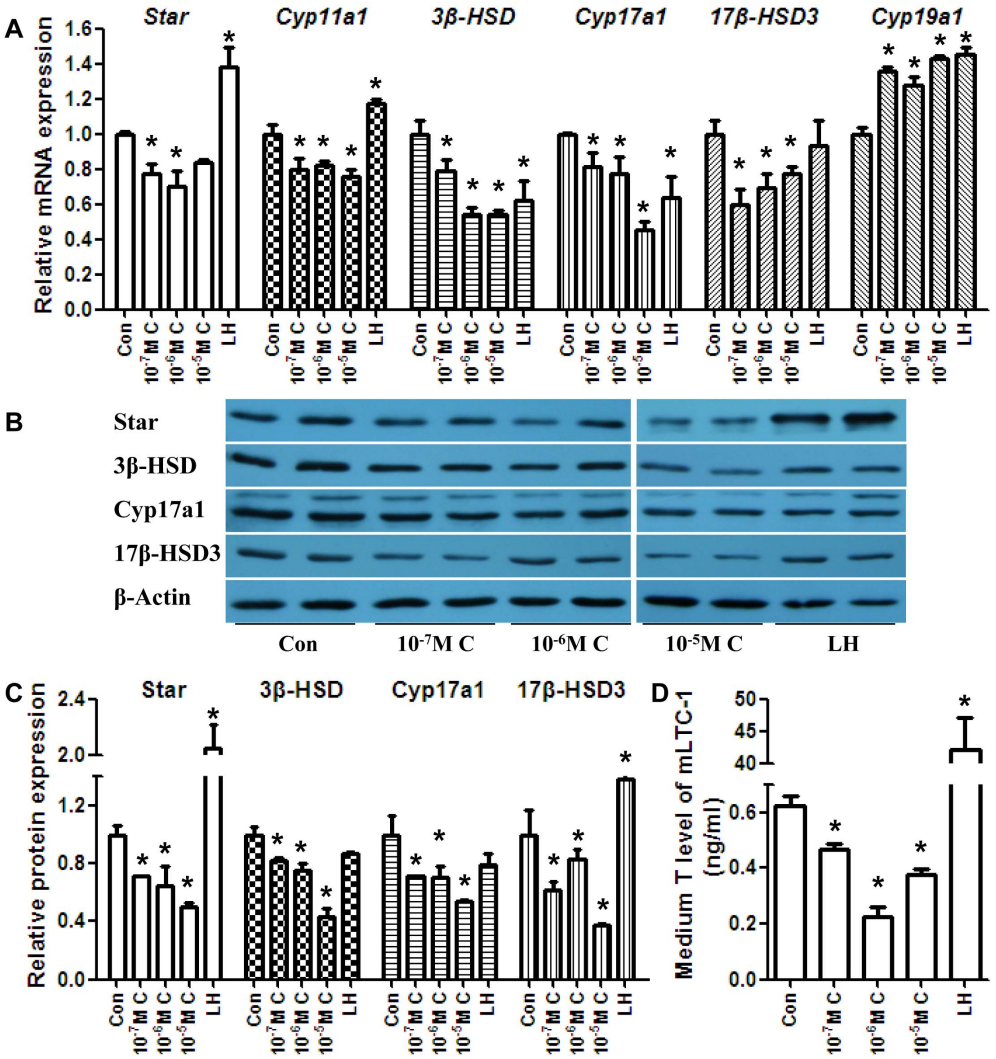

Figure 6. Effects of CYP-treatment on steroidogenesis-related genes and media T levels in mLTC-1 cells. (A) The mRNA levels of mLTC-1 steroidogenesis-related genes. (B) The protein levels of mLTC-1 steroidogenesis-related genes. (C) Quantitative analysis of scanning densitometry of protein levels of mLTC-1 steroidogenesis-related genes from (B). (D) Media T levels of mLTC-1 cells. The mRNA and protein levels of Star, 3β-HSD, Cyp17a1, and 17β-HSD3 were downregulated significantly compared with the control group. The mRNA and protein levels of Cyp19a1 were upregulated after CYP treatment. The media T levels were downregulated. LH was used as a positive control. The data represent the mean ± SEM. *$P<0.05$. C, CYP.
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and the T levels of the two CYP-treated groups were lower than that of the vehicle group (Fig. 4D left), whereas the E_2 level was higher in the CYP groups at PD45.5 and PD90.5 (Fig. 4D right). *AR* was downregulated at PD45.5 and PD90.5 (Figs. 4A–C). In contrast, *ERα* was upregulated at all three time points (Figs. 4A–C). *Pcna* was upregulated at PD45.5 but downregulated at PD90.5 in the CYP groups (Figs. 4A–C). The same results were found with the immunohistological analysis (Figs. 3G–L, 3T). *Insl3*, an important regulatory factor in testicular descent and germ cell apoptosis [43], was also decreased in the CYP groups (Fig. S5).

Because of the altered germ cell number, we investigated the gene expression level of mitosis and meiosis markers. At PD21.5, *Nanos3* and *Cyclin D2* were decreased in the CYP groups (Fig. 5A). At PD45.5, the *Nanos3*, *Stra8*, and *Cyclin A1* mRNA levels were decreased (Fig. 5B). At PD90.5, *Stra8* and *Cyclin A1* were significantly decreased in the CYP groups (Fig. 5C). The number of Pcna-positive proliferating germ cells was decreased in the CYP groups at the three time points (Figs. 3G–L, 3T), whereas the TUNEL assay results indicated increased number of apoptotic germ cells in the CYP group at the three time points (Figs. 3M–R, 3U).

The *in vivo* results indicated the disruption of steroidogenesis after maternal exposure. We treated the mLTC-1 Leydig cell line with CYP (LH was used as a positive control) to determine the effect of CYP on the basal expression level of related genes. LH treatment increased the *Star* mRNA expression level. CYP treatment decreased *Star*, *Cyp11a1*, *3β-HSD*, *Cyp17a1*, and *17β-HSD3* expression, whereas *Cyp19a1* was increased. The Star protein level was elevated significantly post-LH induction. The protein levels of Star, 3β-HSD, Cyp17a1, and 17β-HSD3 were decreased post-CYP treatment (Figs. 6B/C). CYP treatment led to decreased T production (Fig. 6D). The expression of AR was downregulated in the CYP group. In contrast, the expression of ERα was downregulated, and the expression of Pcna was upregulated at both the mRNA and protein levels (Figs. 7A–C). Another set of *in vitro* experiments was also conducted, and these

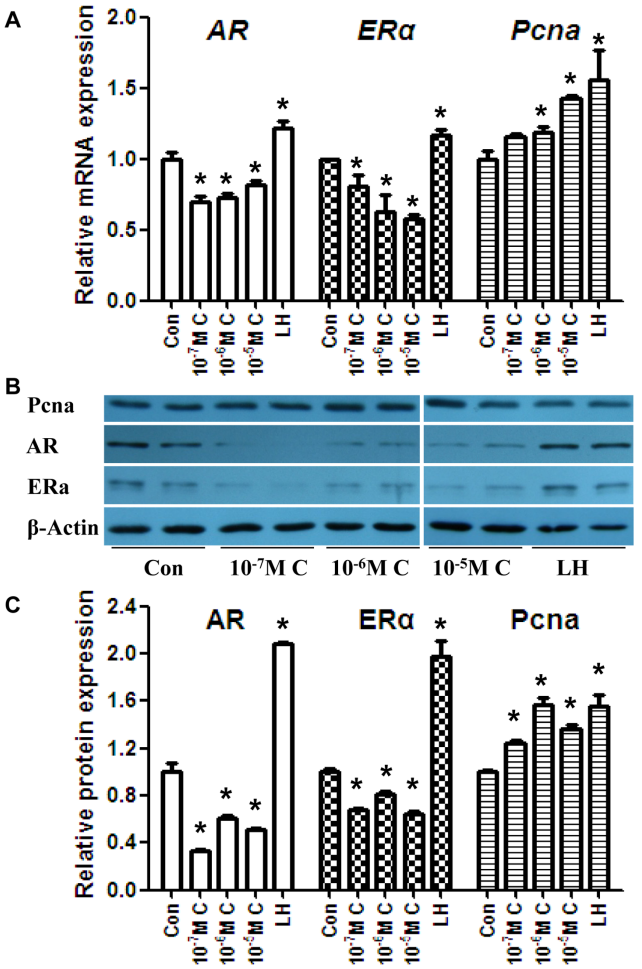

Figure 7. Effects of CYP-treatment on AR, Erα, and Pcna in mLTC-1cells. (A) The mRNA levels of *AR*, *ERα*, and *Pcna* in mLTC-1 cells. (B) The protein levels of AR, Erα, and Pcna in mLTC-1 cells. (C) Quantitative analysis of scanning densitometry of protein levels from (B). The mRNA and protein levels of AR were downregulated after CYP treatment, which is consistent with the T levels. In contrast, the ERα mRNA and protein levels were upregulated. The Pcna mRNA and protein levels suggest that CYP treatment may promote the proliferation of Leydig cells. The data represent the mean ± SEM. *$P<0.05$. C, CYP.
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included a vehicle control group, a vehicle+LH group, and a CYP+LH group (Fig. S6).

Discussion

Most studies have reported that direct exposure to high-dose of CYP impairs male reproduction [10,18,44]. We investigated the low-dose effects of CYP exposure during the perinatal stage and found impaired testicular development and steroidogenesis in the male offspring. The male-o-female ratio, BW, and TW were decreased at PD21.5. The structure of the seminiferous epithelium layer was also changed. The expression levels of steroidogenesis genes and hormone receptors were altered after CYP exposure both *in vivo* and *in vitro*. The mitosis and meiosis markers were also changed. The serum T levels were decreased, and E_2 levels were increased.

Androgen is a prerequisite for normal spermatogenesis and development [45], and the binding of androgen to the AR plays an important role in the induction of the male external genitalia during embryonic differentiation and spermatogenesis [46]. In fetal and neonatal testes, AR expression is restricted to the interstitial compartment [47,48]. Merlet *et al.* observed testicular dysgenesis during the embryonic period of gender differentiation in AR knockout mice [49]. Thus, perinatal CYP exposure may affect the precursors of adult Leydig cells. *Star* and *3β-HSD* were downregulated significantly in the CYP groups; in addition, in these groups, the T level was reduced, and the *Cyp19a1* and E_2 levels were increased. Moreover, the AR hormone receptor was downregulated, and ERα was upregulated. An imbalance of androgenic and estrogenic signals may lead to serious structural abnormalities. Previous results have demonstrated that mice overexpressing human aromatase possess a multitude of structural and functional alterations in the reproductive organs [50,51], and a decreased male-to-female ratio may arise from this overexpression. Taken together, the current results indicate that the inhibition of the androgenic signal during the prenatal and neonatal periods impairs the ability of Leydig cells to produce T in favor of E_2 due to the overexpression of aromatase.

The apoptosis of spermatogonia and spermatocytes occurs in the mitotic phase [52,53]. Studies have also found that deltamethrin and diethylstilboestrol induce a greater degree of apoptosis in adult male testes [54,55]. In this study, we found much greater apoptosis of germ cells in the CYP groups. Sufficient T plays a vital role in the inhibition of germ cell apoptosis [56]. Reduced T levels lead to the separation of germ cells from the epithelium of the seminiferous tubules [57]. In the present study, we found that the serum T levels were decreased significantly by maternal CYP exposure, which will weaken their ability to maintain spermatogenesis. Studies on bisphenol A [58,59] and hexachlorocyclohexane [60] have demonstrated that EDCs can affect mitosis and meiosis, and we also found that the expression of mitosis and meiosis marker genes was altered. The levels of Nanos3, which is important for maintaining undifferentiated spermatogonia [61], and the cell cycle regulator Cyclin D2 [62] were evaluated. We found that the expression of these two genes was decreased in the CYP groups at PD21.5 and PD45.5. The analysis of Stra8, which regulates meiotic initiation in spermatogenesis [63–65], and Cyclin A1, which is a meiosis-specific cyclin [66,67], revealed that these genes were decreased at PD45.5 and PD90.5. Our results illustrated that spermatogenesis and steroidogenesis were affected by CYP treatment and that the vacuolation of germ cells may be a result of the decreased expression of these key proteins and the reduced T levels.

The male-to-female ratio at birth is a marker of parental endocrine disruption [68]. In this study, we found that the sex ratio of the offspring was decreased in a dose-dependent manner. *In utero* exposure is widely considered t the most sensitive exposure time in terms of reproductive effects [69]. Studies have shown that the mammalian hormone levels around the time of conception are associated with the sex of the resulting offspring [70]. Parental exposure to both dioxin and vinclozolin has been shown to cause excess female offspring [69,71] due to altered hormone concentrations. In the present study, we found that the serum E_2/T ratio was higher in the CYP groups, which may account for the decreased male-to-female sex ratio. In addition, the observed fetal death sites *in utero* after CYP treatment indicated that CYP affects male fetal development. Even we could not figure out the precise mechanism in this study, this decreased sex ratio resulting from CYP exposure should arouse the attention of researchers and policy makers. Does CYP affect the proteins that regulate fetal formation and maternal-fetus interface, or could it directly affect

the genes controlling sex? The mechanism should be elucidated further.

Although the gene expression profiles were mostly similar between the *in vivo* and *in vitro* CYP treatment conditions, the gene expression of *ERα* differed between the two conditions. It is known that the localization of *ERα* is different during different testicular development stages [72]. In the fetal testis, *ERα* is present in Leydig cells only [73], whereas in the neonatal testis, *ERα* is present in Leydig cells, rete testis [74], and efferent ductules [74,75]. In the adult testis, *ERα* expression is also found in round spermatids [76]. CYP may not influence *ERα* expression in regions other than Leydig cells, which may explain the difference in the results between the *in vitro* and the *in vivo* conditions.

In summary, our study determined that maternal low-dose CYP exposure during the perinatal stage impairs steroidogenesis and spermatogenesis in male offspring, which may have long-term effects on male fertility. These results have been found in mice, and our findings suggest that CYP may also impair testicular development in humans.

Supporting Information

Figure S1 Sex determination of offspring using through *Sry* gene analysis. The sex of the offspring was determined through morphology and amplification of the *Sry* gene. F1–F8, offspring of one maternal mouse treated with high-dose CYP; a negative and positive control were also used. *L19* was used as an internal control.
(TIF)

Figure S2 Concentration of CYP in the prepared dosages and the mother and offspring mouse sera. The CYP concentrations of all of the samples were determined by ELISA. The concentrations of the prepared dosages were 20,243.5, 214,363.5, and 2,204,083.4 ppb, which are consistent with the expected values (23,980.81, 239,808.1 and 2,398,081 ppb). The serum concentrations of the CYP-treated maternal mice were 5.04, 34.82, and 169.8405 ppb, and those in the offspring ranged from 0.57 to 7.63 ppb (low dosage to high dosage). The residual concentrations in the sera of the mother and offspring mice in the vehicle group were close to zero. The data represent the mean ± SEM.
(TIF)

Figure S3 Fetal death *in utero* of oil- and CYP-treated groups. (A) Utero at E14.5 from oil-treated mouse. (B) Utero at E14.5 from high dose CYP-treated mouse, several fetal death sites (arrow) were observed in CYP-treated mouse. C, CYP.
(TIF)

Figure S4 Time course expression of steroidogenesis genes at PD21.5, PD45.5, and PD90.5. The mRNA levels of *Star*, *Cyp11a1*, *3β-HSD*, *Cyp17a1*, *17β-HSD3*, and *Cyp19a1* in the testes at PD21.5, PD45.5, and PD90.5 were measured using RT-PCR. All of the genes were significantly increased at PD45.5 and PD90.5 compared with the levels observed at PD21.5. The data represent the mean ± SEM. *indicates a significant difference between the group and the PD21.5 group; #indicates a significant difference between PD45.5 and PD90.5, * or $^{\#}P<0.05$.
(TIF)

Figure S5 Effects of maternal perinatal CYP exposure on *Insl3* level of offspring. The mRNA levels of *Insl3* in the different treatment groups at PD21.5, PD45.5, and PD90.5. CYP treatment can decrease the *Insl3* level at the three time points. The data represent the mean ± SEM. *indicates a significant difference between the group and control group, * $P<0.05$.
(TIF)

Figure S6 Effects of CYP+LH treatment on steroidogenesis-related genes and *AR*, *Erα*, and *Pcna* levels in mLTC-1 cells. (A) mRNA levels of steroidogenesis-related genes in mLTC-1 cells. (B) mRNA levels of mLTC-1 *AR*, *ERα*, and *Pcna*. (C) Media T levels of mLTC-1 cells. CYP+LH reduced the induction effect of LH on *Star*, *Cyp11a1*, *AR*, and *ERα*, but the mRNA levels were still higher than those observed in the vehicle group. *3β-HSD*, *cyp17a1*, and *17β-HSD3* were downregulated by CYP+LH treatment, whereas LH alone had a minor inhibitory effect on the expression of these genes. *Cyp19a1* and *Pcna* were upregulated by CYP+LH treatment, whereas LH alone increased the expression of these genes. The media T levels were decreased in the 10^{-6} and 10^{-5} M CYP+LH groups compared with the LH group. The data represent the mean ± SEM. *indicates a significant difference between the group and the control group, #indicates a significant difference between the group and LH group, *or $^{\#}P<0.05$. C, CYP.
(TIF)

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Author Contributions

Conceived and designed the experiments: XL CH. Performed the experiments: CH. Analyzed the data: CH XL. Contributed reagents/materials/analysis tools: XL CH. Wrote the paper: CH XL. Critical revision: XL CH.

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