Maternal Cypermethrin Exposure During Lactation Impairs Testicular Development and Spermatogenesis in Male Mouse Offspring

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Received 24 July 2009; revised 27 November 2009; accepted 6 December 2009

ABSTRACT: Within the last decade, numerous epidemiological studies have demonstrated that endocrine disruptors are a possible cause for a decline in semen quality. Cypermethrin is a widely used pyrethroid insecticide, but little is known about its potentially adverse effects on male reproduction. In the present study, we investigated the effects of maternal cypermethrin exposure during lactation on testicular development and spermatogenesis in male offspring. Maternal mice were administered with cypermethrin (25 mg/kg) by gavage daily from postnatal day 0 (PND0) to PND21. Results showed that the weight of testes at PND21 was significantly decreased in pups whose mothers were exposed to cypermethrin during lactation. Maternal cypermethrin exposure during lactation markedly decreased the layers of spermatogenic cells, increased the inside diameter of seminiferous tubules, and disturbed the array of spermatogenic cells in testes of pups at PND21. In addition, maternal cypermethrin exposure during lactation markedly reduced mRNA and protein levels of testicular P450scc, a testosterone (T) synthetic enzyme. Correspondingly, the level of serum and testicular T at weaning was significantly decreased in pups whose mothers were exposed to cypermethrin during lactation. Although the expression of testicular T synthetic enzymes and serum and testicular T in adulthood had restored to control level, the decreased testicular weight and histological changes were irreversible. Importantly, the number of spermatozoa was significantly decreased in adult male offspring whose mothers were exposed to cypermethrin during lactation. In conclusion, maternal cypermethrin exposure during lactation permanently impairs testicular development and spermatogenesis in male offspring, whereas cypermethrin-induced endocrine disruption is reversible. Environ Toxicol 26: 382–394, 2011.

Keywords: cypermethrin; pyrethroid; testes; spermatogenesis; testosterone

INTRODUCTION

Endocrine disruptors are exogenous substances or mixtures that alter function of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or population. Endocrine disruptors may mimic, block, or modulate the synthesis, release, transport, metabolism and binding or elimination of natural hormones (Caserta et al., 2008). Within the last decade, numerous epidemiological studies have demonstrated that endocrine disruptors are a possible cause for a decline in semen quality and the increased incidence of testicular cancer, undescended testes, and hypospadias (Rignell-Hydbom et al., 2004; De Jager et al., 2006; Carbone et al., 2007; Mocarelli et al., 2008). Phthalates are the widely studied endocrine disruptors. According to an earlier study, gestational and

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Contract grant sponsor: National Natural Science Foundation of China.
Contract grant number: 30671786.
Contract grant sponsor: Key Project of Chinese Ministry of Education.
Contract grant number: 208060.

Published online 3 February 2010 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/tox.20566

lactational exposure of rats to butyl benzyl phthalate caused a decrease in the weight of testes and sperm production (Sharpe et al., 1995). Several recent studies showed that phthalates induced female-like anal–genital distance, retained nipples, undescended testes, and hypospadias in male offspring whose mothers were exposed to these chemicals during pregnancy (Parks et al., 2000; Akingbemi et al., 2004; Swan et al., 2005; McKee et al., 2006; Mahood et al., 2007; Scott et al., 2008; Hu et al., 2009). Vinclozolin, a commonly used fungicide, is another important endocrine disruptor. Several studies showed that prenatal vinclozolin exposure reduced the number of sperm and fertility at adulthood and increased the incidence of hypospadias and retained nipples in male offspring (Gray et al., 1994; Anway et al., 2005, 2006; Chang et al., 2006; Christiansen et al., 2008).

Cypermethrin [(R,S)-α-cyano-3-phenoxybenzyl (1R,S)-*cis*,trans-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropane], a synthetic pyrethroid insecticide, has been widely used to control noxious insects in agriculture, forestry, households, horticulture, and the public health. Like other synthetic pyrethroid insecticides, cypermethrin is a mutagen (Bhunya and Pati, 1988; Surrallés et al., 1995). An earlier study showed that a single oral dose of cypermethrin significantly increased the frequency of sister chromatid exchange in mouse bone marrow cells (Chauhan et al., 1997). A recent study found that cypermethrin induced sister chromatid exchange, chromosomal aberrations, and micronucleus formation in human peripheral lymphocytes (Kocaman and Topaktaş, 2009). However, relatively few studies have investigated potentially adverse effects of pyrethroid insecticides including cypermethrin on male reproduction and endocrine disruption. According to an earlier report, fertility was significantly reduced in male rats exposed to cypermethrin. A significant reduction in the number of implantation sites and viable fetuses was also observed in females impregnated by males exposed to cypermethrin. In addition, epididymal and testicular sperm counts as well as daily sperm production were significantly decreased in males exposed to cypermethrin (Elbetieha et al., 2001). A recent study showed that the reduced semen quality and the increased sperm DNA damage were associated with the level of urinary pyrethroid metabolites in 207 men recruited from an infertility clinic (Meeker et al., 2008).

In the present study, we investigated the effects of maternal cypermethrin exposure during lactation on testicular development and spermatogenesis in mice. T is essential in normal testicular development and spermatogenesis. Steroidogenic acute regulatory (StAR) protein is limiting factor in T biosynthesis, responsible for the transport of cholesterol into mitochondria (Miller, 2007). T synthetic enzymes including cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc), cytochrome P450 17α-hydroxysteroid dehydrogenase ($P450_{17\alpha}$), and 17β-hydroxysteroid dehydrogenase (17β-HSD) play a critical role in the synthesis of T in Leydig cells. Therefore, the present study also investigated the effects of maternal cypermethrin exposure during lactation on T synthesis and the expression of StAR, P450scc, $P450_{17\alpha}$, and 17β-HSD in testes.

MATERIALS AND METHODS

Chemicals

Cypermethrin was purchased from Sigma Chemical (St Louis, MO). All other reagents were from Sigma or as indicated in the specified methods.

Animals and Treatments

The ICR mice (8- to 10-week-old; male mice: 28–30 g; female mice: 24–26 g) were purchased from Beijing Vital River (Beijing, China) whose foundation colonies were all introduced from Charles River Laboratories (Wilmington, MA, USA). 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 for a period of 1 week before use. For mating purposes, four females were housed overnight with two males starting at 9:00 p.m. Females were checked by 7:00 a.m. the next morning, and the presence of a vaginal plug was designated as gestational day (gd) 0. Within 24 h after birth, excess pups were removed so that ten pups were kept per dam. Maternal mice were administered with cypermethrin (25 mg/kg) by gavage daily from postnatal day 0 (PND0) to weaning (PND21). The corn oil-treated pregnant mice served as controls.

At postnatal day (PND) 21, twelve male pups from six litters each group were terminated. Blood serum was collected for measurement of T. Testes were excised, dissected, weighed, and then divided into two parts: left one was kept at −80°C for subsequent measurement of T, RT-PCR, and immunoblotting; the other part of the testes was immersed in modified Davidson's fluid (mDF) for 12–24 h for testicular histology and apoptosis analysis (Latendresse et al., 2002). At postnatal day (PND) 70, twelve male pups from six litters each group were slaughtered. Blood serum was collected for measurement of T. Cauda epididymides were removed for analysis of sperm quality. Testes were excised, dissected, weighed, and then divided into two parts: left one was kept at −80°C for subsequent measurement of T, RT-PCR, and Western blot; the other part of the testes was immersed in mDF for 12–24 h for testicular histology and apoptosis analysis. 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.

Sperm Analysis

The cauda epididymides were removed and immediately immersed into the F12 medium supplemented with 0.1%

TABLE I. 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	42	310
P450scc	Forward: 5′-AGGTGTAGCTCAGGACTTCA-3′; Reverse: 5′-AGGAGGCTATAAAGGACACC-3′	94	56	72	39	370
P450 17α	Forward: 5′-CCAGGACCCAAGTGTGTTCT-3′; Reverse: 5′-CCTGATACGAAGCACTTCTCG-3′	94	56	72	39	250
17β-HSD	Forward: 5′- ATTTTACCAGAGAAGACATCT-3′; Reverse: 5′- GGGGTCAGCACCTGAATAATG-3′	94	52	72	43	367

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 (World Health Organization, 1999).

Testicular Histology and Immunohistochemistry

Mouse testes were embedded in paraffin using standard procedures at the Pathological Lab of 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 goat polyclonal antibody against 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 rabbit antigoat (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, Carpinteria, California, USA). Immunostaining was developed by application of diaminobenzidine (liquid DAB$^+$; Dako, Carpinteria, California, USA), 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 twelve randomly selected fields from each slide at a magnification of 400×.

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 radioimmunoassay (RIA) kits from Beijing North Institute of Biological Technology (Beijing, China). For measuring testicular T in mice, testes 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 to dryness in a fume hood, and 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/testis.

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 were determined by measuring the 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 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 1XFlexi buffer. The primers were synthesized by Sangon Biological Technology (Shanghai, China), according to sequence designs described by the literature (Akingbemi et al., 2003). The primer pairs, the number of cycles, and annealing temperature are shown in Table I. 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.

Immunoblotting

Testicular samples 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 dodecylsylphate (SDS), and 1 mM phenylmethylsulfonyl fluoride (PMSF). Samples were then centrifuged at 15,000 $\times$ 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, Bedford, MA) 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 mouse StAR, P450scc, $P450_{17\alpha}$, and 17β-HSD (Santa Cruz Biotechnology, USA) or β-actin (Beijing Biosynthesis Biotechnology, Beijing, China) at room temperature. After washes in DPBS containing 0.05% Tween-20 four times for 10 min each, the membranes were incubated with goat antirabbit 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).

Terminal dUTP Nick-End Labeling Staining

For the detection of apoptosis, paraffin-embedded sections were stained with the terminal dUTP nick-end labeling (TUNEL) technique using an *in situ* apoptosis detection kit (Promega) according to the manufacturer's protocols. For fetal testis, the number of TUNEL-positive cells was counted in four randomly selected fields from each slide at a magnification of 400×. For adult testis, the number of TUNEL-positive cells was counted in twelve randomly selected fields from each slide at a magnification of 400×.

Statistical Analysis

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

RESULTS

Effects of Maternal Cypermethrin Exposure During Lactation on Testicular Development

No signs of maternal toxicity were observed in dams treated with cypermethrin. In addition, maternal cypermethrin exposure during lactation had little effect on body weight of pups at PND21 (data not shown). The effects of maternal cypermethrin exposure during lactation on the weight of testes in male pups at weaning (PND21) were analyzed. Results showed that the weight of testes at weaning was significantly decreased in pups whose mothers were exposed to cypermethrin during lactation as compared with controls (56.4 $\pm$ 2.6 mg *vs* 45.1 $\pm$ 1.1 mg, $n = 12$, $P < 0.01$).

Effects of Maternal Cypermethrin Exposure During Lactation on Histology, the Numbers of Leydig Cells, and Apoptosis in Testes at Weaning

The morphology of testes in control mice at weaning was normal [Fig. 1(A)]. Maternal cypermethrin exposure during lactation markedly decreased the layers of spermatogenic cells, increased the inside diameter of seminiferous tubules, and disturbed the array of spermatogenic cells in testicular sections of pups at PND21 [Fig. 1(B)]. Leydig cells in testes at weaning were determined by immunostaining for 3β-HSD [Fig. 1(C,D)]. No significant difference on the number of Leydig cells in testes at PND21 was observed between two groups [Fig. 1(G)]. Testicular cell apoptosis was detected using TUNEL [Fig. 1(E,F)]. There was no significant difference on the number of apoptotic cells in testes at PND21 between two groups [Fig. 1(H)].

Effects of Maternal Cypermethrin Exposure during Lactation on the Levels of Serum and Testicular T in Male Pups at Weaning

The effects of maternal cypermethrin exposure during lactation on the level of serum and testicular T at weaning were analyzed. Results showed that the level of serum T at weaning was significantly decreased in pups whose mothers were exposed to cypermethrin during lactation (0.77 $\pm$ 0.26 ng/mL *vs* 0.15 $\pm$ 0.07 ng/mL, $n = 12$, $P < 0.01$). Correspondingly, maternal cypermethrin

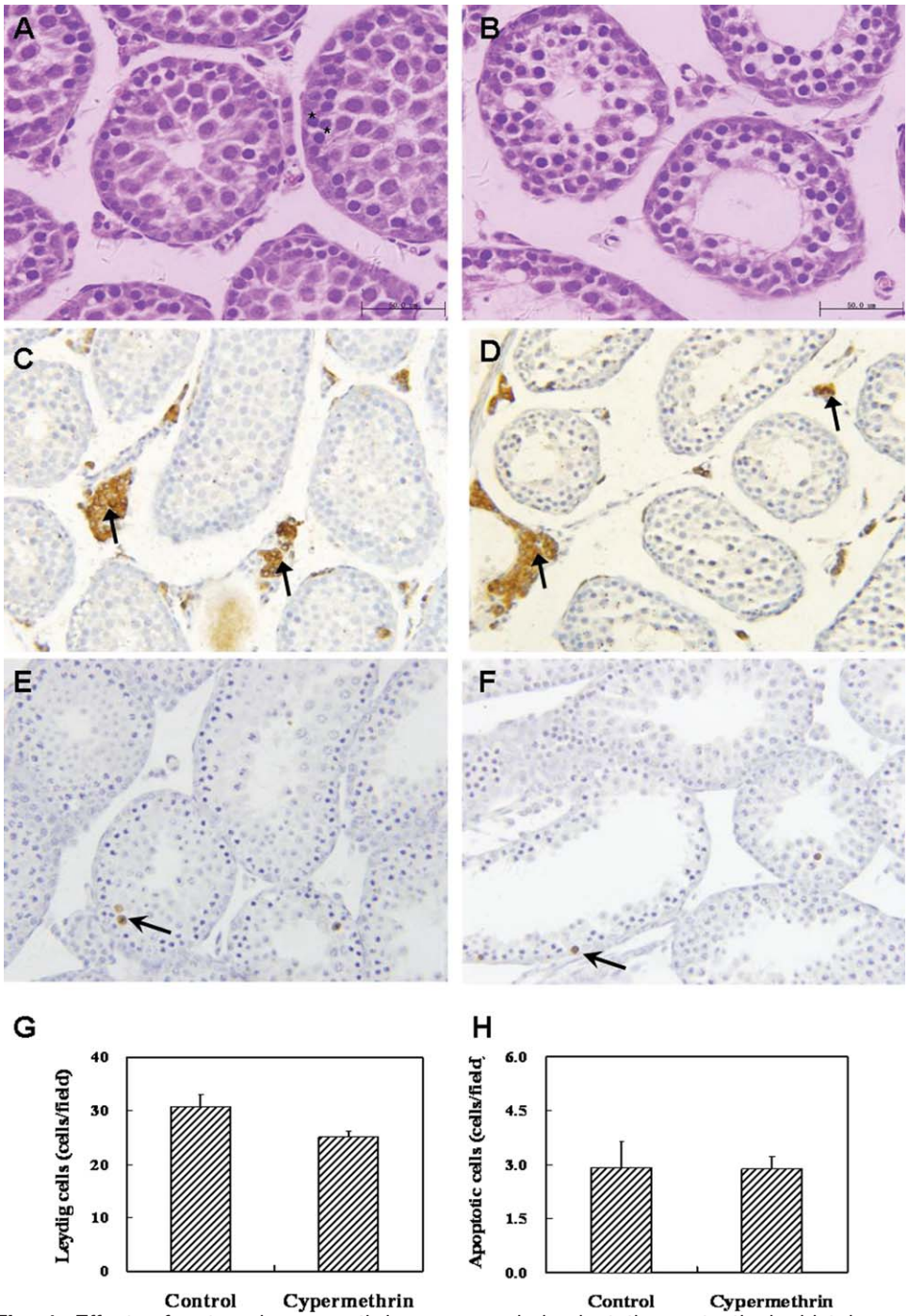

Fig. 1. Effects of maternal cypermethrin exposure during lactation on testicular histology, the number of Leydig cells, and testicular apoptosis in testes of young pups. Maternal mice were administered with cypermethrin (25 mg/kg) by gavage daily from PND0 to PND21. Testes were collected at PND21. Testicular cross sections from (A) control and (B) cypermethrin-treated mice were stained with H&E at 640× magnification. Leydig cells in testes of (C) control and (D) cypermethrin-treated mice were immunolocalized by staining with a polyclonal antibody specific for 3β-HSD. Apoptosis was analyzed with TUNEL staining of testis sections from (E) control and (F) cypermethrin-treated mice. The numbers of (G) Leydig cells and (H) TUNEL$^+$ cells in testes were counted in 12 randomly selected fields from each slide at 400× magnification. Data were expressed as means ± SEM of twelve samples from six litters. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

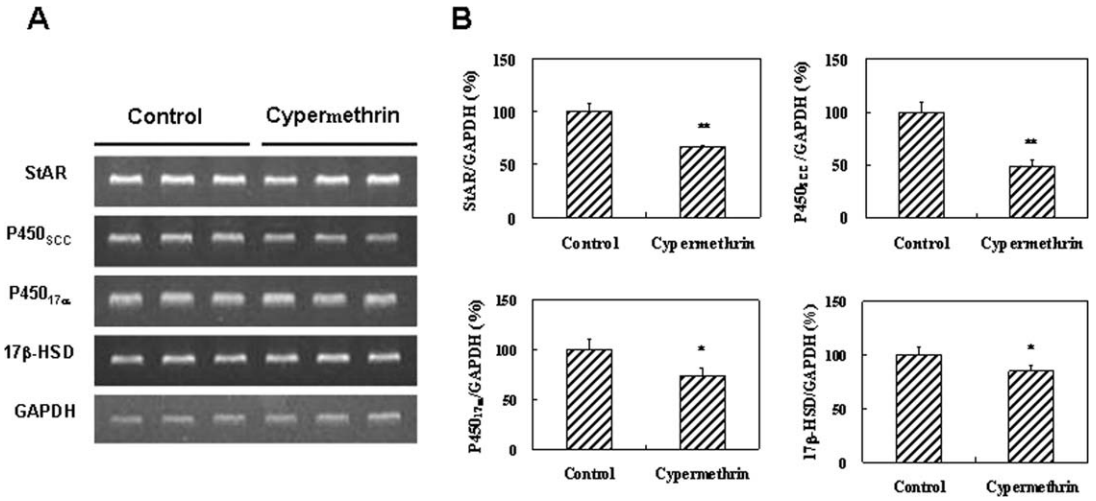

Fig. 2. Effects of maternal cypermethrin exposure during lactation on mRNA levels of testicular StAR and T biosynthetic enzymes in pups at weaning. Maternal mice were administered with cypermethrin (25 mg/kg) by gavage daily from PND0 to PND21. Testes were collected at PND21. (A) mRNA level of StAR, P450scc, P450$_{17\alpha}$, and 17β-HSD was measured using RT-PCR. (B) Quantitative analysis of StAR, P450scc, P450$_{17\alpha}$, and 17β-HSD mRNA from six individual RNA samples at each point was performed. StAR, P450scc, P450$_{17\alpha}$, and 17β-HSD mRNA levels were normalized to GAPDH mRNA level in the same samples. StAR, P450scc, P450$_{17\alpha}$, and 17β-HSD mRNA levels of the control were assigned as 100%. All data were expressed as means ± SEM of six samples from six litters. $^{*}P < 0.05$, $^{**}P < 0.01$ significantly different as compared with controls.

exposure during lactation significantly decreased the level of testicular T in pups at weaning (0.64 ± 0.17 ng/testis *vs* 0.30 ± 0.08 ng/testis, $n = 12$, $P < 0.05$).

Effects of Maternal Cypermethrin Exposure During Lactation on the Expression of Testicular StAR and T Biosynthetic Enzymes in Male Pups at Weaning

The effects of maternal cypermethrin exposure during lactation on mRNA level of testicular StAR and T biosynthetic enzymes at weaning are presented in Figure 2. The levels of testicular P450scc mRNA at weaning were significantly decreased in pups whose mothers were exposed to cypermethrin during lactation. The effects of maternal cypermethrin exposure during lactation on protein expression of testicular StAR and T biosynthetic enzymes at weaning are presented in Figure 3. Results showed that protein level of testicular P450scc at weaning was significantly decreased in pups whose mothers were exposed to cypermethrin during lactation. Although mRNA levels of testicular StAR, 17β-HSD, and P450$_{17\alpha}$ were slightly decreased, maternal cypermethrin exposure during lactation had little effect on protein expression of testicular StAR, 17β-HSD, and P450$_{17\alpha}$ at weaning (Figs. 2 and 3).

Effects of Maternal Cypermethrin Exposure During Lactation on the Weight of Testes and Sperm Quality in Adult Male Offspring

The effects of maternal cypermethrin exposure during lactation on the weight of testes and sperm quality in adult male offspring were analyzed. Results showed that the weight of testes in adult male offspring whose mothers were exposed to cypermethrin during lactation was lighter than that of controls (250.5 ± 10.2 mg *vs* 228.3 ± 6.3 mg, $n = 12$, $P < 0.05$). In addition, maternal cypermethrin exposure during lactation significantly decreased the number of spermatozoa in adult male offspring (19.4×10^6 *vs* 15.8×10^6, $n = 12$, $P < 0.01$).

Effects of Maternal Cypermethrin Exposure During Lactation on Histology and the Numbers of Leydig Cells and Apoptosis in Testes of Adult Male Offspring

The effects of maternal cypermethrin exposure during lactation on testicular histology of adult male pups are shown in Figure 4. The morphology of testes in control mice in adulthood was normal [Fig. 4(A)]. Maternal cypermethrin exposure markedly increased the inside diameter of seminiferous tubules and disturbed the array of spermatogenic cells in testicular sections of adult male offspring

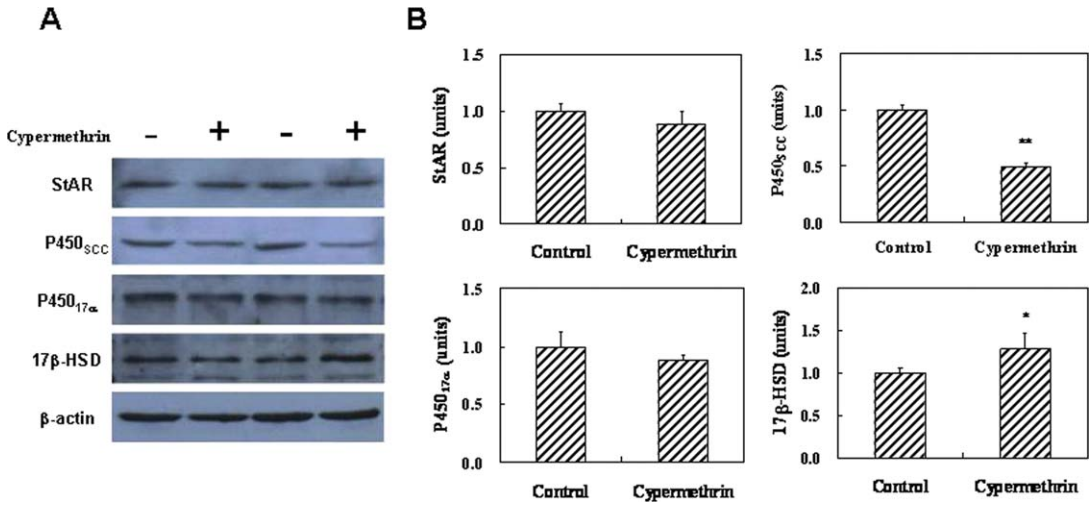

Fig. 3. Effects of maternal cypermethrin exposure during lactation on protein expressions of StAR and T biosynthetic enzymes in pups at weaning. Maternal mice were administered with cypermethrin (25 mg/kg) by gavage daily from PND0 to PND21. Testes were collected at PND21. (A) The protein expression of StAR, P450scc, $P450_{17\alpha}$, and 17β-HSD was measured using immunoblotting. (B) Quantitative analysis of scanning densitometry on four samples at each time point was performed. StAR, P450scc, $P450_{17\alpha}$, and 17β-HSD were normalized to β-actin level in the same sample. The densitometry unit of the control was assigned as 1. All data were expressed as means $\pm$ SEM of six samples from six litters. $^{*}P < 0.05$, $^{**}P < 0.01$ significantly different as compared with controls. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

[Fig. 4(B)], whereas there was no significant difference on the number of Leydig cells in testes of adult male offspring between two groups [Fig. 4(C,D,G)]. There was no significant difference on the number of apoptotic cells in testes between controls and pups whose mothers were exposed to cypermethrin during lactation [Fig. 4(E,F,H)].

Effects of Maternal Cypermethrin Exposure During Lactation on Serum and Testicular T in Adult Male Offspring

The effects of maternal cypermethrin exposure during lactation on the level of serum and testicular T in adult male offspring are analyzed. Results showed that maternal cypermethrin exposure during lactation had little effect on the level of serum T in adult male offspring (19.1 $\pm$ 6.6 ng/mL *vs* 33.7 $\pm$ 8.5 ng/mL, $n = 12$, $P > 0.05$). In addition, maternal cypermethrin exposure during lactation did not affect the level of testicular T in adult male offspring (97.2 $\pm$ 22.1 ng/testis *vs* 156.5 $\pm$ 22.6 ng/testis, $n = 12$, $P > 0.05$).

Effects of Maternal Cypermethrin Exposure During Lactation on the Expression of StAR and T Biosynthetic Enzymes in Testes of Adult Male Offspring

The effects of maternal cypermethrin exposure during lactation on mRNA level of testicular StAR and T biosynthetic enzymes in adult male offspring are presented in Figure 5. There was no significant difference on steady state mRNA level of testicular P450scc, $P450_{17\alpha}$, and 17β-HSD at adulthood, whereas a slight decrease in mRNA level of testicular StAR was observed between controls and mice whose mothers were exposed to cypermethrin during lactation. The effects of maternal cypermethrin exposure during lactation on protein expression of testicular StAR and T biosynthetic enzymes at adulthood are presented in Figure 6. There was a slight decrease in protein expression of testicular StAR in adult male offspring whose mothers were exposed to cypermethrin during lactation. No significant difference on protein expression of testicular P450scc, $P450_{17\alpha}$, and 17β-HSD in adult male offspring was observed between controls and mice whose mothers were exposed to cypermethrin during lactation.

Effects of Maternal Cypermethrin Exposure During Lactation on Fertility of Male Offspring

To investigate the effects of maternal cypermethrin exposure during lactation on the fertility of male offspring, twelve males whose mothers were exposed to cypermethrin during lactation were housed with untreated females. Fertility analysis showed that all twelve males in control group mated successfully with control females, 75% (9/12) of which were found to be fertilized and complete the pregnancy. Ten of eleven males (91%) whose mothers were

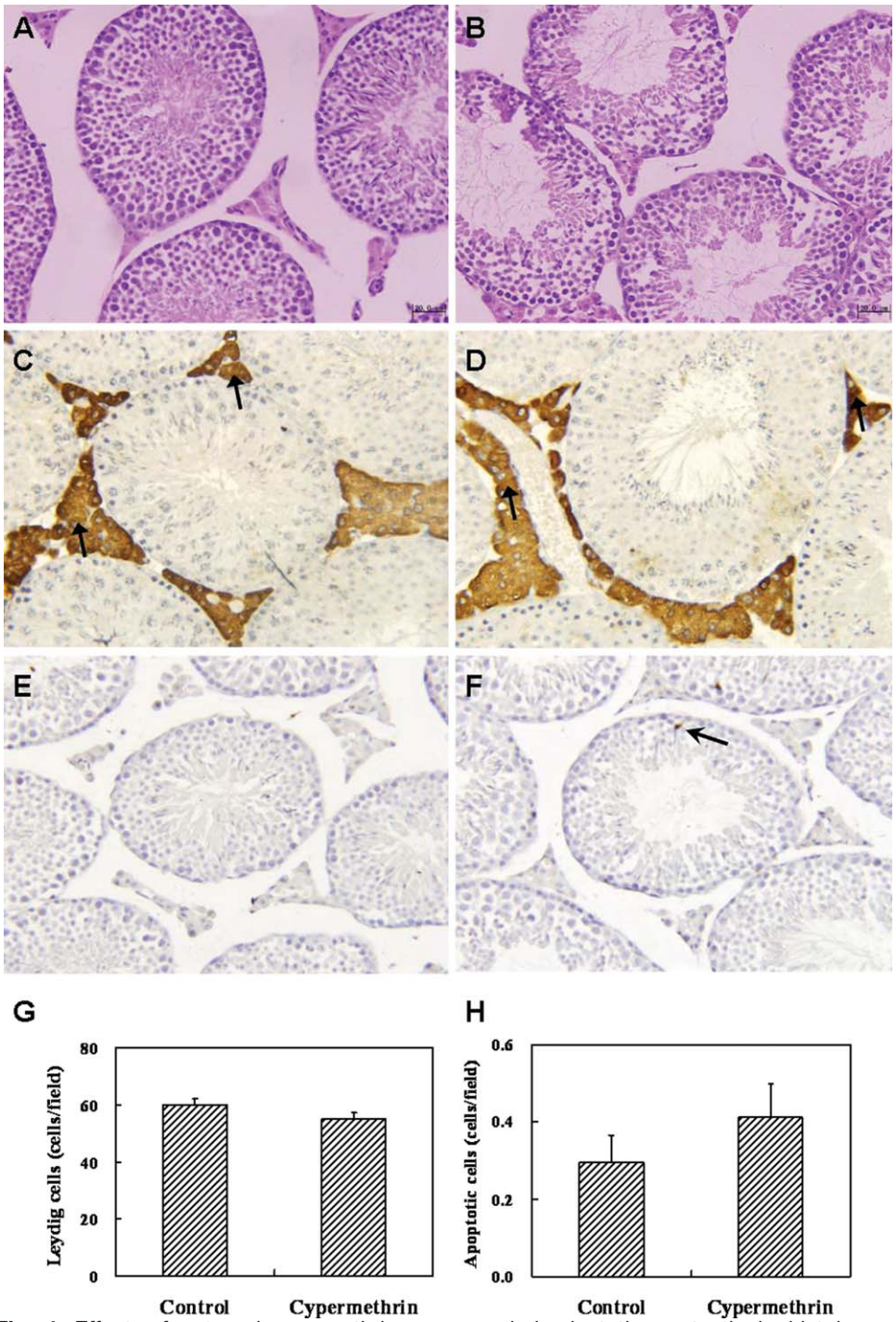

Fig. 4. Effects of maternal cypermethrin exposure during lactation on testicular histology, the number of Leydig cells, and apoptosis in testes of adult male offspring. Maternal mice were administered with cypermethrin (25 mg/kg) by gavage daily from PND0 to PND21. Testes were collected at PND70. Testicular cross sections from (A) control and (B) cypermethrin-treated mice were stained with H&E at 320× magnification. Leydig cells in testes of (C) control and (D) cypermethrin-treated mice were immunolocalized by staining with a polyclonal antibody specific for 3β-HSD. Apoptosis was analyzed with TUNEL staining of testis sections from (E) control and (F) cypermethrin-treated mice. The numbers of (G) Leydig cells and (H) TUNEL$^+$ cells in testes were counted in 12 randomly selected fields from each slide at 400× magnification. Data were expressed as means ± SEM of twelve samples from six litters. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

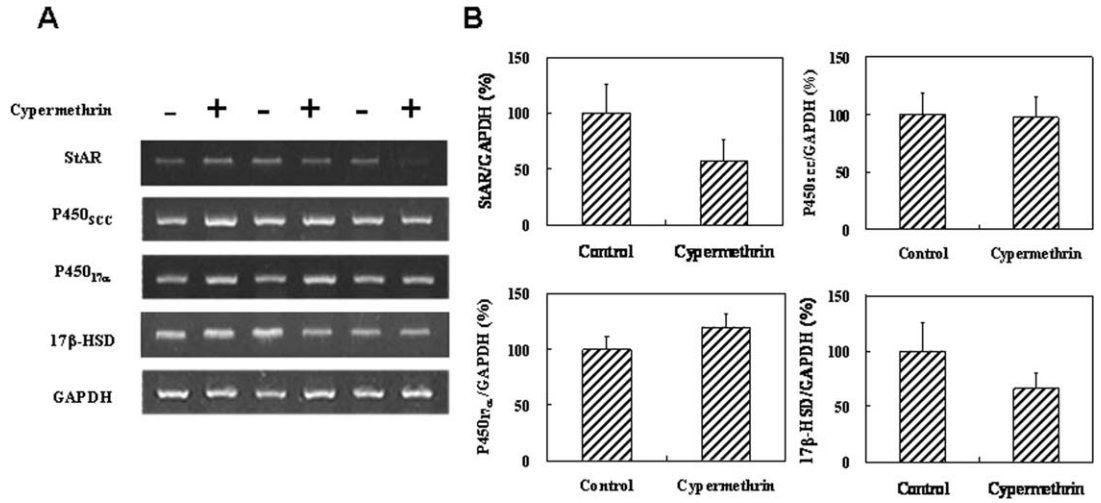

Fig. 5. Effects of maternal cypermethrin exposure during lactation on mRNA levels of testicular StAR and T biosynthetic enzymes in adult male offspring. Maternal mice were administered with cypermethrin (25 mg/kg) by gavage daily from PND0 to PND21. Testes were collected from adult male offspring at PND70. (A) StAR, P450scc, P450$_{17\alpha}$, and 17β-HSD mRNA was measured using RT-PCR. (B) Quantitative analysis of StAR, P450scc, P450$_{17\alpha}$, and 17β-HSD mRNA from six individual RNA samples at each point was performed. The levels of StAR, P450scc, P450$_{17\alpha}$, and 17β-HSD mRNA were normalized to GAPDH mRNA level in the same sample. StAR, P450scc, P450$_{17\alpha}$, and 17β-HSD mRNA levels of the control were assigned as 100%. All data were expressed as means ± SEM of six samples from six litters.

exposed to cypermethrin exposure during lactation mated successfully with control females, all of which were found to be fertilized and complete the pregnancy (Table II). There was no significant difference on the numbers of implantation sites per litter, resorptions, live fetuses, and dead fetuses between cypermethrin-treated mice and

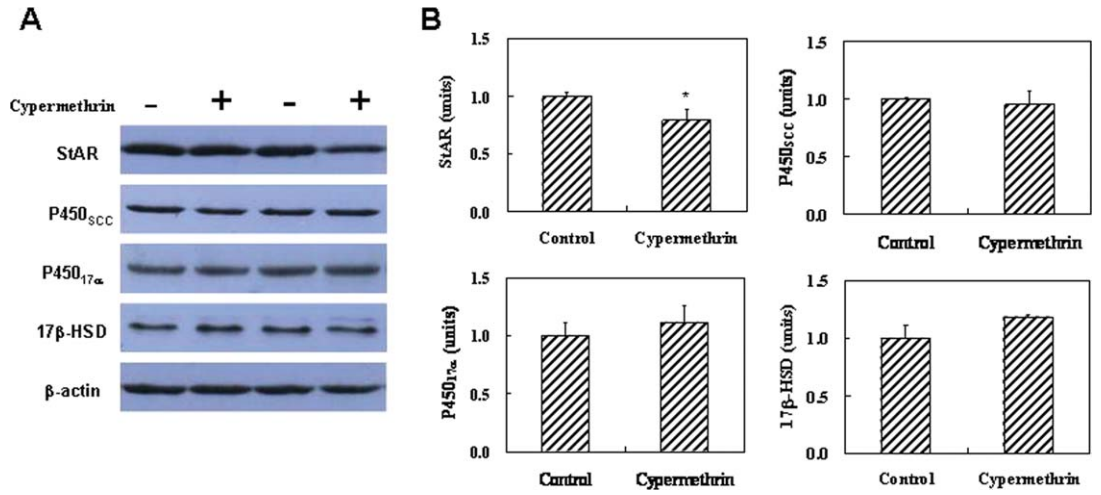

Fig. 6. Effects of maternal cypermethrin exposure during lactation on protein expressions of testicular StAR and T biosynthetic enzymes in adult male offspring. Maternal mice were administered with cypermethrin (25 mg/kg) by gavage daily from PND0 to PND21. Testes were collected from adult male offspring at PND70. (A) The protein expressions of StAR, P450scc, P450$_{17\alpha}$, and 17β-HSD were measured using immunoblotting. (B) Quantitative analysis of scanning densitometry at each time point was performed. StAR, P450scc, P450$_{17\alpha}$, and 17β-HSD were normalized to β-actin level in the same samples. The densitometry unit of the control was assigned as 1. All data were expressed as means ± SEM of six samples from six litters. *$P < 0.05$ as compared with controls. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE II. Effects of maternal cypermethrin exposure during lactation on the fertility of male offsprings

Groups	No. of Tested Males	Males Mated Successfully		Fertile Males	
		n	%	*n*	%
Control	12	12	100	9	75
Cypermethrin	11	10	91	10	100

controls (Table III). In addition, no significant difference on the average litter size, male/female sex ratios, body weight, and crown length in F2 generation was observed between cypermethrin-treated mice and controls (Tables IV and V).

DISCUSSION

Several studies showed that administration of adult male animals with cypermethrin was associated with the poor semen quality (Elbetieha et al., 2001; Ahmad et al., 2009). In the present study, we for the first time investigated the effects of maternal cypermethrin exposure during lactation on testicular development and spermatogenesis in male offspring. We found that the weight of testes at weaning was significantly decreased in pups whose mothers were exposed to cypermethrin during lactation. In addition, maternal cypermethrin exposure during lactation markedly decreased the layers of spermatogenic cells, increased the inside diameter of seminiferous tubules, and disturbed the array of spermatogenic cells in testes of male pups at PND21. Importantly, the decreased testicular weight and histological damage were irreversible. The number of spermatozoa was significantly decreased in adult offspring whose mothers were exposed to cypermethrin during lactation. These results suggest that an indirect exposure to cypermethrin or its metabolites through milk may persistently impair testicular development and spermatogenesis in male offspring.

Androgens, primarily T, which act through the somatic cells to regulate germ cell differentiation, are a prerequisite for normal testicular development and spermatogenesis (Holdcraft and Braun, 2004). Several studies showed that a Sertoli cell-selective knockout of the androgen receptor resulted in spermatogenic arrest in meiosis and infertility with defective spermatogenesis and hypotestosteronemia (Chang et al., 2004; De Gendt et al., 2004). The present study investigated the effects of maternal cypermethrin exposure during lactation on T biosynthesis in testes of male pups at weaning. We found that serum T in young pups at PND21 was significantly decreased when their mothers were exposed to cypermethrin from PND0 to PND21. Correspondingly, maternal exposure to cypermethrin during lactation markedly reduced the level of testicular T in pups at weaning. These results suggest that maternal cypermethrin exposure during lactation decreases T synthesis in testes of male offspring.

StAR and T biosynthetic enzymes play a critical role in the biosynthesis of T in Leydig cells. StAR is an essential and limiting factor in T biosynthesis, responsible for the transport of cholesterol into mitochondria (Miller, 2007). A recent study showed that permethrin, another pyrethroid insecticide, disrupted T synthesis via downregulating the expression of testicular StAR in adult mice (Zhang et al., 2007). In the present study, we investigated the effects of maternal cypermethrin exposure during lactation on the expression of testicular StAR in male pups. Our results showed that maternal cypermethrin exposure during lactation had little effect on protein expression of testicular StARat weaning, whereas mRNA levels of testicular StAR-were slightly decreased in pups whose mothers were exposed to cypermethrin during lactation.

Testicular T synthetic enzymes, primarily P450scc, $P450_{17\alpha}$, and 17β-HSD, play a critical role in T synthesis in Leydig cells. P450scc initiates the first enzymatic step in T biosynthesis in the inner mitochondrial membrane of Leydig cells, where cholesterol is converted to pregnenolone. Pregnenolone is catalyzed by $P450_{17\alpha}$ to produce 17-hydroxyprogesterone and androstenedione, with the latter then being converted to T by 17β-HSD (Payne and Youngblood, 1995). The present study showed that the levels of testicular P450scc mRNA at PND21 were significantly decreased in pups whose mothers were exposed to cypermethrin from PND0 to PND21. Consistent with the decreased mRNA, P450scc protein was significantly decreased in testes of male pups whose mothers were exposed to cypermethrin during lactation. Although the levels of testicular 17β-HSD and $P450_{17\alpha}$ mRNA at weaning were slightly decreased, maternal cypermethrin exposure during lactation had little effect on protein expression of 17β-HSD and $P450_{17\alpha}$ in testes at weaning. These results

TABLE III. The fetal outcomes

Groups	No. of Litters (*n*)	Implantation Sites per Litter ($\bar{x}$ ± SEM)	Resorptions per Litter ($\bar{x}$ ± SEM)	Live Fetuses per Litter ($\bar{x}$ ± SEM)	Dead Fetuses per Litter ($\bar{x}$ ± SEM)
Control	9	13.11 ± 0.59	0.67 ± 0.24	11.89 ± 0.63	0.56 ± 0.44
Cypermethrin	10	11.81 ± 1.24	0.90 ± 0.28	10.60 ± 1.19	0.30 ± 0.15

TABLE IV. Effects of maternal cypermethrin exposure during lactation on the litter size and sex ratio in F2 generation

Groups	No. of Litters (*n*)	Litter Size			Male/Female Ratio
		Male	Female	Total	
Control	9	5.3 ± 0.6	6.6 ± 0.6	11.9 ± 0.6	0.90 ± 0.16
Cypermethrin	10	4.6 ± 0.5	6.0 ± 1.0	10.6 ± 1.2	0.90 ± 0.18

suggest that the decreased testicular T synthesis in pups whose mothers were exposed to cypermethrin during lactation may be attributed to downregulation of P450scc expression in testes.

Aside from the expression of StAR and T biosynthetic enzymes, we searched for other possible factors involved in the decreased T production in pups whose mothers were exposed to cypermethrin during lactation. Recently, several studies showed that abnormal Leydig cell aggregation in the fetal testis of rats exposed to di(*n*-butyl)phthalate or di(2-ethylhexyl)phthalate, the two endocrine disruptors, was associated with the decreased level of testicular T (Mahood et al., 2005, 2007; Lin et al., 2008). In the present study, we analyzed the number of leydig cells in testes of pups at weaning. We found that no significant difference in the number of Leydig cells in testes was observed between controls and mice whose mothers were exposed to cypermethrin during lactation. However, the present study does not exclude that maternal cypermethrin exposure during lactation disrupts testicular T synthesis via abnormal Leydig cell aggregation. Additional work is required to determine whether maternal cypermethrin exposure during lactation induces abnormal Leydig cell aggregation in testes of male pups.

In the present study, we found that maternal cypermethrin exposure during lactation induced a persistent impairment on testicular histology and spermatogenesis in male offspring. To investigate whether maternal cypermethrin exposure during lactation permanently disrupts testicular T synthesis in male offspring, we detected the level of serum and testicular T in adult male offspring. We found that serum and testicular T in adulthood had restored to control level. Correspondingly, the expression of testicular T biosynthetic enzymes had also fully recovered, although the level of testicular StAR in cypermethrin-treated mice remained lower than that of controls. These results suggest that the disruptive effects of maternal cypermethrin exposure during lactation on testicular T synthesis are reversible.

Numerous testicular toxicants including some endocrine-disrupting compounds (EDCs), such as lindane, mono(2-ethylhexyl)phthalate, di(2-ethylhexyl)phthalate, and para-nonylphenol, have been demonstrated to induce germ cell apoptosis in testes (Ichimura et al., 2003; Borch et al., 2005; Sobarzo et al., 2006; McClusky et al., 2007; Saradha et al., 2009). To elucidate whether germ cell apoptosis is involved in mediating testicular toxicity of cypermethrin, the present study evaluated apoptosis in testes. Our results showed that no significant difference in the number of apoptotic cells was observed in testes between controls and pups whose mothers were exposed to cypermethrin during lactation. In addition, maternal cypermethrin exposure during lactation did not affect the number of apoptotic cells in testes of adult male offspring. These results suggest that cypermethrin-mediated impairment on testicular development and spermatogenesis is independent of germ cell apoptosis.

Relatively few studies have investigated potentially adverse effects of pyrethroid insecticides on male fertility. An earlier study showed that fertility was significantly reduced in male rats exposed to cypermethrin. A significant reduction in the number of implantation sites and viable fetuses was also observed in females impregnated by males exposed to cypermethrin (Elbetieha et al., 2001). In the present study, we investigated the effects of maternal cypermethrin exposure during lactation on the fertility of male offspring. We found that 91% of males whose mothers were exposed to cypermethrin during lactation mated successfully with control females, all of which were found to be fertilized and complete the pregnancy. There was no significant difference on the numbers of implantation sites per litter, resorptions, live fetuses, and dead fetuses between cypermethrin-treated mice and controls. In addition, maternal cypermethrin exposure during lactation had

TABLE V. The fetal weight and crown length

Groups	No. of Litters (*n*)	Weight (g, $\bar{x}$ ± SEM)			Crown Length (mm, $\bar{x}$ ± SEM)		
		Male	Female	Total	Male	Female	Total
Control	9	1.33 ± 0.03	1.21 ± 0.03	1.26 ± 0.03	25.4 ± 0.18	24.9 ± 0.21	25.1 ± 0.15
Cypermethrin	10	1.41 ± 0.05	1.33 ± 0.02	1.39 ± 0.05	25.9 ± 0.26	25.3 ± 0.16	25.8 ± 0.25

little effect on the average litter size, male/female sex ratios, body weight, and crown length in F2 generation.

In summary, the present results allow us to reach the following conclusions. First, maternal cypermethrin exposure during lactation permanently impairs testicular development and spermatogenesis; second, maternal cypermethrin exposure during lactation induces a reversible decrease in testicular T synthesis via downregulating the expression of testicular P450scc in male offspring.

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