Available online at www.sciencedirect.com
ScienceDirect
Theriogenology 72 (2009) 599–611
Theriogenology
www.theriojournal.com

Beta-cypermethrin impairs reproductive function in male mice by inducing oxidative stress

X.-Z. Wang, S.-S. Liu, Y. Sun, J.-Y. Wu, Y.-L. Zhou, J.-H. Zhang *

Chongqing Key Laboratory of Forage & Herbivore, College of Animal Science and Technology, Southwest University, Chongqing 400716, China

Received 9 January 2009; received in revised form 15 March 2009; accepted 11 April 2009

Abstract

Cypermethrin (CYP), an insecticide, has deleterious effects on male reproductive function. The objective was to identify whether the effects of beta-CYP on male reproductive organs were associated with oxidative stress. Three doses of beta-CYP (1, 10, and 20 mg/kg) were administered to male mice for 35 d, with or without vitamin E (20 mg/kg). The moderate (10 mg/kg) and high (20 mg/kg) doses of beta-CYP not only decreased body weight and the weight of the testes, epididymides, seminal vesicles, and prostate ($P < 0.05$) but also reduced serum testosterone concentration and the expression of steroidogenic acute regulatory protein ($P < 0.05$), in addition to damaging the seminiferous tubules and sperm development. Furthermore, moderate and high doses of beta-CYP administration decreased sperm number, sperm motility, and intact acrosome rate ($P < 0.05$). Based on ultrastructural analyses, high doses of beta-CYP produced swelling and degeneration of mitochondria and the smooth endoplasmic reticulum of Leydig cells and caused the formation of concentric circles. These toxic effects of beta-CYP may be mediated by increasing oxidative stress, as the moderate and high doses of this compound increased malondialdehyde and nitric oxide in testes ($P < 0.05$); reduced the activity of catalase, glutathione peroxidase (GSH-Px), and superoxide dismutase ($P < 0.05$); and activated ERK1/2 ($P < 0.05$). Vitamin E reversed the effects of beta-CYP on testosterone production and testis damage ($P < 0.05$ vs. the high-dose group). Therefore, we inferred that beta-CYP damaged the structure of testes and decreased sperm output by inducing oxidative stress.

Keywords: Beta-cypermethrin; Oxidative stress; Pyrethroid insecticide; Reproductive function; Testis

1. Introduction

Cypermethrin (CYP) is a synthetic pyrethroid insecticide that has been widely used over the past 30 yr in China and other countries against pests, particularly Lepidoptera, cockroaches, and termites. In animals, cypermethrin has been used as a chemotherapeutic agent against ectoparasite infestations [1]. Beta-CYP is a mixture of the alpha and theta forms of the insecticide. Its activity is lower than that of alpha-CYP but higher than other CYPs. Beta-CYP has been applied widely for agricultural pest control in China and comprises more than 50% of the total pyrethroid market production [2]. Although cypermethrin was considered safe and was widely used on agricultural crops and forests as well as in public and animal health [3], there is accumulating evidence that chronic exposure or high-dose CYP has toxic effects on humans and animals.

Cypermethrin can be found in trace amounts or at higher concentrations in soil and air. In mammals, CYP can accumulate in body fat, skin, liver, kidneys, adrenal glands, ovaries, lung, blood, and heart [4–6]. However, the main target for CYP is the central nervous system.

* Corresponding author. Tel.: +86 23 68250215; fax: +86 23 68251196.
E-mail address: swu_jhz@yahoo.com.cn (J.H. Zhang).

0093-691X/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2009.04.016

Symptoms of CYP toxicity in laboratory animals include pawing, burrowing, salivation, tremors, writhing, and seizures. In humans, high doses of CYP result in twitching, drowsiness, coma, and seizures [7]. Cypermethrin exerts its neurotoxic effect through voltage-dependent sodium channels and integral protein ATPases in the neuronal membrane [8,9].

In addition to neurons, reproductive organs are another toxic target of CYP [10,11]. Cypermethrin decreases the weight of testosterone-sensitive organs, increases the height of seminal gland epithelium, and reduces sperm count and motility in male mice [11–15]. Moreover, CYP significantly reduced serum concentrations of testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) [11], in addition to decreasing the number of implantation sites and viable fetuses in females mated with these male mice [11]. Taken together, it is evident that CYP disrupted male reproductive function.

The mechanism by which CYP affects male reproduction is unclear: Pyrethroids are rapidly metabolized in mammals, and several studies have shown that CYP damages the brain, liver, and erythrocytes by causing oxidative stress [16–18]. However, there are no studies that have investigated how oxidative stress mediates CYP-induced deficits in male reproduction. Consequently, the current study examined the role of oxidative stress in beta-CYP–induced damage to the testes and the possible protective effects of vitamin E. Vitamin E is a fat-soluble vitamin with potent antioxidant properties that scavenges intracellular free radicals and maintains cell membrane integrity by inhibiting lipid peroxidation induced by reactive oxygen species (ROS) [19].

2. Materials and methods

2.1. Materials

Beta-CYP (>99% pure) was obtained from Nanjing Panfeng Chem Ltd. (Nanjing, Jiangsu, China). The steroidogenic acute regulatory protein (StAR) antibody was kindly provided by Professor D.M. Stocco (Texas Tech University Science Center, Lubbock, TX, USA), and antibodies for phosphorylated (p)-ERK (E-4) and ERK were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The ^{125}I-testosterone radioimmunoassay (RIA) kit was purchased from the China Institute of Atomic Energy (Beijing, China). Total (Cu-Zn and Mn) superoxide dismutase (T-SOD) assay kit, malondialdehyde equivalents assay kit, nitrite assay kit, total nitric oxide synthase (NOS) assay kit, and specific NOS assay kit were purchased from Nanjin Jiancheng Biotechnology CO, Ltd (Nanjing, Jiangsu, China). Glutathione peroxidase (GSH-Px) assay kit and catalase (CAT) activity assay kit were purchased from Beyotime Institute of Biotechnology, China (Shanghai, China). All other reagents used in the analyses were of analytical grade and were obtained locally.

2.2. Animals and experimental design

Kunbai male mice (outbred strain) weighing ~30 to 35 g were provided by a local veterinary research institute. All animal treatment procedures were approved by the Animal Care Committee of Southwest University. These animals were adapted to the laboratory conditions before experiments and were housed in a standard animal facility under controlled temperature (22 °C), relative humidity (50% to 60%), and photoperiod (12 h light/12 h dark), with ad libitum access to water and food pellets.

According to the doses used by others [5,15] and a preliminary test, the highest dose in the study was 20 mg/kg body weight. Sixty adult Kunbai male mice were randomly allocated into six groups (N = 10/group). Doses were determined according to body weight, which did not differ between groups, and delivered periorally every day for 35 d. Both beta-CYP and vitamin E were dissolved in 0.1 mL peanut oil and administered to animals by gavage. Groups were as follows: (I) vehicle control: 0.1 mL (peanut oil); (II) low dose: 1 mg/kg beta-CYP; (III) moderate dose: 10 mg/kg beta-CYP; (IV) high dose: 20 mg/kg beta-CYP; (V) 20 mg/kg beta-CYP + 20 mg/kg vitamin E; and (VI) 20 mg/kg vitamin E.

At 36 d after the start of treatment, all mice were anesthetized with halothane and killed by aseptically severing the neck vessels.

2.3. Hormone assay

Blood samples were taken from the eye sockets of animals under anesthesia using a 1-mm syringe. Blood samples were centrifuged at 6111 × g for 4 min, and serum samples were stored at–70 °C until analysis. Serum hormone concentrations were assayed using RIA, as per kit instructions. Sensitivity (0.01 ng/mL) and coefficients of variation (intra-assay 5%, interassay 9%) were regarded as satisfactory.

2.4. Ultrastructure of Leydig cells and histologic structure of testes and epididymides

Samples of testes and epididymides were immersion-fixed in Bouin's solution for histopathology and

embedded in paraffin. Sections (5 μm thick) were cut, stained with hematoxylin and eosin, mounted with Dextran Plasticizer Xylene (DPX, St. Louis, MO, Sigma), and examined using light microscopy. To detect the effect of beta-CYP on the number of Leydig cells, Leydig cells were quantified in the interstitium between seminiferous tubules stained by hematoxylin and eosin (H&E). During counting, fields were randomly selected and were without overlap. Thirty to 40 areas per section and 10 sections per animal were scored [20].

For ultrastructure analysis, some testes samples were cut into 2-mm-thick slices and fixed in ice-cold fixative consisting of 4% paraformaldehyde, 0.25% glutaraldehyde, and 0.15 M HEPES-KOH buffer (pH 7.4) for 30 min. For ultrastructure analysis of Leydig cells, testes were postfixed in 2% osmium tetroxide, dehydrated, and embedded in Araldite 502. Ultrathin sections (70 to 90 nm thick) of the blocks were picked up on copper grids; sections were stained with uranyl acetate and lead citrate and were analyzed under transmission electron microscope at 80 kV.

2.5. Sperm collection and analysis of sperm output

The cauda epididymis was cleared of blood and adipose tissue and opened to extract sperm; sperm were collected by centrifugation with saline-merthiolate-Triton (SMT [1 mL/10 mg tissue] 0.9% [w/v] NaCl; 0.01% [w/v] merthiolate; 0.05% [v/v] Triton X-100). The number of sperm was measured using a hemocytometer: A sample was placed into a hemocytometer and at least three chambers were counted for each spermatid sample. If the totals were not within 10% of each other, the samples were recounted. One thousand sperm per each epididymis were assayed for viability and malformation. Sperm viability was assessed by eosin Y stain. This supravital stain determines whether the membrane is physically intact; if the membrane is damaged or broken, the dye is able to stain the sperm, but if the membrane is intact, it is unstained. The percentage of viable sperm was calculated as follows: Number of viable sperm/[(number of viable sperm) + (number of nonviable sperm)] ×100%. Sperm malformation rate was assayed by the motility of sperm and intact nature of the acrosome. The motility of sperm samples was estimated under a light microscope at 200× magnification, following activation of 2 μL of sperm in 20 μL of water on a microscope slide. The integrity of the acrosome was assessed using Wright-Giemsa stain [11,21–23].

2.6. Antioxidant enzyme activity and oxidative stress assays

Testes tissue was homogenized in 5 mL ice-cold Tris-HCl buffer (0.01 mol/L, pH 7.4) containing 0.01% EDTA-2Na, 0.01 mol/L saccharose, and 0.8% NaCl. The homogenization procedure was carried out for 2 min at 12,861 × g. All procedures were performed at 4 °C. Homogenate, supernatant, and extracted samples were prepared to determine the activities of CAT [24], GSH-Px, T-SOD [25,26], malondialdehyde (MDA), NO, and NOS.

To detect lipid peroxidation, malondialdehyde concentrations of testes tissue homogenates were assayed according to a modified method based on the reaction with thiobarbituric acid. Briefly, malondialdehyde or MDA-like substances and TBA react to produce a pink pigment with a maximum absorption of 532 nm. Malondialdehyde concentrations in tissue was expressed as nmol/g protein [27]. Total nitrite/nitrate concentrations were measured using the Griess reagent (0.1% N-1-napthylethylenediamine dihydrochloride in water, 1% sulfanilamide in 5% phosphoric acid, and 0.1 M sodium nitrite in water) after the cadmium-mediated reduction of nitrate to nitrite. Nitrite concentrations were measured at 550 nm and NO content expressed as μmol/mL [28]. According to the base principle of Asano [29] and the protocol of the manufacturer, the activities of total NOS (T-NOS) and inducible NOS (iNOS) were detected. Briefly, NOS can produce NO by catalyzing a five-electron oxidation of a guanidino nitrogen of L-arginine (L-Arg). Then, NO can bind a nucleophilic substrate; the change in absorbance at 530 nm is monitored with a spectrophotometer. Adding an iNOS inhibitor, S-methylisothiourea sulfate, to samples eliminates iNOS activity, which was calculated by subtracting non-iNOS activity from T-NOS activity. Total nitric oxide synthase (T-NOS) and inducible nitric oxide synthase (iNOS) activity concentrations were expressed as units/mg protein.

2.7. Western blot analysis

For isolation of protein from testicular tissue, testes were snap-frozen in liquid nitrogen and then homogenized in lysis buffer (5 mM phosphate buffer [pB], pH 7.2, containing 0.1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 1 mg/L chymostatin). Next, cells were lysed in lysis buffer (pBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 2 μg/mL aprotinin). The protein content of the supernatant from centrifugation was determined by spectro-

Table 1
Effect of beta-CYP on mean (±SEM) body weight gain and the weight of testosterone-sensitive organs in mice (n = 10/group).

Group	Weight (g)				
	Body weight gain	Testis	Epididymis	Seminal vesicles	Prostate
Vehicle control	5.08 ± 1.6	0.146 ± 0.003	0.052 ± 0.01	0.158 ± 0.019	0.069 ± 0.003
1 mg/kg beta-CYP	4.44 ± 0.87	0.138 ± 0.006	0.051 ± 0.01	0.136 ± 0.011	0.068 ± 0.002
10 mg/kg beta-CYP	2.43 ± 1.47*	0.115 ± 0.005*	0.049 ± 0.01*	0.114 ± 0.010*	0.067 ± 0.002
20 mg/kg beta-CYP	0.94 ± 0.42*	0.089 ± 0.004*	0.047 ± 0.01*	0.066 ± 0.005*	0.062 ± 0.002*
20 mg/kg beta-CYP + 20 mg/kg vitamin E	3.18 ± 0.97†	0.133 ± 0.005†	0.050 ± 0.01†	0.138 ± 0.008†	0.069 ± 0.002†
20 mg/kg vitamin E	4.8 ± 1.43	0.144 ± 0.009	0.050 ± 0.02	0.156 ± 0.018	0.070 ± 0.003

Within a column: *$P < 0.05$ versus control ($P < 0.05$); †$P < 0.05$ versus 20 mg/kg beta-CYP group.

photometry using bovine serum albumin as a standard. Approximately 40 μg total protein per lane was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). After blocking in 5% nonfat milk in PBS containing 0.1% Tween-20 (pBST), membranes were incubated in a 1:500 dilution of primary antibody in 5% milk/PBST at 4 °C overnight. Primary antibodies were anti-StAR, anti-phospho-ERK1/2, anti-ERK1/2, and anti-beta-actin. The membranes were washed three times and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2500) at room temperature for 1 h. Reactive bands were visualized by SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL, USA), and the membranes were then subjected to X-ray autoradiography. Band intensities were determined by Quantity One software (Bio-Rad).

2.8. Statistical analysis

All data are reported as means ± SEM. Statistical analyses were performed using SPSS (Version 16.0; SPSS Inc, Chicago, IL, USA). Data were analyzed by one-way ANOVA and the Fisher's least significant difference (LSD) method to determine treatment differences. All percentage data were subjected to arc-sine transformation before statistical analysis. A probability of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of beta-CYP on body weight and testosterone-sensitive organ weights

The three doses of beta-CYP decreased body weight gain and weight of testosterone-sensitive organs, such as testes, epididymides, seminal vesicles, and prostate. These measures were reduced in mice given 10 or 20 mg/kg beta-CYP compared with that for the vehicle control ($P < 0.05$). Vitamin E ameliorated the effect of CYP; body and testosterone-sensitive organ weight was higher in the 20 mg/kg beta-CYP group that received vitamin E than that in the 20 mg/kg beta-CYP group that did not receive vitamin E ($P < 0.05$; Table 1). Vitamin E alone had no effect on body or testosterone-sensitive organ weights, indicating a true rescue effect ($P > 0.05$; Table 1).

3.2. Beta-CYP negatively affects sperm output and quality

All doses of beta-CYP used decreased sperm count, viability, and intact acrosome rate. Compared with controls, sperm count, viability, and motility decreased with moderate and high doses of beta-CYP ($P < 0.05$, for both) while increasing the rate of acrosome deformity by approximately 183% and 296%, respectively ($P < 0.05$). Vitamin E reduced the effect of CYP by increasing sperm count and decreasing the rate of malformation ($P < 0.05$; Table 2).

3.3. Effect of beta-CYP on histologic structure of testes and epididymides

Testes from the control group were in various stages of spermatogenesis; spermatozoa and Leydig cells were abundant in the interstitium (Fig. 1A). Qualitative analyses revealed that the low dose (1 mg/kg) of beta-CYP decreased the number of interstitial Leydig cells but did not affect the intratubular compartment of seminiferous tubules ($P < 0.05$; Fig. 1B). As the concentration of beta-CYP increased, the number of spermatids and cells in the seminiferous tubules appeared to decrease; moreover, some cells began to slough ($P < 0.05$; Fig. 1C). At the high beta-CYP dose,

Table 2
Effect of beta-CYP on mean (±SEM) sperm count and quality in mice (n = 10/group).

Treatment group	Sperm count (10^8/g epididymidis)	Viability (%)	Sperm motility (%)	Intact acrosome rate (%)
Vehicle control	6.60 ± 1.25	88.8 ± 4.3	66.9 ± 8.9	87.6 ± 6.6
1 mg/kg beta-CYP	6.54 ± 0.87	85.5 ± 2.8	58.7 ± 7.1	75.5 ± 8.4
10 mg/kg beta-CYP	5.22 ± 0.85*	74.3 ± 1.8*	49.9 ± 6.7*	64.9 ± 6.7*
20 mg/kg beta-CYP	2.96 ± 0.64*	64.0 ± 1.5*	38.8 ± 6.5*	50.9 ± 5.1*
20 mg/kg beta-CYP + 20 mg/kg vitamin E	6.35 ± 0.88†	78.4 ± 2.1†	62.4 ± 8.3†	79.4 ± 5.5†
20 mg/kg vitamin E	6.63 ± 1.09	87.3 ± 3.04	71.3.8 ± 9.1	88.8 ± 6.3

Within a column: *$P < 0.05$ versus control; †$P < 0.05$ versus 20 mg/kg beta-CYP group.

only primary spermatocytes were observed; these had vacuolization (Fig. 1D). All types of spermatids were nearly absent from the lumen of the seminiferous tubules. Coadministering beta-CYP with vitamin E to mice appeared to prevent these effects, as various types of spermatocytes and spermatids were observed ($P < 0.05$; Fig. 1E). Vitamin E alone had no effect compared with controls ($P > 0.05$; Fig. 1F).

Sperm were numerous in the lumens of the epididymides in controls (Fig. 2A), and the low dose of beta-CYP did not significantly affect these numbers (Fig. 2B). With the higher dose of beta-CYP, the number of sperm in the lumen tended to diminish (Fig. 2C). At the highest dose (20 mg/kg), the lumens of the seminiferous tubules were nearly devoid of sperm (Fig. 2D). Vitamin E prevented the decline in sperm number caused by beta-CYP (Fig. 2E) but had no effect on this measure when administered alone (Fig. 2F).

3.4. Effect of beta-CYP on serum testosterone concentration and StAR expression

Serum testosterone was not affected by the low dose (1 mg/kg) of beta-CYP ($P > 0.05$) but was reduced to approximately 65% and 30% of control with the moderate (10 mg/kg) and high doses (20 mg/kg), respectively ($P < 0.05$ for each). Vitamin E reduced the effect of beta-CYP on testosterone concentration ($P < 0.05$) but had no effect when administered alone ($P > 0.05$; Fig. 3A).

Concentrations of steroidogenesis acute regulatory protein (StAR) were not affected by the low dose (1 mg/kg) of beta-CYP ($P > 0.05$) but were reduced to approximately 5.3% of control with a high dose (20 mg/kg) ($P < 0.05$). Vitamin E blocked the beta-CYP–induced decrease in StAR expression ($P < 0.05$; Fig. 3B).

3.5. Effects of beta-CYP on the ultrastructure of Leydig cells

Vehicle-treated controls had Leydig cells with normal smooth endoplasmic reticulum (sER) and mitochondria profiles (Fig. 4A). Beta-CYP treatment caused mitochondria and sER to swell, and the boundary of the nuclear membrane became indistinct (Fig. 4B). As the concentration of beta-CYP was elevated, smooth endoplasmic reticula degenerated, and mitochondria swelled and tended to become transparent (Fig. 4C). At the high dose of beta-CYP, smooth endoplasmic reticula formed concentric circles (Fig. 4D). Vitamin E prevented these structural changes when coadministered with beta-CYP (Fig. 4E) but had no effect when administered alone (Fig. 4F).

3.6. Effect of beta-CYP on oxidative stress

Beta-CYP at 1 mg/kg had no effect on MDA concentrations compared with control ($P > 0.05$; Table 3). At 10 and 20 mg/kg, beta-CYP increased both MDA and NO (for both, $P < 0.05$ compared with controls), but changes were reversed with vitamin E treatment ($P < 0.05$ compared with 20 mg/kg beta-CYP group; Table 3). All concentrations of beta-CYP tested increased T-NOS and iNOS activity in a dose-dependent manner; that is, the largest changes occurred at 20 mg/kg ($P < 0.05$). Vitamin E reduced the effect of beta-CYP on these enzymes ($P < 0.05$ compared with the 20 mg/kg beta-CYP group; Table 3).

3.7. Beta-CYP decreases the activity of antioxidant enzymes

The low dose of beta-CYP had no effect on antioxidant enzymes ($P > 0.05$). Compared with controls, the activity concentrations of CAT, GSH-Px, and

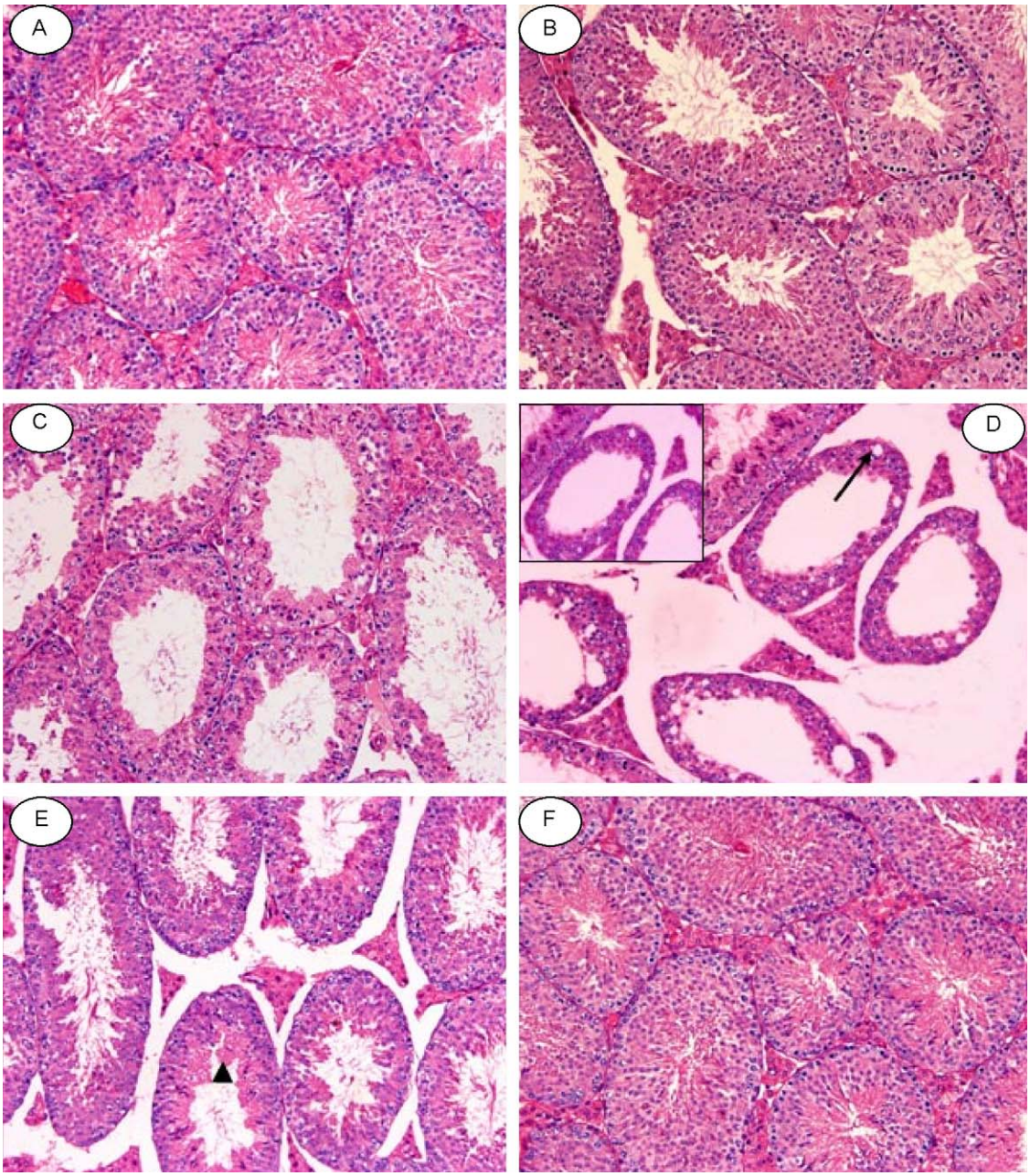

Fig. 1. Effect of beta-CYP on histologic structure of the testes. Mice were given three doses of beta-CYP for 35 d, and then the histologic structure of the testes was examined by hematoxylin and eosin. Photomicrographs show the testes of mice from each treatment group: (A) vehicle control; (B) 1 mg/kg beta-CYP; (C) 10 mg/kg beta-CYP; (D) 20 mg/kg beta-CYP; (E) 20 mg/kg beta-CYP + 20 mg/kg vitamin E; and (F) 20 mg/kg vitamin E. Arrow and arrowhead indicate vacuolization and cell sloughing, respectively (×200). Inset in (D) is a magnified view of the same image (×400).

T-SOD were reduced in groups that received the moderate and high doses of beta-CYP (for all, $P < 0.05$). Vitamin E restored the activity of these three enzymes nearly to control concentrations ($P > 0.05$, compared with control; Table 4).

3.8. Effect of beta-CYP on ERK activity

Compared with controls, concentrations of p-ERK1 and p-ERK2 protein were elevated with increasing doses of beta-CYP ($P < 0.05$); adding vitamin E

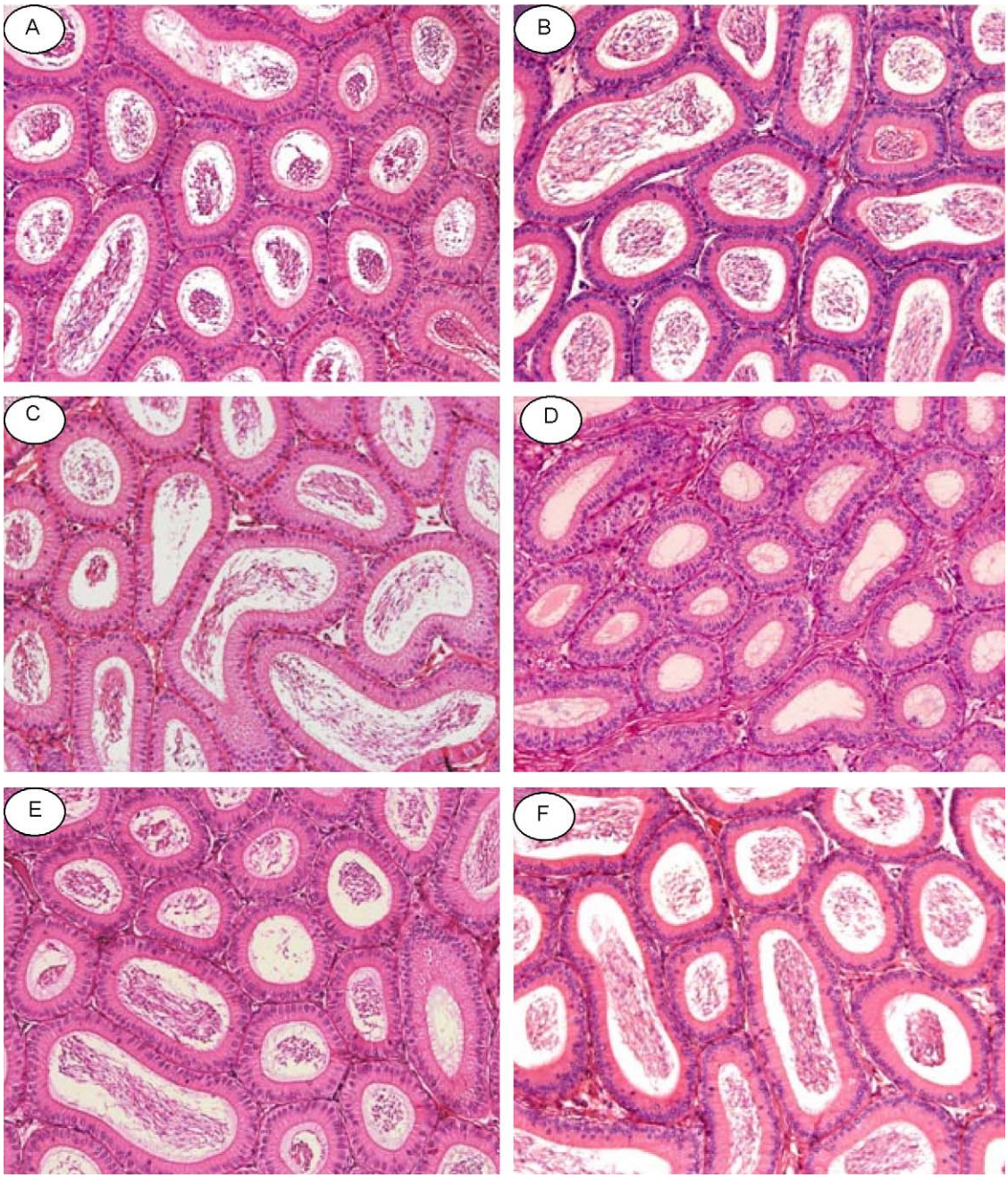

Fig. 2. Effect of beta-CYP on histologic structure of the epididymides by hematoxylin and eosin. Epididymides of mice from each treatment group: (A) vehicle control; (B) 1 mg/kg beta-CYP; (C) 10 mg/kg beta-CYP; (D) 20 mg/kg beta-CYP; (E) 20 mg/kg beta-CYP + 20 mg/kg vitamin E; and (F) 20 mg/kg vitamin E. Scale bars = 50 μm (×200).

prevented this increase ($P < 0.05$ compared with 20 mg/kg beta-CYP group; Fig. 5).

4. Discussion

In this study, beta-CYP had profound negative effects on male reproductive function; however, these changes were prevented by administering an antioxidant. The high dose (20 mg/kg) of beta-CYP significantly reduced the weight of the reproductive organs including testes, epididymides, seminal vesicles, and prostate and diminished sperm number, development, and quality. Beta-CYP also reduced serum testosterone concentrations, possibly by reducing StAR expression

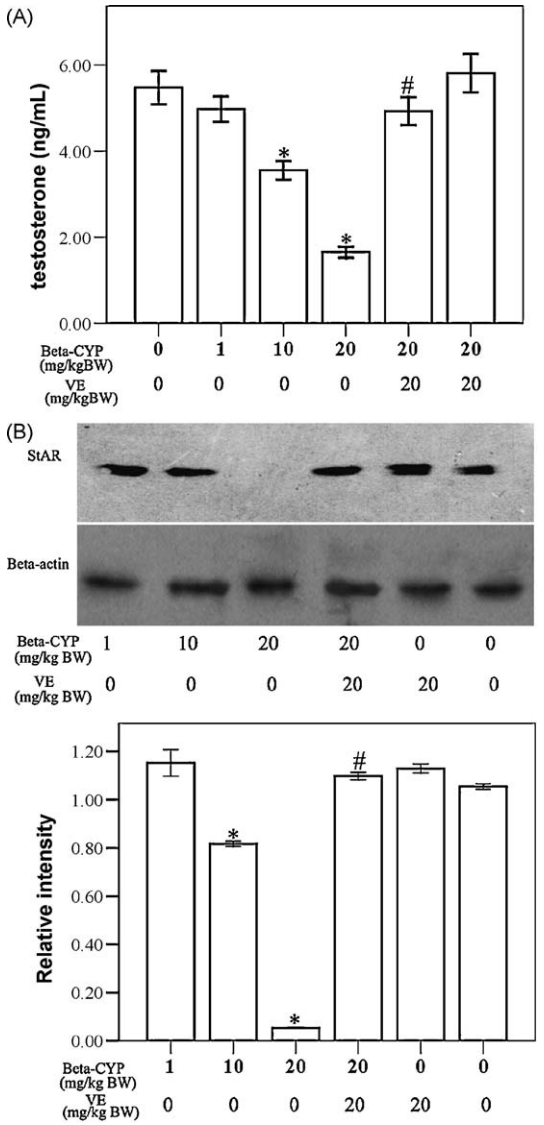

Fig. 3. Effect of beta-CYP on serum testosterone and StAR expression in mice. (A) Serum testosterone concentrations decreased with beta-CYP treatment in a dose-dependent manner (n = 10/group), whereas vitamin E (VE) prevented this decrease. (B) Representative Western blot for the StAR protein (n = 3/group). The relative intensity was determined by ratio of StAR to beta-actin, as measured by densitometry. *$P < 0.05$ compared with control; #$P < 0.05$ compared with 20 mg/kg beta-CYP group.

and damaging the seminiferous tubules. These toxic effects of beta-CYP may be caused by oxidative stress, as they appeared concomitant with increases in markers for oxidative stress and decreases in the activity of antioxidant enzymes. Furthermore, the antioxidant vitamin E reversed the deleterious effects of beta-CYP on testosterone production and testes.

Pyrethrins such as CYP and cyfluthrin can interact with androgen receptors to exert antiandrogenic activity [30], which varies among optical isomers. Cyfluthrin, beta-cyfluthrin, and CYP can significantly inhibit dihydrotestosterone (DHT)-induced androgen receptor activity; however, beta-CYP does not have antiandrogenic activity in vitro or in vivo [15]. Despite its lack of effect on androgen receptors, beta-CYP reduced serum testosterone and sperm count and viability while increasing rates of sperm malformation in male rats, consistent with another report [14]. Furthermore, in the current study, beta-CYP decreased the weight of male reproductive organs and reduced serum testosterone concentrations in mice. We inferred that this compound acted directly on the testes and affected the androgen biosynthesis in Leydig cells; this decrease in testosterone attenuated the function of steroid-sensitive organs, thus reducing their weight.

Beta-CYP also damaged the structure of the testes and epididymides, resulting in primary spermatocyte vacuolization. Testosterone has a crucial role in spermatogenesis; exogenous testosterone administration can decrease intratesticular concentrations of the hormone, which in turn reduces the number of pachytene spermatocytes and round spermatids by increasing germ cell apoptosis at all stages [31]. We hypothesized that the decrease in serum testosterone concentrations in the current study suppressed spermatogenesis.

Leydig cells play a crucial role in testosterone synthesis. The rate-determining step of this synthesis is the transport of the precursor, cholesterol, from intracellular sources into mitochondria. Steroidogenesis acute regulatory protein is an important factor regulating the cholesterol transport process, and it is necessary for steroid synthesis in order to maintain the structural integrity of Leydig cell mitochondria [32]. Diminished concentrations of serum testosterone could result from decreases in the number of or damage to the ultrastructure of Leydig cells. Based on histologic analysis, beta-CYP decreased the number of Leydig cells, and ultrastructural examination revealed the formation of concentric circles in the rough endoplasmic reticulum (RER), as well as mitochondrial swelling, in these cells. The impairment of mitochondria could affect the processing and maturation of StAR; accordingly, StAR expression was reduced in this study.

The mechanism underlying beta-CYP effects on spermatogenesis and testosterone synthesis is unclear. It is known that CYP could induce oxidative stress and as a hydrophobic compound may accumulate in

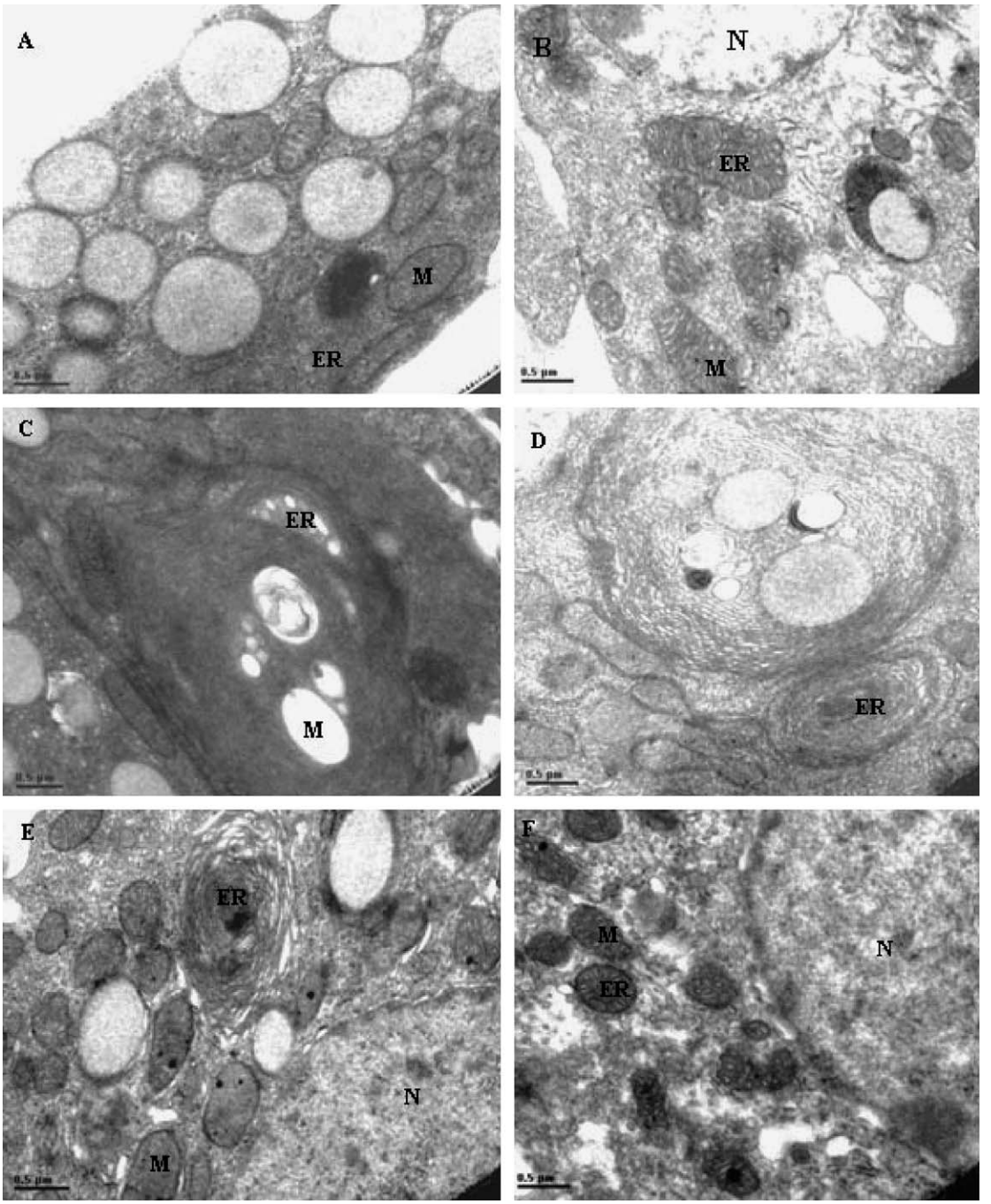

Fig. 4. Effects of beta-CYP on the ultrastructure of Leydig cells in mice stained with uranyl acetate and lead citrate. Ultrastructure of Leydig cells of mice from each of the treatment groups: (A) vehicle control; (B) 1 mg/kg beta-CYP; (C) 10 mg/kg beta-CYP; (D) 20 mg/kg beta-CYP; (E) 20 mg/kg beta-CYP + 20 mg/kg vitamin E; and (F) 20 mg/kg vitamin E. Magnification was 17,500× for all panels except (C), which was 15,000 × . M: mitochondrion; ER: endoplasmic reticulum; N: nucleus.

cell membranes and disturb membrane structure [33]. Administering certain doses of CYP to mice for 60 d caused oxidative stress in a dose- and time-dependent manner [34]. In the current study, beta-CYP increased MDA concentrations, indicating the induction of lipid peroxidation, which can lead to loss of membrane structure and function. These results were similar to those of other studies [18,35]. Nitric oxide synthase

Table 3
Effect of beta-CYP on mean (±SEM) measures of oxidative stress in mice (n = 10/group).

Treatment group	MDA (nmol/mg protein)	NO (μmol/L)	T-NOS (unit/mg protein)	iNOS (unit/mg protein)
Vehicle control	0.44 ± 0.08	0.144 ± 0.032	1.44 ± 0.32	0.98 ± 0.15
1 mg/kg beta-CYP	0.61 ± 0.06	0.273 ± 0.048	2.73 ± 0.48	1.93 ± 0.26*
10 mg/kg beta-CYP	1.79 ± 0.19*	0.385 ± 0.051*	3.85 ± 0.51*	2.11 ± 0.36*
20 mg/kg beta-CYP	3.27 ± 0.24*	0.485 ± 0.062*	4.87 ± 0.62*	3.53 ± 0.67*
20 mg/kg beta-CYP + 20 mg/kg vitamin E	0.60 ± 0.06†	0.192 ± 0.033†	1.92 ± 0.33†	1.33 ± 0.19†
20 mg/kg vitamin E	0.40 ± 0.07	0.127 ± 0.014	1.27 ± 0.14	1.10 ± 0.19

Within a column: *$P < 0.05$ versus control; †$P < 0.05$ versus 20 mg/kg beta-CYP.

Table 4
Effect of beta-CYP on mean (±SEM) antioxidant enzyme activity in mice (n = 10/group).

Treatment group	CAT (μmol min^{-1} mg $protein^{-1}$)	GSH-Px (units/mg protein)	T-SOD (units/mg protein)
Vehicle control	0.520 ± 0.087	2.004 ± 0.485	1.085 ± 0.105
1 mg/kg beta-CYP	0.548 ± 0.068	1.825 ± 0.420	0.929 ± 0.208
10 mg/kg beta-CYP	0.302 ± 0.041*	0.845 ± 0.162*	0.428 ± 0.135*
20 mg/kg beta-CYP	0.07 ± 0.016*	0.285 ± 0.066*	0.311 ± 0.144*
20 mg/kg beta-CYP + 20 mg/kg vitamin E	0.486 ± 0.031†	1.760 ± 0.366†	1.0471 ± 0.063†
20 mg/kg vitamin E	0.604 ± 0.102	2.205 ± 0.532	1.229 ± 0.158

Within a column: *$P < 0.05$ versus control; †$P < 0.05$ versus 20 mg/kg beta-CYP group.

inhibitors can increase serum testosterone concentrations, whereas NO donors or increased NO concentrations can decrease testosterone [36,37]. Interestingly, beta-CYP increased the concentrations of NO, which regulates sperm motility, viability, capacitation, and zona pellucida–binding ability [38–40]. We inferred that the increase in NO could have caused the reduction in testosterone. The concentration of NO in the cell is regulated by NOS. In testes, iNOS was present in the cytoplasm of rat Leydig cells, and iNOS mRNA expression and NO production were enhanced by interleukin 1-beta, round spermatid factor, and bacterial lipopolysaccharide [41–43]. In the current study, beta-CYP also increased the concentration of iNOS, as well as T-NOS, and thus raised NO concentrations.

Antioxidants can inhibit free radical formation. Both SOD and CAT can dismutate O_2 and decompose H_2O_2, resulting in a decrease in oxidative stress, which is the effective way of protecting cells from damage. It is well established that GSH-Px is found mainly in the cytosol and mitochondria and is widely affected by xenobiotics [44]. Low doses of beta-CYP increased the activity of SOD, CAT, and GSH-Px within a few days [18,35], whereas administration of CYP (1/10 LD50) for 30 d decreased SOD and CAT activity in rat liver [5]. In this study, delivering beta-CYP for 35 d decreased T-SOD, GSH-Px, and CAT activity, consistent with the study of Manna et al. [5]. However, results of the current study differed from those of other's study, most likely due to our higher doses and more prolonged treatment [18,35]. The high dose and long duration of exposure of mice to CYP could be the main cause of damage to the cell structure and decreases in SOD and CAT activity.

Further evidence that oxidative stress was involved in beta-CYP's effects on reproductive function was provided by the observation that the antioxidant vitamin E protected against the toxic effects of the pesticide. Vitamin E is fat-soluble and prevents oxidative damage by blocking the oxidation of polyunsaturated fatty acids [45,46] and has been shown to ameliorate aluminum-induced testicular damage in rats [47]. The negative effects on reproductive function and elevated oxidative stress caused by another toxic agent, Aroclor 1254, were also reversed by vitamin E [19]. Similarly, vitamin E demonstrated a protective effect against CYP-induced elevation of thiobarbituric acid reactive substances (TBARS) by decreasing MDA concentrations in liver, brain, and kidney [35,48]. In the current study, vitamin E prevented the effects of treatment with beta-CYP: cell sloughing and vacuolation; decreased T-SOD, GSH-Px, and CAT activity; and increased MDA concentrations. Thus, beta-CYP impaired reproductive function in male mice by inducing oxidative stress.-

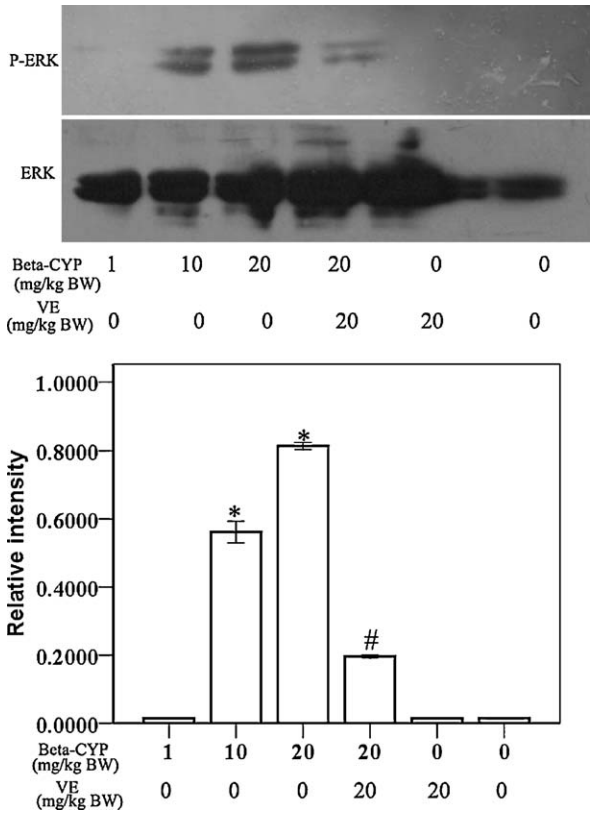

Fig. 5. Effect of beta-CYP on ERK activity in mice (n = 3/group). Testes tissue homogenates were analyzed for p-ERK1/2 or ERK1/2 (loading control) by immunoblotting. The relative intensity was determined by ratio of p-ERK to total ERK as measured by densitometry. $P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with 20 mg/kg beta-CYP group.

Reactive oxygen species as second messengers are known to induce signaling cascades such as ERK1/2, which is a prototype member of the mitogen-activated protein kinase (MAPK) family and regulates numerous cellular processes [49]. Although ERK1/2 generally regulates cell growth and differentiation, the JNK (Jun N-terminal kinase) and p38 family MAPKs preferentially mediate stress. There is now increasing evidence that ERK1/2 can also be stimulated by oxidative stress [50–52]. In the current study, beta-CYP at the two high doses activated ERK1/2 and vitamin E inhibited this effect, suggesting that ROS could be involved as a second messenger in beta-CYP's effects on testis function. Whether ERK1/2 activation is the main cause of reduced testosterone synthesis is unclear. To address this question, further investigations will be necessary to study the effect of beta-CYP on cultured mouse Leydig cells.

In conclusion, high doses of beta-CYP damaged the structure of the testes and decreased sperm output by inducing oxidative stress. Although beta-CYP is highly toxic to insects and has low potency in mammals, high doses and prolonged exposures to beta-CYP can be harmful to the reproductive systems of experimental animals. Thus, beta-CYP use should be radically reduced to protect mammalian health.

Acknowledgments

We thank Dr. Zhong-Xian Lu for his valuable and constructive criticism and Mr. J. Hodo Bedell for editorial assistance with the manuscript. This study was supported by a grant from the National Natural Science Foundation of China (No. 30270955).

References

[1] Velisek J, Wlasow T, Gomulka P, Svobodova Z, Dobsikova R, Novotny L, Dudzik M. Effects of cypermethrin on rainbow trout (Oncorhynchus mykiss). Vet Med (Praha) 2006;51:469–76.
[2] Zhang L, Gao X, Liang P. Beta-cypermethrin resistance associated with high carboxylesterase activities in a strain of house fly, Musca domestica (Diptera: Muscidae). Pestic Biochem Physiol 2007;89:65–72.
[3] Igbedioh SO. Effects of agricultural pesticides on humans, animals, and higher plants in developing countries. Arch Environ Health 1991;46:218–24.
[4] Hall BE, Vickers JA., Hopkins JA. A study to determine the bioaccumulation of 14C-cypermethrin radioactivity in the rat following repeated oral administration.WHO, 1980. Report No.2487-72/20.
[5] Manna S, Bhattacharyya D, Mandal TK, Das S. Repeated dose toxicity of alfa-cypermethrin in rats. J Vet Sci 2004;5:241–5.
[6] Wielgomas B, Krechniak J. Toxicokinetic interactions of alpha-cypermethrin and chlorpyrifos in rats. Pol J Environ Stud 2007;16:267–74.
[7] He F, Wang S, Liu L, Chen S, Zhang Z, Sun J. Clinical manifestations and diagnosis of acute pyrethroid poisoning. Arch Toxicol 1989;63:54–8.
[8] Kakko I, Toimela T, Tahti H. The synaptosomal membrane bound ATPase as a target for the neurotoxic effects of pyrethroids, permethrin and cypermethrin. Chemosphere 2003;51:475–80.
[9] Soderlund DM, Bloomquist JR. Neurotoxic actions of pyrethroid insecticides. Annu Rev Entomol 1989;34:77–96.
[10] Valentine WM. Toxicology of selected pesticides, drugs, and chemicals. Short-chain alcohols. Vet Clin North Am Small Anim Pract 1990;20:515–23.
[11] Elbetieha A, Da'as SI, Khamas W, Darmani H. Evaluation of the toxic potentials of cypermethrin pesticide on some reproductive and fertility parameters in the male rats. Arch Environ Contam Toxicol 2001;41:522–8.
[12] Bhunya SP, Pati PC. Genotoxic effects of a synthetic pyrethroid insecticide, cypermethrin, in mice in vivo. Toxicol Lett 1988;41:223–30.
[13] Rodriguez H, Tamayo C, Inostroza J, Soto C, Bustos-Obregon E, Paniagua R. Cypermethrin effects on the adult mice seminal glands. Ecotoxicol Environ Saf 2009;72:658–62.
[14] Song L, Wang YB, Sun H, Yuan C, Hong X, Qu JH, et al. Effects of fenvalerate and cypermethrin on rat sperm motility patterns in

vitro as measured by computer-assisted sperm analysis. J Toxicol Environ Health A 2008;71:325–32.
[15] Wu W, Zhang J, Zhu W, Zheng YF, Zhu HJ, Xu M, Zhu XQ. [Antiandrogenic effects of cypermethrin and beta-cypermethrin] (in Chinese, with English abstract). Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi 2008;26:193–7.
[16] Zegura B, Lah TT, Filipic M. The role of reactive oxygen species in microcystin-LR-induced DNA damage. Toxicology 2004; 200:59–68.
[17] Giray B, Gurbay A, Hincal F. Cypermethrin-induced oxidative stress in rat brain and liver is prevented by vitamin E or allopurinol. Toxicol Lett 2001;118:139–46.
[18] Kale M, Rathore N, John S, Bhatnagar D. Lipid peroxidative damage on pyrethroid exposure and alterations in antioxidant status in rat erythrocytes: a possible involvement of reactive oxygen species. Toxicol Lett 1999;105:197–205.
[19] Senthil kumar J, Banudevi S, Sharmila M, Murugesan P, Srinivasan N, Balasubramanian K, et al. Effects of vitamin C and E on PCB (Aroclor 1254) induced oxidative stress, androgen binding protein and lactate in rat Sertoli cells. Reprod Toxicol 2004; 19:201–8.
[20] Tae HJ, Jang BG, Ahn DC, Choi EY, Kang HS, Kim NS, et al. Morphometric studies on the testis of Korean ring-necked pheasant (Phasianus colchicus karpowi) during the breeding and non-breeding seasons. Vet Res Commun 2005; 29:629–43.
[21] Kaneko T, Yamamura A, Ide Y, Ogi M, Yanagita T, Nakagata N. Long-term cryopreservation of mouse sperm. Theriogenology 2006;66:1098–101.
[22] Yildiz C, Fleming C, Ottaviani P, McKerlie C. Fresh and frozen-thawed sperm quality, nuclear DNA integrity, in vitro fertility, embryo development, and live-born offspring of N-ethyl-N-nitrosourea (ENU) mice. Cryobiology 2008;57:156–62.
[23] Krzanowska H, Styrna J, Wabik-Sliz B. Analysis of sperm quality in recombinant inbred mouse strains: correlation of sperm head shape with sperm abnormalities and with the incidence of supplementary spermatozoa in the perivitelline space. J Reprod Fertil 1995;104:347–54.
[24] Lick H. Catalase. In: Bergemeuer HU, editor. Methods of Enzymatic Analysis. Verlag Chemie; 1963. p. 885–8.
[25] Flohe L, Gunzler WA. Assays of glutathione peroxidase. Methods Enzymol 1984;105:114–21.
[26] Bannister JV, Calabrese L. Assays for superoxide dismutase. Methods Biochem Anal 1987;32:279–312.
[27] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95: 351–8.
[28] Fonseca ES, Massoco CO, Palermo-Neto J. Effects of prenatal stress on stress-induced changes in behavior and macrophage activity of mice. Physiol Behav 2002;77:205–15.
[29] Asano K, Chee CB, Gaston B, Lilly CM, Gerard C, Drazen JM, Stamler JS. Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells. Proc Natl Acad Sci U S A 1994;91:10089–93.
[30] Zhang J, Zhu W, Zheng YF, Yang J, Zhu XQ. The antiandrogenic activity of pyrethroid pesticides cyfluthrin and beta-cyfluthrin. Reprod Toxicol 2008;25:491–6.
[31] Lue Y, Wang C, Liu YX, Hikim AP, Zhang XS, Ng CM, et al. Transient testicular warming enhances the suppressive effect of testosterone on spermatogenesis in adult cynomolgus monkeys (Macaca fascicularis). J Clin Endocrinol Metab 2006;91: 539–45.
[32] Jefcoate C. High-flux mitochondrial cholesterol trafficking, a specialized function of the adrenal cortex. J Clin Invest 2002; 110:881–90.
[33] Grajeda-Cota P, Ramirez-Mares MV, de Mejia EG. Vitamin C protects against in vitro cytotoxicity of cypermethrin in rat hepatocytes. Toxicol In Vitro 2004;18:13–9.
[34] Kanbur M, Liman BC, Eraslan G, Altinordulu S. Effects of cypermethrin, propetamphos, and combination involving cypermethrin and propetamphos on lipid peroxidation in mice. Environ Toxicol 2008;23:473–9.
[35] Atessahin A, Yilmaz S, Karahan I, Pirincci I, Tasdemir B. The effects of vitamin E and selenium on cypermethrin-induced oxidative stress in rats. Turk J Vet Anim Sci 2005;29:385–91.
[36] Pomerantz DK, Pitelka V. Nitric oxide is a mediator of the inhibitory effect of activated macrophages on production of androgen by the Leydig cell of the mouse. Endocrinology 1998;139:922–31.
[37] Adams ML, Meyer ER, Sewing BN, Cicero TJ. Effects of nitric oxide-related agents on rat testicular function. J Pharmacol Exp Ther 1994;269:230–7.
[38] Hellstrom WJ, Bell M, Wang R, Sikka SC. Effect of sodium nitroprusside on sperm motility, viability, and lipid peroxidation. Fertil Steril 1994;61:1117–22.
[39] Perera DM, Katz M, Heenbanda SR, Marchant S. Nitric oxide synthase inhibitor NG-monomethyl-L-arginine preserves sperm motility after swim-up. Fertil Steril 1996;66:830–3.
[40] Sengoku K, Tamate K, Yoshida T, Takaoka Y, Miyamoto T, Ishikawa M. Effects of low concentrations of nitric oxide on the zona pellucida binding ability of human spermatozoa. Fertil Steril 1998;69:522–7.
[41] Fujisawa M, Tatsumi N, Fujioka H, Kanzaki M, Okuda Y, Arakawa S, Kamidono S. Nitric oxide production of rat Leydig and Sertoli cells is stimulated by round spermatid factor(s). Mol Cell Endocrinol 2000;160:99–105.
[42] Tatsumi N, Fujisawa M, Kanzaki M, Okuda Y, Okada H, Arakawa S, Kamidono S. Nitric oxide production by cultured rat Leydig cells. Endocrinology 1997;138:994–8.
[43] Reddy MM, Mahipal SVK, Subhashini J, Reddy MC, Roy KR, Reddy GV, et al. Bacterial lipopolysaccharide-induced oxidative stress in the impairment of steroidogenesis and spermatogenesis in rats. Reprod Toxicol 2006;22:493–500.
[44] Uner N, Oruc EO, Canli M, Sevgiler Y. Effects of cypermethrin on antioxidant enzyme activities and lipid peroxidation in liver and kidney of the freshwater fish, Oreochromis niloticus and Cyprinus carpio (L.). Bull Environ Contam Toxicol 2001;67: 657–64.
[45] Traber MG, Packer L. Vitamin E: beyond antioxidant function. Am J Clin Nutr 1995;62:1501S–9.
[46] Aldana L, Tsutsumi V, Craigmill A, Silveira MI. Gonzalez de Mejia E. alpha-Tocopherol modulates liver toxicity of the pyrethroid cypermethrin. Toxicol Lett 2001;125:107–16.
[47] Kutlubay R, Oguz EO, Can B, Guven MC, Smik Z, Tuncay OL, Vitamin. E protection from testicular damage caused by intraperitoneal aluminium. Int J Toxicol 2007;26:297–306.
[48] Vannucchi H, Jordao Junior AA, Iglesias AC, Morandi MV, Chiarello PG. Effect of different dietary levels of vitamin E on lipid peroxidation in rats. Arch Latinoam Nutr 1997;47: 34–7.
[49] Zhu D, Tan KS, Zhang X, Sun AY, Sun GY, Lee JC. Hydrogen peroxide alters membrane and cytoskeleton properties and increases intercellular connections in astrocytes. J Cell Sci 2005;118:3695–703.

[50] Boulton TG, Yancopoulos GD, Gregory JS, Slaughter C, Moomaw C, Hsu J, Cobb MH. An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. Science 1990;249:64–7.

[51] Akimoto T, Nonaka T, Harashima K, Sakurai H, Ishikawa H, Mitsuhashi N. Radicicol potentiates heat-induced cell killing in a human oesophageal cancer cell line: the Hsp90 chaperone complex as a new molecular target for enhancement of thermosensitivity. Int J Radiat Biol 2004;80:483–92.

[52] Ng DC, Bogoyevitch MA. The mechanism of heat shock activation of ERK mitogen-activated protein kinases in the interleukin 3-dependent ProB cell line BaF3. J Biol Chem 2000;275:40856–6.