Food and Chemical Toxicology 48 (2010) 1160–1169

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Maternal fenvalerate exposure during pregnancy persistently impairs testicular development and spermatogenesis in male offspring

Heng Zhang [1], Hua Wang [1], Yan-Li Ji, Ying Zhang, Tao Yu, Huan Ning, Cheng Zhang, Xian-Feng Zhao, Qun Wang, Ping Liu, De-Xiang Xu *

Department of Toxicology, Anhui Medical University, Hefei 230032, China

ARTICLE INFO

Article history:
Received 17 July 2009
Accepted 1 February 2010

Keywords:
Fenvalerate
Fetus
Testis
Spermatogenesis
Testosterone
Endocrine disruptor

ABSTRACT

Fenvalerate, a widely used pyrethroid insecticide, has been associated with poor semen quality. As yet, little is known about the effects of prenatal fenvalerate exposure on testicular development. The present study investigated the effects of prenatal fenvalerate exposure on testicular development and spermatogenesis. The pregnant mice were administered fenvalerate (30 mg/kg) by gavage daily from gestational day (gd) 13 to gd 18. The weights of testes and epididymides were significantly decreased in mice whose mothers were exposed to fenvalerate during pregnancy. Importantly, maternal fenvalerate exposure during pregnancy markedly decreased the number of mature seminiferous tubules (stages VII and VIII) in testes of adult male offspring. In addition, maternal fenvalerate exposure during pregnancy significantly reduced the number of epididymal spermatozoa in adult male offspring. Additional experiments showed that the level of serum testosterone (T) was significantly decreased in male fetuses whose mothers were exposed to fenvalerate during pregnancy. Correspondingly, mRNA and protein levels of $P450_{17\alpha}$, a T synthetic enzyme, were significantly decreased in fetal testes. Moreover, the disruptive effect of prenatal fenvalerate exposure on testicular T synthesis was irreversible. In conclusion, prenatal fenvalerate exposure irreversibly impairs testicular development and spermatogenesis at least into early adulthood.

1. 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, cryptorchidism and hypospadias (Rignell-Hydbom et al., 2004; De Jager et al., 2006; Carbone et al., 2007; Mocarelli et al., 2008).

Fenvalerate [(R, S)-α-cyano-3-phenoxybenzyl (R, S)-2-(4-chlorophenyl) -3-methylbutyric ester] is a synthetic pyrethroid insecticide, which has been widely used to control noxious insects in agriculture, forestry and horticulture (WHO, 1990). As a possible contaminant of food, fenvalerate could be usually ingested by human being. A recent study showed that the frequencies of sex chromosome aneuploidy and numerical chromosome aberration in spermatozoa were significantly increased among workers exposed to fenvalerate (Xia et al., 2004). In addition, an increase in sperm DNA fragmentation was observed among workers exposed to fenvalerate (Bian et al., 2004). According to an epidemiological investigation, occupational fenvalerate exposure was associated with the poor semen in male workers (Tan et al., 2006). Our recent study showed that maternal fenvalerate exposure during lactation markedly disrupted spermatogenesis and steroidogenesis in male offspring (Zhang et al., 2009). An earlier study demonstrated that perinatal fenvalerate exposure during the critical periods of male brain sexual differentiation significantly decreased sexual behavior and increased immobility in the open field. Importantly, a significant reduction in ductus deferens and seminal vesicle weights was also observed in male offspring of rats exposed to fenvalerate (Moniz et al., 1999). Although fenvalerate has been demonstrated to be a reproductive toxicant in human beings and rodent animals, little is known about the mechanism of its reproductive toxicity.

The purpose of the present study was to investigate the effects of prenatal fenvalerate exposure on testicular development and

Abbreviations: EDCs, endocrine disrupting chemicals; 3β-HSD, 3β-hydroxysteroid dehydrogenase; $P450_{17\alpha}$, cytochrome P450 17α-hydroxysteroid dehydrogenase; P450scc, cytochrome P450 cholesterol side-chain cleavage enzyme; StAR, steroidogenic acute regulatory protein; T, testosterone; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; TDS, testicular dysgenesis syndrome.

* Corresponding author. Tel.: +86 551 5167923; fax: +86 551 5161179.
E-mail address: xudex@126.com (D.-X. Xu).

[1] These authors contributed equally to this work.

0278-6915/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fct.2010.02.003

spermatogenesis. We paid attention to explore the role of the decreased T synthesis in fetal testes in fenvalerate-induced impairment on testicular development and spermatogenesis in mice. In addition, we were also to observe the reversibility of maternally fenvalerate-induced impairment on testicular development and spermatogenesis in male offspring.

2. Materials and methods

2.1. Chemicals

Fenvalerate 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

The CD-1 mice (8–10 week-old; male mice: 28–30 g; female mice: 24–26 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 for a period of 1 week before use. According to our previous experience, the male mice can be effectively used if four females and two males were randomly placed together in one cage. Importantly, pregnancy rate might be higher in mice caging with four females and two males per cage. 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. next morning, and the presence of a vaginal plug was designated as gestational day (gd) 0. According to our previous experience, the dose of 30 mg/kg, about 1/8 LD_{50} of the fenvalerate, was chose in the preliminary study. Our preliminary data showed that no signs of maternal toxicity were observed in dams who were treated with fenvalerate during pregnancy. Fenvalerate treatment had little effect on body weight gain of pregnant mouse. In addition, maternal fenvalerate exposure during pregnancy did not affect the weight of fetuses. Therefore, in the present study, the pregnant mice were administered with fenvalerate (30 mg/kg, 6 mg/ml dissolved in corn oil) by gavage daily from gd 13 to gd 18. However, other doses were not tested. The control pregnant mice were administered with corn oil by gavage daily from gd 13 to gd 18. Twelve dams each group were euthanized on gd 18. Fetuses were dissected under a binocular microscope. Sex was determined by the morphology of the gonalds. Fetal sera from male fetuses were collected from trunk blood by decapitation of fetal mice and kept at −80 °C for subsequent determination of T. All the left fetal testes were aseptically removed for RT-PCR or immunoblotting. All the right fetal testes were immersed in modified Davidson's fluid (mDF) for 6–12 h for histology, TUNEL and immunohistochemistry (Howroyd et al., 2005).

For remaining pregnant mice, natural birth occurred between gd 18 at 16:00 and gd 19 at 18:00. Within 24 h after birth, pups were randomly adjusted in each group, so that only five male pups and five female pups were kept per litter. At postnatal day (PND) 80, twelve young adult male mice from 6 l in each group were euthanized under thiopental anesthesia, always in the morning. Sera were collected and kept at −80 °C for subsequent measurement of T (testosterone). The epididymides were removed, left one for weighing and the right one for sperm count. Testes were excised, dissected, weighted, and then divided in two parts: left one was kept at −80 °C for subsequent measurement of T, RT-PCR and immunoblotting. All the right testes were immersed in mDF for 12–24 h for histology, TUNEL and immunohistochemistry (Latendresse et al., 2002). 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.

2.3. Testicular histology and immunohistochemistry

For histological examination, testes immersed in mDF were dehydrated and embedded in paraffin. Sections were cut, mounted on glass slides, deparaffinized with xylene, and stained with hematoxylin and eosin (H&E) using standard procedures for morphological analyses. The method of classification was adopted to define the stages of seminiferous tubules (Oakberg, 1956; Chiou et al., 2008). For adult testes, the tubules were classified into three stage groups: stages I–VI, stages VII–VIII, and stages IX–XII. More than 150 tubules were classified for each section from a litter, and each experiment was repeated three times.

Leydig cells in testes were identified by immunostaining for 3β-hydroxysteroid dehydrogenase (3β-HSD). Sections were mounted onto coated slides, dewaxed, and rehydrated. Antigen retrieval was performed by microwave irradiation for 5 min in 0.01 M citrate buffer (pH 6.0). Slides were incubated for 30 min in 3% (vol/vol) hydrogen peroxide in methanol to block endogenous peroxidase activity and then washed in Tris-buffered saline [TBS; 0.05 M Tris, 0.85% (wt/vol) NaCl (pH 7.4)]. Nonspecific binding sites were blocked with an appropriate normal serum diluted 1:5 in TBS containing 5% (wt/vol) 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 anti-goat (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). For fetal testicular sections, quantification of LC clustering was performed using the public domain NIH Image J Program. Specimens immunostained for 3β-HSD were of sufficient homogeneity, high contrast and low background to allow computer-assisted counting of LC (Leydig cell) clusters and determination of LC cluster area. The cluster number and the proportion of each section occupied by LC clusters were obtained as previously described (Mahood et al., 2007). LC clusters were then assigned to one of three groups: small clusters, accounting for $\leqslant$5% of the total LC cluster area per testis; medium clusters, accounting for 5.1–14.9%; and large clusters, which individually accounted for $\geqslant$15% of the total LC cluster area per testis. For adult testicular sections, the number of 3β-HSD-positive cells per testis was counted by the method as previously described (Mendis-Handagama, 1992).

2.4. Radioimmunoassay (RIA)

Blood were collected from male mice. Fetal blood was pooled from each litter. Sera were 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, testes were homogenized in 0.5 ml PBS (PH7.4). T was extracted from homogenate using diethyl ether. After extraction, the organic phase was evaporated into dryness in a fume hood. T was solubilized in an aliquot of PBS and measured using ^{125}I-based RIA kits (Beijing, China) following the manufacturer's protocols. The lower limit of detection for the assay was 0.1 ng/ml. There was no more than 10% and 15% in the coefficient of variation for intra- and inter-assay. Testicular T was expressed as ng/mg testis.

2.5. 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

Table 1
Primers 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	37[a],35[b]	340
StAR	Forward: 5′-TGTCAAGGAGATCAAGGTCCTG-3′ Reverse: 5′-CGATAGGACCTGGTTGATGAT-3′	94	57	72	34[a], 35[b]	310
P450scc	Forward: 5′-AGGTGTAGCTCAGGACTTCA-3′ Reverse: 5′-AGGAGGCTATAAAGGACACC-3′	94	56	72	34 [a], 38 [b]	370
$P450_{17\alpha}$	Forward: 5′-CCAGGACCCAAGTGTGTTCT-3′ Reverse: 5′-CCTGATACGAAGCACTTCTCG-3′	94	56	72	34 [a], 38 [b]	250
17β-HSD	Forward: 5′-ATTTTACCAGAGAAGACATCT-3′ Reverse: 5′-GGGGTCAGCACCTGAATAATG-3′	94	52	72	37 [a], 40 [b]	367

[a] The optimal number of cycles for cDNA in fetal testes.
[b] The optimal number of cycles for cDNA in testes from adult mice.

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 described by the literature (Akingbemi et al., 2004). 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.

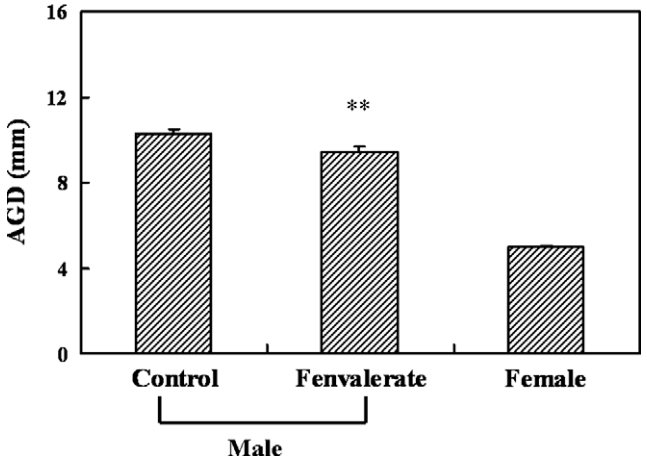

Fig. 1. Effects of maternal fenvalerate exposure during pregnancy on anogenital distance (AGD) of male fetuses. ** $P < 0.01$ significantly different as compared with control male values.

2.6. Immunoblotting

Samples from fetal testes or adult 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, Massachusetts, 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 steroidogenic acute regulatory (StAR), cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc), cytochrome P450 17α-hydroxysteroid dehydrogenase ($P450_{17\alpha}$) and 17β-hydroxysteroid dehydrogenase (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 anti-rabbit IgG antibody for 2 h. The membranes were then washed for

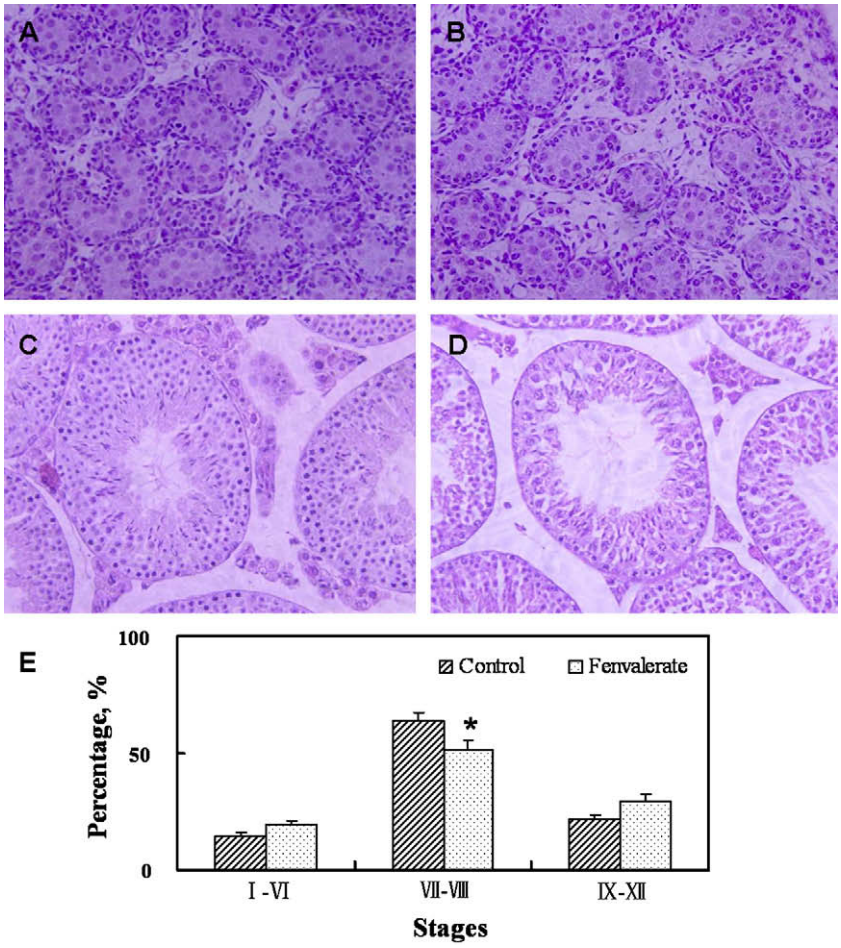

Fig. 2. Effects of maternal fenvalerate exposure during pregnancy on histology for testes of male offspring. (A) and (B) Fetal testes were collected at 8 h after the last administration of fenvalerate. Testicular sections from (A) control and (B) fenvalerate-treated mice were stained with H&E at 400× magnification. (C) and (D) Testes of adult offspring were collected at PND80. Testicular sections from (C) control and (D) fenvalerate-treated mice were stained with H&E at 400× magnification. (E) Changes on stages of seminiferous tubules in mouse testes at adulthood. Testicular cross sections were stained by hematoxylin/eosin staining. The stages of the seminiferous tubules were classified into three stage groups: stages I–VI, stages VII–VIII, and stages IX–XII. Data were expressed as mean ± SEM of five sections from five litters. More than 750 tubules per group were examined. * $P < 0.05$ significantly different as compared with the controls.

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).

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

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

2.8. Sperm analysis

The 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 suspensions were analyzed for sperm count according to WHO laboratory manual 4th Edition (1999).

2.9. Statistical analysis

For RT-PCR, StAR, P450scc, $P450_{17\alpha}$ and 17β-HSD mRNA level was 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 immunoblotting, 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 ± SEM. Student's *t* test was used to determine differences between fenvalerate-treated mice and controls.

3. Results

3.1. Maternal toxicity and fetal development

To investigate the effects of maternal fenvalerate exposure during pregnancy on fetal development, the pregnant mice were orally administered with fenvalerate (30 mg/kg) daily on gd 13–18. No signs of maternal toxicity were observed in dams treated with fenvalerate. Fenvalerate treatment had little effect on body weight gain of dams during pregnancy. In addition, maternal fenvalerate exposure during pregnancy did not affect the weight of fetuses (data not shown). No significant difference in male-to-female sex ratio or the number of live fetuses per dam was observed between fenvalerate-treated mice and controls (data not shown). However, maternal fenvalerate exposure during pregnancy obviously reduced anogenital distance (AGD) in male fetuses as compared with the controls (Fig. 1). It is a pity that we ignored the details about fetal testicular weight (volume) in different groups.

3.2. Histology and apoptosis in testes

The effects of maternal fenvalerate exposure during pregnancy on histology for testes of male offspring are presented in Fig. 2. No abnormal morphology was observed in fetal testes of fenvalerate-treated mice (Fig. 2B). Figs. 2C–E show that the percent of the

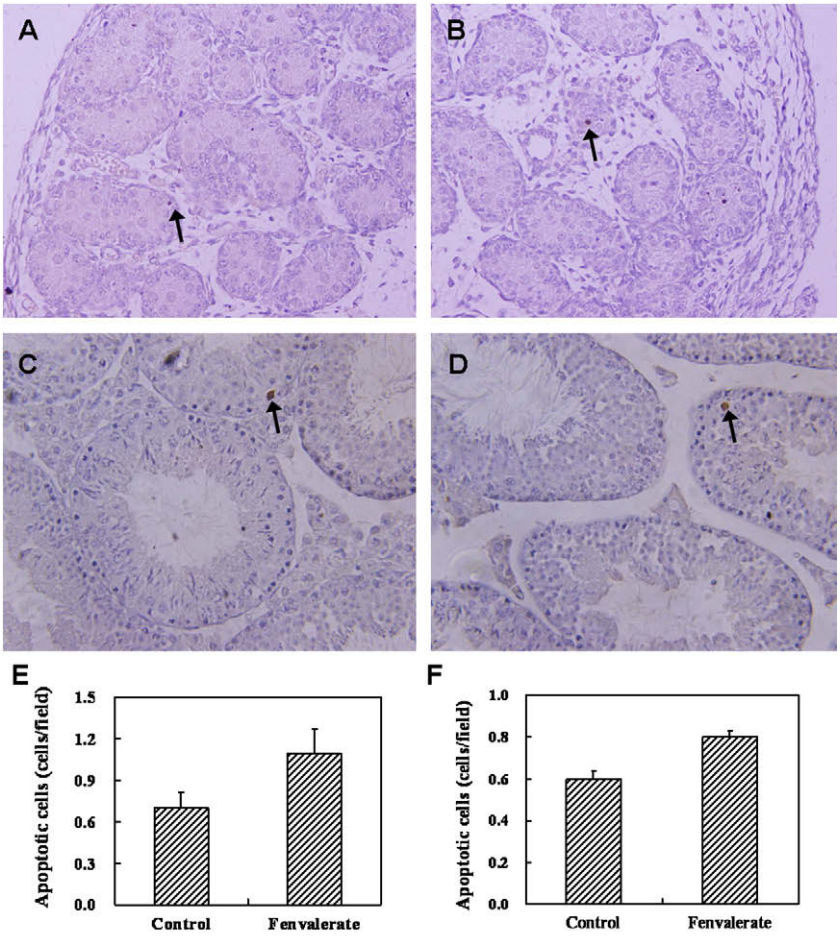

Fig. 3. Effects of maternal fenvalerate exposure during pregnancy on the number of apoptosis in testes. (A) and (B) Fetal testes were collected at 8 h after the last administration of fenvalerate. Apoptosis in fetal testes was analyzed with TUNEL staining of testicular sections from (A) control and (B) fenvalerate-treated mice. (C) and (D) Testes of adult offspring were collected at PND80. Apoptosis in testes of adult offspring was analyzed with TUNEL staining of testicular sections from (C) control and (D) fenvalerate-treated mice. (E) The number of $TUNEL^+$ cells in fetal testes was counted in 3 randomly selected fields from each slide at 400× magnification. (F) The number of $TUNEL^+$ cells in testes of adult offspring was counted in twelve randomly selected fields from each slide at 400× magnification. All data were expressed as means ± SEM of six samples from six litters.

cycle of the seminiferous tubules in different stages. In control males, 14.3%, 64.0%, 21.8% of the seminiferous tubules were in stages I–VI, VII–VIII, and IX–XII, respectively. In fenvalerate-treated males, 19.5%, 51.1%, 29.5% of the seminiferous tubules were in stages I–VI, VII–VIII, and IX–XII, respectively. The effects of maternal fenvalerate exposure during pregnancy on apoptosis in testes of male offspring were determined using TUNEL assay. The results are presented in Fig. 3. No significant increase in the number of apoptotic cells was observed in testes of fetuses whose mothers were exposed to fenvalerate during pregnancy (Fig. 3E). In addition, maternal fenvalerate exposure during pregnancy had little effect on the number of apoptotic cells in testes of adult male offspring (Fig. 3F).

3.3. Serum and testicular T

The effects of maternal fenvalerate exposure during pregnancy on serum T in male fetuses were analyzed. Results showed that maternal fenvalerate exposure during pregnancy significantly decreased the level of serum T in male fetuses (1.29 ± 0.22 ng/ml vs. 0.84 ± 0.13 ng/ml, $n = 12$, $P < 0.05$). However, it is a pity that we ignored the details about fetal testicular T in different groups. The effects of maternal fenvalerate exposure during pregnancy on serum and testicular T in adult male offspring were analyzed. Results showed that the level of serum T in adulthood remained significantly decreased in mice whose mothers were exposed to fenvalerate during pregnancy (14.19 ± 3.09 ng/ml vs. 6.64 ± 2.68 ng/ml, $n = 12$, $P < 0.05$). Correspondingly, the level of testicular T in adulthood was also significantly decreased in mice whose mothers were exposed to fenvalerate during pregnancy (1.28 ± 0.23 ng/mg testis vs. 0.67 ± 0.21 ng/mg testis, $n = 12$, $P < 0.05$).

3.4. Testicular StAR and T synthetic enzymes

The effects of maternal fenvalerate exposure during pregnancy on mRNA level of testicular StAR and T synthetic enzymes are presented in Fig. 4. Results showed that maternal fenvalerate exposure during pregnancy significantly down-regulated mRNA level of

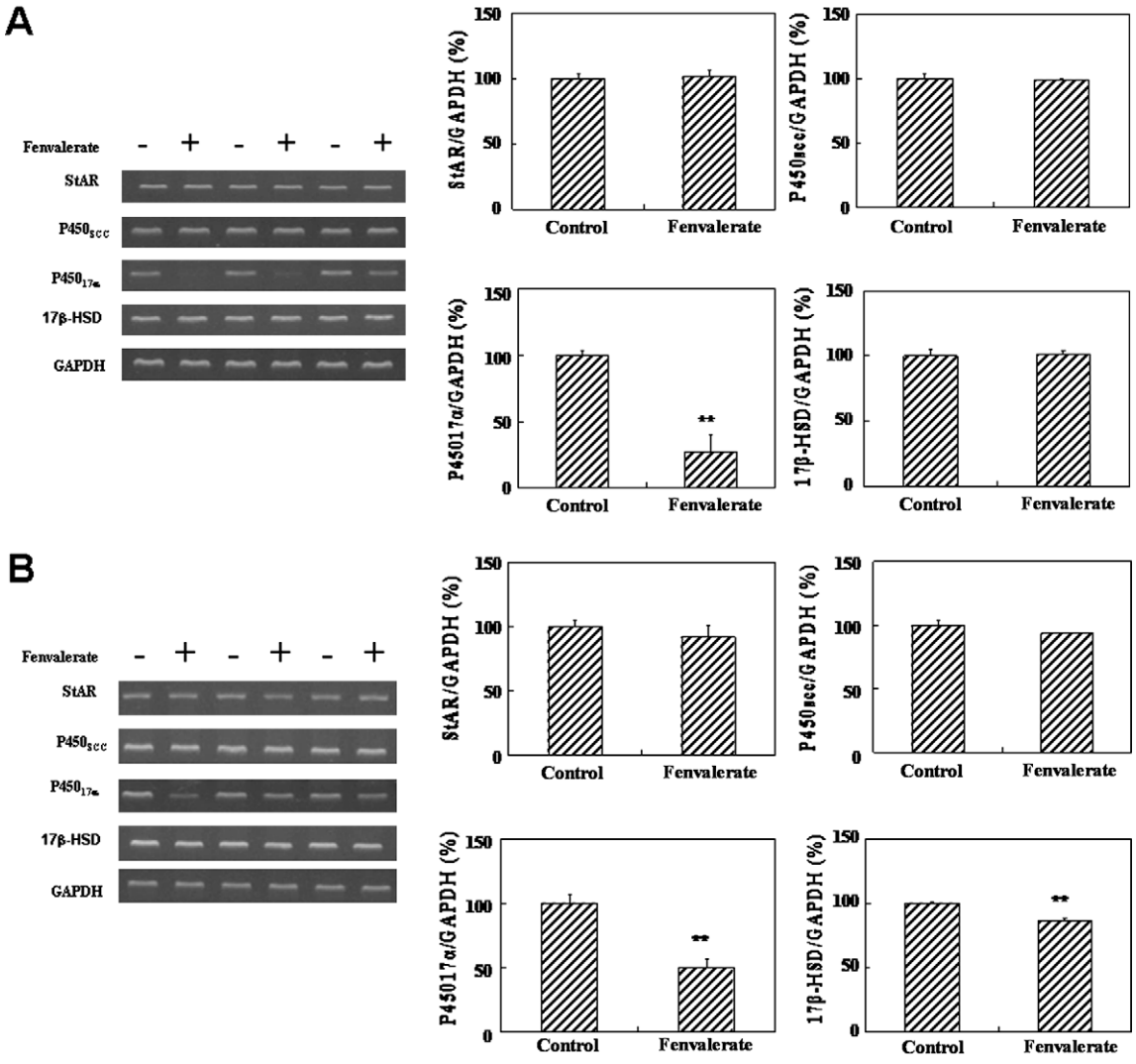

Fig. 4. Effects of maternal fenvalerate exposure during pregnancy on mRNA levels of testicular StAR and T biosynthetic enzymes. (A) Fetal testes were collected at 8 h after the last administration of fenvalerate. (B) Testes of adult offspring were collected on PND80. StAR, P450scc, P450$_{17\alpha}$ and 17β-HSD mRNA was measured using RT-PCR. Quantitative analysis of StAR, P450scc, P450$_{17\alpha}$ and 17β-HSD mRNA level from six individual RNA samples from six litters was performed. StAR, P450scc, P450$_{17\alpha}$ and 17β-HSD mRNA was 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 litters. $^{**}P < 0.01$ significantly different as compared with controls.

$P450_{17\alpha}$ in fetal testes (Fig. 4A). Surprisingly, the level of testicular $P450_{17\alpha}$ mRNA in adult male offspring was obviously decreased in mice whose mothers were exposed to fenvalerate during pregnancy (Fig. 4B). As shown in Fig. 4A, maternal fenvalerate exposure during pregnancy had little effect on mRNA level of StAR, P450scc and 17β-HSD in fetal testes. In addition, maternal fenvalerate exposure during pregnancy did not affect mRNA level of testicular StAR and P450scc in adult offspring, whereas the level of 17β-HSD mRNA was slightly decreased in testes of adult offspring whose mothers were exposed to fenvalerate during pregnancy (Fig. 4B).

The effects of maternal fenvalerate exposure during pregnancy on protein level of testicular StAR and T synthetic enzymes are presented in Fig. 5. Results showed that maternal fenvalerate exposure during pregnancy significantly down-regulated protein expression of $P450_{17\alpha}$ in fetal testes (Fig. 5A). As shown in Fig. 5B, protein level of $P450_{17\alpha}$ remained decreased in testes of adult offspring whose mothers were exposed to fenvalerate during pregnancy. However, maternal fenvalerate exposure during pregnancy did not affect protein level of StAR, P450scc and 17β-HSD in testes of adult offspring (Fig. 5B).

3.5. Leydig cells

Leydig cells in testes were identified by immunostaining for 3β-HSD (Fig. 6). As shown in Fig. 6E, changes in Leydig cells distribution were markedly occurred in testes of fetuses whose mothers were exposed to fenvalerate during pregnancy. Maternal fenvalerate exposure during pregnancy significantly decreased small LC clusters as compared with the controls. In addition, a significant increase in medium LC clusters was occured in fetuses whose mothers were exposed to fenvalerate during pregnancy. The effects of prenatal fenvalerate exposure on the number of Leydig cells in adult testis were shown in Fig. 6C–F. No significant increase in the number of Leydig cells per testis was observed in adult offspring whose mothers were exposed to fenvalerate during pregnancy (Fig. 6F).

3.6. Weights of male reproductive organs and sperm quality

The effects of maternal fenvalerate exposure during pregnancy on the weights of testes and epididymides in adult male offspring

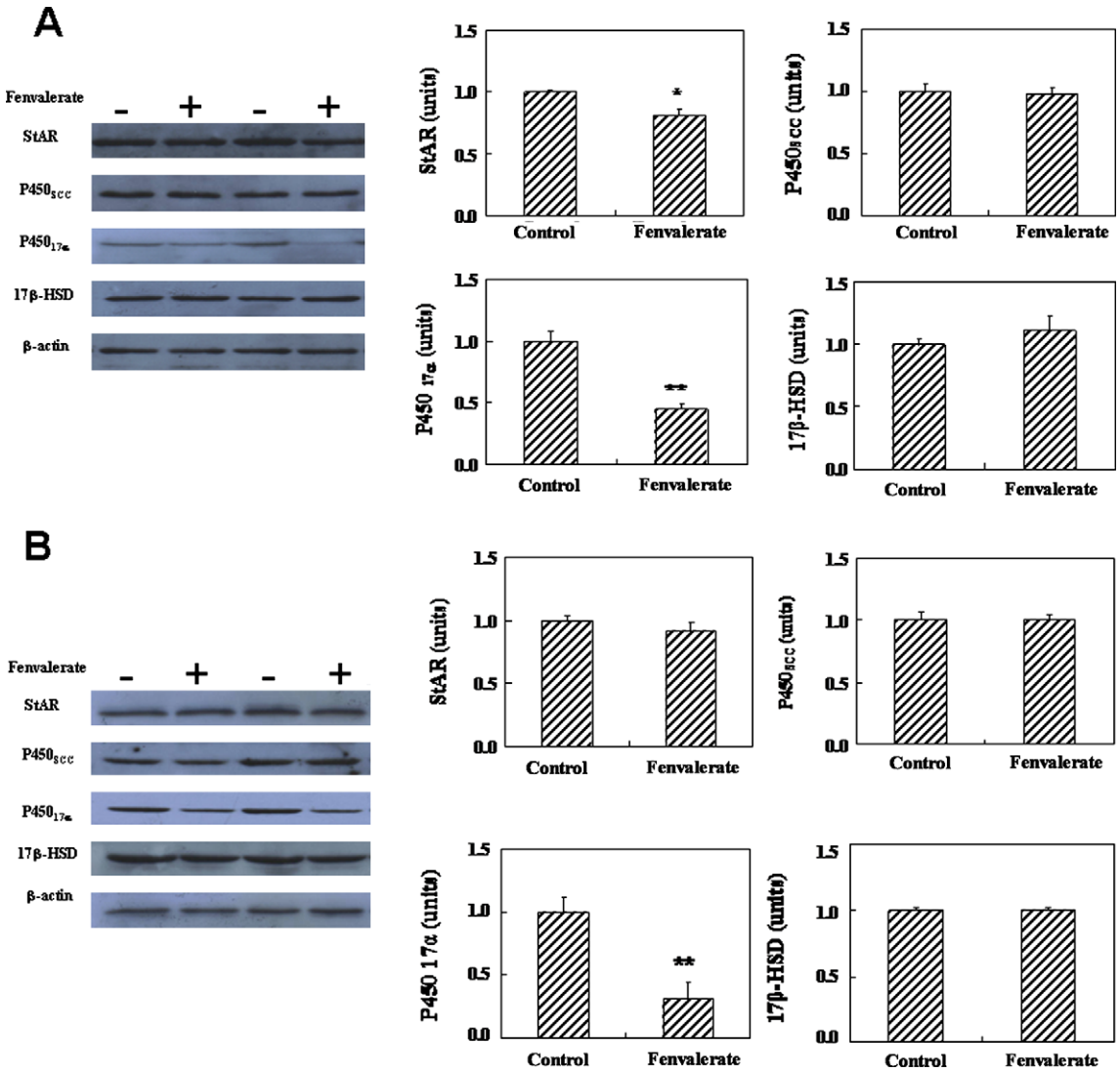

Fig. 5. Effects of maternal fenvalerate exposure during pregnancy on protein expressions of testicular StAR and T biosynthetic enzymes. (A) Fetal testes were collected at 8 h after the last administration of fenvalerate. (B) Testes of adult offspring were collected on PND80. The protein expression of StAR, P450scc, $P450_{17\alpha}$ and 17β-HSD was measured using immunoblotting. Quantitative analysis of scanning densitometry on six samples from six litters was performed. StAR, P450scc, $P450_{17\alpha}$ and 17β-HSD was normalized to β-actin level in the same samples. The densitometry units of the control were assigned as 1. 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.

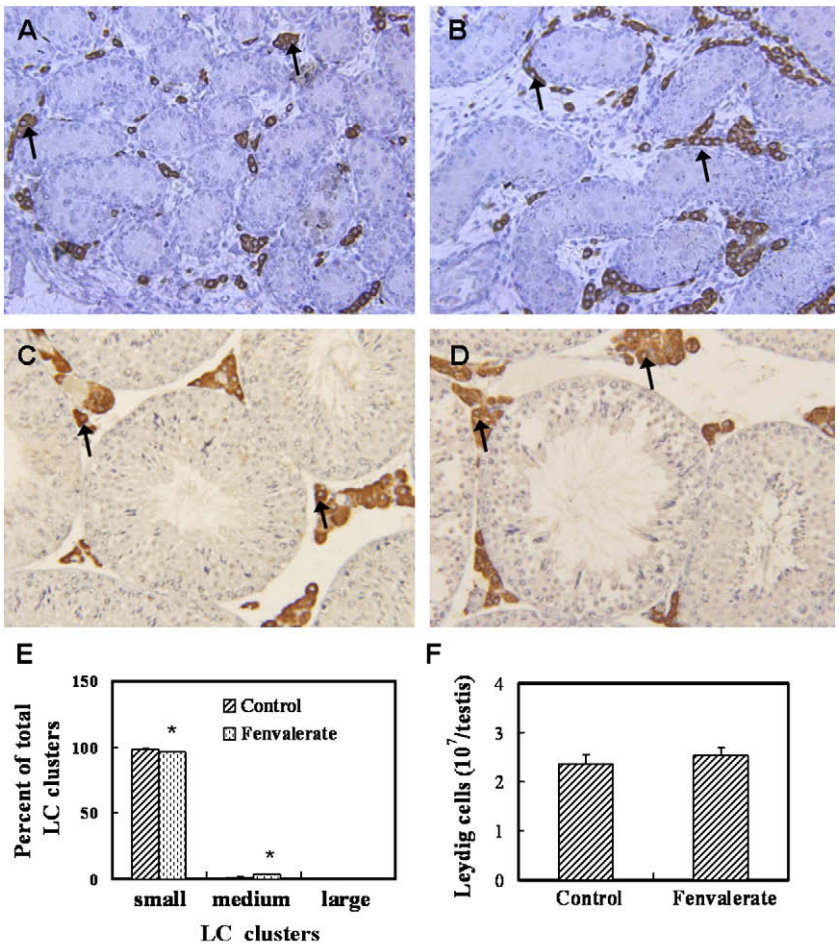

Fig. 6. Effects of maternal fenvalerate exposure during pregnancy on the distribution and number of Leydig cells in testes. (A) and (B) Fetal testes were collected at 8 h after the last administration of fenvalerate. Leydig cells in fetal testes from (A) control and (B) fenvalerate-treated mice were immunolocalized by staining with a polyclonal antibody specific for 3β-HSD. (C) and (D) Testes of adult offspring were collected at PND80. Leydig cells in testes of adult offspring from (C) control and (D) fenvalerate-treated mice were immunolocalized by staining with a polyclonal antibody specific for 3β-HSD. (E) Distribution (aggregation) of Leydig cell (LC) clusters in fetal testes was analyzed. Small clusters account for ⩽5% of the total LC cluster area per testis, medium clusters for 5.1–14.9%, and large clusters for ⩾15%. (F) The number of Leydig cells per testis in adult offspring was counted. All data were expressed as means ± SEM of six samples from six litters. $^{*}P < 0.05$ significantly different as compared with controls.

were analyzed. Results showed that the absolute weight of testes at PND80 was significantly decreased in mice whose mothers were exposed to fenvalerate during pregnancy (0.27 ± 0.03 g vs. 0.24 ± 0.03 g, $n = 12$, $P < 0.05$). In addition, maternal fenvalerate exposure during pregnancy significantly reduced the absolute weight of epididymides in adult male offspring (90 ± 4.0 mg vs. 80 ± 4.0 mg, $n = 12$, $P < 0.05$). The effects of maternal fenvalerate exposure during pregnancy on sperm quality are presented in Fig. 7. Results showed that the number of spermatozoa in epididymides at PND80 was significantly decreased in mice whose mothers were exposed to fenvalerate during pregnancy (23.6×10^6 vs. 17.2×10^6, $n = 12$, $P < 0.01$).

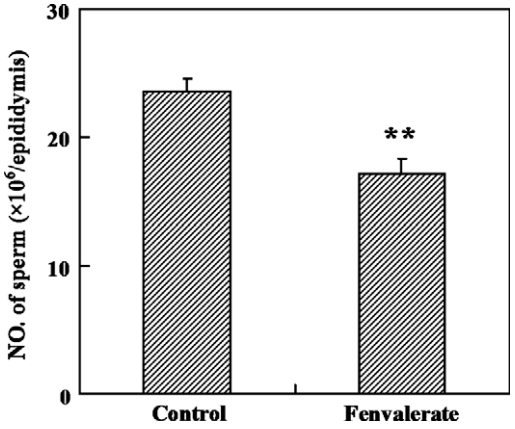

Fig. 7. Effects of maternal fenvalerate exposure during pregnancy on sperm count in adult male offspring. All data were expressed as means ± SEM of twelve samples from six litters. $^{**}P < 0.01$ significantly different as compared with controls.

3.7. Fertility of male offspring

To investigate the effects of maternal fenvalerate exposure during pregnancy on the fertility of adult male offspring (PND80), twelve males whose mothers were exposed to fenvalerate during

Table 2
Effects of maternal fenvalerate exposure during pregnancy on the fertility of male offspring.

Groups	No. tested males	Males mated successfully		Fertile males	
		n	%	*n*	%
Control	12	12	100	11	91.67
Fenvalerate	12	9	75	8	66.67

Table 3
The fetal outcomes.

Groups	Number 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	11	12.89 ± 0.42	0.56 ± 0.29	12.27 ± 0.52	0.11 ± 0.11
Fenvalerate	8	13.33 ± 0.88	0.83 ± 0.40	11.88 ± 1.13	0.50 ± 0.17

pregnancy were housed with untreated females. Fertility analysis showed that all twelve males in control group mated successfully with control females, 91.7% (11/12) of which were found to be fertilized and completed the pregnancy. 75% (9/12) males whose mothers were exposed to fenvalerate exposure during pregnancy mated successfully with control females, 66.7% (8/12) of which were found to be fertilized and completed the pregnancy (Table 2). There was no significant difference on the numbers of implantation sites, resorptions, live fetuses and dead fetuses per litter between fenvalerate-treated mice and controls (Table 3). 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 fenvalerate-treated mice and controls (Tables 4 and 5).

4. Discussion

Several studies showed that administration of fenvalerate to male adult rodents decreased semen quality (el-Demerdash et al., 2004; Arena et al., 2008). In the present study, we found that the weights of testes and epididymides were significantly decreased in mice whose mothers were exposed to fenvalerate during pregnancy. Importantly, maternal fenvalerate exposure during pregnancy markedly decreased the number of mature seminiferous tubules (stages VII and VIII) in testes of adult male offspring. In addition, maternal fenvalerate exposure during pregnancy significantly reduced the number of spermatozoa in epididymides in adult male offspring. These results suggest that maternal fenvalerate exposure during pregnancy irreversibly impairs testicular development and spermatogenesis in male offspring.

During embryonic development, T, which acts through the somatic cells to regulate germ cell differentiation, is the most important hormone controlling the masculinization of reproductive tract and the genitalia, normal testicular development and spermatogenesis (Holdcraft and Braun, 2004). An earlier study showed that a Sertoli cell-selective knockout of the androgen receptor (AR) resulted in infertility with defective spermatogenesis and hypotestosteronemia (Chang et al., 2004). In addition, various studies found that AR was present in fetal germ cells and demonstrated that male fetal germ cells were direct targets for T that physiologically inhibited germ cell proliferation (Cupp and Skinner, 2001; Merlet et al., 2007), although there were some reports of its absence in male fetal germ cell (Williams et al., 2001). To explore the association between testicular T synthesis and fenvalerate-induced impairments on reproductive development and spermatogenesis, the present study investigated the effects of maternal fenvalerate exposure during pregnancy on testicular T synthesis and AGD in male fetuses. We found that the level of serum T was significantly decreased in male fetuses whose mothers were exposed to fenvalerate during pregnancy. In addition, prenatal exposure to fenvalerate markedly induced reduction of the male AGD to phenotype of a typical female. Importantly, the level of serum T remained lower in adult male offspring whose mothers were exposed to fenvalerate during pregnancy. In addition, the level of testicular T at PND80 was also decreased in mice whose mothers were exposed to fenvalerate during pregnancy. These results suggest that maternal fenvalerate during pregnancy persistently disrupts testicular T synthesis in male offspring. The decreased testicular T synthesis might be associated with impairments on testicular development and spermatogenesis in male offspring whose mothers were exposed to fenvalerate during pregnancy.

StAR is an essential in testicular T synthesis, responsible for the transport of cholesterol into mitochondria (Miller, 2007). Testicular T synthetic enzymes, primarily P450scc, $P450_{17\alpha}$ and 17β-HSD, play a critical role on 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). A recent study found that permethrin, another pyrethroid insecticide, down-regulated the expression of StAR in testes of adult mice. The present study showed that mRNA and protein levels of $P450_{17\alpha}$ were significantly decreased in testes of fetuses whose mothers were exposed to fenvalerate during pregnancy. Surprisingly, the present study found that mRNA and protein levels of testicular $P450_{17\alpha}$ remained significantly decreased in adult male offspring whose mothers were exposed to fenvalerate during pregnancy. These results suggest that prenatal fenvalerate exposure irreversibly downregulates the expression of testicular $P450_{17\alpha}$ in mice. The downregulation of $P450_{17\alpha}$ may, at least partially, contribute to the decreased T synthesis in testes of mice whose mothers were exposed to fenvalerate during pregnancy.

Several studies showed that abnormal Leydig cell aggregation in the fetal testis of rats exposed to di-(*n*-butyl) phthalate (DBP) or di-(2-ethylhexyl) phthalate (DEBP), two endocrine disruptors, was associated with decrease in testicular T synthesis (Mahood

Table 4
Effec0ts of maternal fenvalerate exposure during pregnancy on the litter size and sex ratio in F2 generation.

	No. litters	Litter size			Male/female ratio
		Male	Female	Total	
Control	11	5.45 ± 0.58	6.82 ± 0.40	12.27 ± 0.43	0.86 ± 0.13
Fenvalerate	8	5.38 ± 1.00	6.50 ± 0.87	11.88 ± 0.83	0.95 ± 0.20

Table 5
The weight and crown length of fetuses in F2 generation.

Groups	Litters	Weight (g, $\bar{x}$ ± SEM)			Crown length (mm, $\bar{x}$ ± SEM)		
		Male	Female	Total	Male	Female	Total
Control	11	1.39 ± 0.05	1.25 ± 0.05	1.31 ± 0.04	25.3 ± 0.02	24.8 ± 0.02	25.1 ± 0.02
Fenvalerate	8	1.31 ± 0.02	1.25 ± 0.02	1.28 ± 0.02	24.6 ± 0.04	24.2 ± 0.03	24.8 ± 0.03

et al., 2005, 2007; Lin et al., 2008). In the present study, we also found that maternal fenvalerate exposure during pregnancy significantly decreased small LC clusters as compared with the controls. In addition, a significant increase in medium LC clusters was occured in fetuses whose mothers were exposed to fenvalerate during pregnancy. These results suggested that the decreased T synthesis might, at least partially, contribute to fenvalerate-induced abnormal Leydig cell aggregation in fetal testes of mice.

Several studies showed that some endocrine disruptors induced germ cell apoptosis in testes (Sobarzo et al., 2006; McClusky et al., 2007; Saradha et al., 2009). An in vitro study found that mono-(2-ethylhexyl) phthalate (MEHP), a well-known endocrine disruptor, reduced the number of germ cells by increasing apoptosis without change in T production in human fetal testes (Lambrot et al., 2009). In the present study, we investigated the effect of maternal fenvalerate exposure during pregnancy on apoptosis in fetal testes. Our results showed that maternal fenvalerate exposure during pregnancy had little effect on the number of apoptotic cells in fetal testes. In addition, no abnormal morphology was observed in testes of fetuses whose mothers were exposed to fenvalerate during pregnancy. These results suggest that impairment of maternal fenvalerate exposure during pregnancy on testicular development and spermatogenesis can not be attributed to its direct effect on germ cell in fetal testes.

In summary, the present study indicates that maternal fenvalerate exposure during pregnancy irreversibly impairs testicular development and spermatogenesis in male offspring. The decreased T synthesis might, at least partially, contribute to fenvalerate-induced impairment on testicular development and spermatogenesis, and the reduction in fertility.

Recently, a series of studies demonstrated that prenatal exposure to endocrine disruptors persistently disrupted sexual differentiation and increased the incidence of cryptorchidism and hypospadias in male offspring via reducing testicular T synthesis (Sharpe et al., 1995; 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). Therefore, Additional work is required to determine whether maternal fenvalerate exposure during pregnancy disrupts sexual differentiation and results in the incidence of cryptorchidism and hypospadias in male offspring.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by National Natural Science Foundation of China (30671786) and the Key Project of Chinese Ministry of Education (208060).

References

Akingbemi, B.T., Ge, R., Klinefelter, G.R., Zirkin, B.R., Hardy, M.P., 2004. Phthalate-induced Leydig cell hyperplasia is associated with multiple endocrine disturbances. Proc. Natl. Acad. Sci. USA 101, 775–780.

Arena, A.C., Fernandez, C.D., Porto, E.M., Bissacot, D.Z., Pereira, O.C., Kempinas, W.G., 2008. Fenvalerate, a pyrethroid insecticide, adversely affects sperm production and storage in male rats. J. Toxicol. Environ. Health A 71, 1550–1558.

Bian, Q., Xu, L.C., Wang, S.L., Xia, Y.K., Tan, L.F., Chen, J.F., Song, L., Chang, H.C., Wang, X.R., 2004. Study on the relation between occupational fenvalerate exposure and spermatozoa DNA damage of pesticide factory workers. Occup. Environ. Med. 61, 999–1005.

Carbone, P., Giordano, F., Nori, F., Mantovani, A., Taruscio, D., Lauria, L., Figà-Talamanca, I., 2007. The possible role of endocrine disrupting chemicals in the aetiology of cryptorchidism and hypospadias: a population-based case-control study in rural Sicily. Int. J. Androl. 30, 3–13.

Caserta, D., Maranghi, L., Mantovani, A., Marci, R., Maranghi, F., Moscarini, M., 2008. Impact of endocrine disruptor chemicals in gynaecology. Hum. Reprod. Update 14, 59–72.

Chang, C., Chen, Y.T., Yeh, S.D., Xu, Q., Wang, R.S., Guillou, F., Lardy, H., Yeh, S., 2004. Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in Sertoli cells. Proc. Natl. Acad. Sci. USA 101, 6876–6881.

Chiou, T.J., Chu, S.T., Tzeng, W.F., Huang, Y.C., Liao, C.J., 2008. Arsenic trioxide impairs spermatogenesis via reducing gene expression levels in testosterone synthesis pathway. Chem. Res. Toxicol. 21, 1562–1569.

Cupp, A.S., Skinner, M.K., 2001. Actions of the endocrine disruptor methoxychlor and its estrogenic metabolite on in vitro embryonic rat seminiferous cord formation and perinatal testis growth. Reprod. Toxicol. 15, 317–326.

De Jager, C., Farias, P., Barraza-Villarreal, A., Avila, M.H., Ayotte, P., Dewailly, E., Dombrowski, C., Rousseau, F., Sanchez, V.D., Bailey, J.L., 2006. Reduced seminal parameters associated with environmental DDT exposure and *p,p′*-DDE concentrations in men in Chiapas, Mexico: a cross-sectional study. J. Androl. 27, 16–27.

el-Demerdash, F.M., Yousef, M.I., Kedwany, F.S., Baghdadi, H.H., 2004. Role of alpha-tocopherol and beta-carotene in ameliorating the fenvalerate-induced changes in oxidative stress, hemato-biochemical parameters, and semen quality of male rats. J. Environ. Sci. Health B 39, 443–459.

Holdcraft, R.W., Braun, R.E., 2004. Hormonal regulation of spermatogenesis. Int. J. Androl. 27, 335–342.

Howroyd, P., Hoyle-Thacker, R., Lyghyt, O., Williams, D., Kleymenova, E., 2005. Morphology of the fetal rat testis preserved in different fixatives. Toxicol. Pathol. 33, 300–304.

Hu, G.X., Lian, Q.Q., Ge, R.S., Hardy, D.O., Li, X.K., 2009. Phthalate-induced testicular dysgenesis syndrome: Leydig cell influence. Trends Endocrinol. Metab. 20, 139–145.

Lambrot, R., Muczynski, V., Lecureuil, C., Angenard, G., Coffigny, H., Pairault, C., Moison, D., Frydman, R., Habert, R., Rouiller-Fabre, V., 2009. Phthalates impair germ cell development in the human fetal testis in vitro without change in testosterone production. Environ. Health Perspect. 117, 32–37.

Latendresse, J.R., Warbrittion, A.R., Jonassen, H., Creasy, D.M., 2002. Fixation of testes and eyes using a modified Davidson's fluid: comparison with Bouin's fluid and conventional Davidson's fluid. Toxicol. Pathol. 30, 524–533.

Lin, H., Ge, R.S., Chen, G.R., Hu, G.X., Dong, L., Lian, Q.Q., Hardy, D.O., Sottas, C.M., Li, X.K., Hardy, M.P., 2008. Involvement of testicular growth factors in fetal Leydig cell aggregation after exposure to phthalate in utero. Proc. Natl. Acad. Sci. USA 105, 7218–7222.

Mahood, I.K., Hallmark, N., McKinnell, C., Walker, M., Fisher, J.S., Sharpe, R.M., 2005. Abnormal Leydig Cell aggregation in the fetal testis of rats exposed to di(*n*-butyl) phthalate and its possible role in testicular dysgenesis. Endocrinology 146, 613–623.

Mahood, I.K., Scott, H.M., Brown, R., Hallmark, N., Walker, M., Sharpe, R.M., 2007. In utero exposure to di(*n*-butyl) phthalate and testicular dysgenesis: comparison of fetal and adult end points and their dose sensitivity. Environ. Health Perspect. 115 (Suppl. 1), 55–61.

McClusky, L.M., de Jager, C., Bornman, M.S., 2007. Stage-related increase in the proportion of apoptotic germ cells and altered frequencies of stages in the spermatogenic cycle following gestational, lactational, and direct exposure of male rats to *p*-nonylphenol. Toxicol. Sci. 95, 249–256.

McKee, R.H., Pavkov, K.L., Trimmer, G.W., Keller, L.H., Stump, D.G., 2006. An assessment of the potential developmental and reproductive toxicity of di-isoheptyl phthalate in rodents. Reprod. Toxicol. 21, 241–252.

Mendis-Handagama, S.M., 1992. Estimation error of Leydig cell numbers in atrophied rat testes due to the assumption of spherical nuclei. J. Microsc. 168 (Pt 1), 25–32.

Merlet, J., Racine, C., Moreau, E., Moreno, S.G., Habert, R., 2007. Male fetal germ cells are targets for androgens that physiologically inhibit their proliferation. Proc. Natl. Acad. Sci. USA 104, 3615–3620.

Miller, W.L., 2007. StAR search – what we know about how the steroidogenic acute regulatory protein mediates mitochondrial cholesterol import. Mol. Endocrinol. 21, 589–601.

Mocarelli, P., Gerthoux, P.M., Patterson Jr., D.G., Milani, S., Limonta, G., Bertona, M., Signorini, S., Tramacere, P., Colombo, L., Crespi, C., Brambilla, P., Sarto, C., Carreri, V., Sampson, E.J., Turner, W.E., Needham, L.L., 2008. Dioxin exposure, from infancy through puberty, produces endocrine disruption and affects human semen quality. Environ. Health Perspect. 116, 70–77.

Moniz, A.C., Cruz-Casallas, P.E., Oliveira, C.A., Lucisano, A., Florio, J.C., Nicolau, A.A., Spinosa, H.S., Bernardi, M.M., 1999. Perinatal fenvalerate exposure: behavioral and endocrinology changes in male rats. Neurotoxicol. Teratol. 21, 611–618.

Oakberg, E.F., 1956. A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. Am. J. Anat. 99, 391–413.

Parks, L.G., Ostby, J.S., Lambright, C.R., Abbott, B.D., Klinefelter, G.R., Barlow, N.J., Gray Jr., L.E., 2000. The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. Toxicol. Sci. 58, 339–349.

Payne, A.H., Youngblood, G.L., 1995. Regulation of expression of steroidogenic enzymes in Leydig cells. Biol. Reprod. 52, 217–225.

Rignell-Hydbom, A., Rylander, L., Giwercman, A., Jonsson, B.A., Nilsson-Ehle, P., Hagmar, L., 2004. Exposure to CB-153 and *p,p′*-DDE and male reproductive function. Hum. Reprod. 19, 2066–2075.

Saradha, B., Vaithinathan, S., Mathur, P.P., 2009. Lindane induces testicular apoptosis in adult Wistar rats through the involvement of Fas-FasL and mitochondria-dependent pathways. Toxicology 255, 131–139.

Scott, H.M., Hutchison, G.R., Jobling, M.S., McKinnell, C., Drake, A.J., Sharpe, R.M., 2008. Relationship between androgen action in the "male programming window", fetal sertoli cell number, and adult testis size in the rat. Endocrinology 149, 5280–5287.

Sharpe, R.M., Fisher, J.S., Millar, M.M., Jobling, S., Sumpter, J.P., 1995. Gestational and lactational exposure of rats to xenoestrogens results in reduced testicular size and sperm production. Environ. Health Perspect. 103, 1136–1143.

Sobarzo, C.M., Lustig, L., Ponzio, R., Denduchis, B., 2006. Effect of di-(2-ethylhexyl) phthalate on N-cadherin and catenin protein expression in rat testis. Reprod. Toxicol. 22, 77–86.

Swan, S.H., Main, K.M., Liu, F., Stewart, S.L., Kruse, R.L., Calafat, A.M., Mao, C.S., Redmon, J.B., Ternand, C.L., Sullivan, S., Teague, J.L., 2005. Study for future families research team. Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ. Health Perspect. 113, 1056–1061.

Tan, L., Wang, S., Ji, J., Sun, X., Li, Y., Wang, Q., Chen, L., 2006. Effects of fenvalerate exposure on semen quality among occupational workers. Contraception 73, 92–96.

Williams, K., McKinnell, C., Saunders, P.T., Walker, M., Fisher, J.S., Turner, K.J., Atanassova, N., Sharpe, M., 2001. Neonatal exposure to potent and environmental oestrogens and abnormalities of the male reproductive system in the rat: evidence for importance of the androgen–oestrogen balance and assessment of the relevance to man. Hum. Reprod. Update 7, 236–247.

World Health Organization, 1990. Environmental Health Criteria No. 95: Fenvalerate. World Health Organization, Geneva.

World Health Organization, 1999. WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction, fourth ed. Cambridge University Press, Cambridge, United Kingdom.

Xia, Y., Bian, Q., Xu, L., Cheng, S., Song, L., Liu, J., Wu, W., Wang, S., Wang, X., 2004. Genotoxic effects on human spermatozoa among pesticide factory workers exposed to fenvalerate. Toxicology 203, 49–60.

Zhang, H., Wang, H., Ji, Y.L., Ning, H., Yu, T., Zhang, C., Zhang, Y., Zhao, X.F., Wang, Q., Liu, P., Meng, X.H., Xu, D.X., 2009. Lactational fenvalerate exposure permanently impairs testicular development and spermatogenesis in mice. Toxicol. Lett. 191, 47–56.