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Permethrin May Disrupt Testosterone Biosynthesis via Mitochondrial Membrane Damage of Leydig Cells in Adult Male Mouse

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Permethrin, a popular synthetic pyrethroid insecticide used to control noxious insects in agriculture, forestry, households, horticulture, and public health throughout the world, poses risks of environmental exposure. Here we evaluate the reproductive toxicity of *cis*-permethrin in adult male ICR mice that were orally administered *cis*-permethrin (0, 35, or 70 mg/kg·d) for 6 wk. Caudal epididymal sperm count and sperm motility in the treated groups were statistically reduced in a dose-dependent manner. Testicular testosterone production and plasma testosterone concentration were significantly and dose-dependently decreased with an increase in LH, and a significant regression was observed between testosterone levels and *cis*-permethrin residues in individual mice testes after exposure. However, no significant changes were observed in body weight, reproductive organ absolute and relative weights, sperm morphology, and plasma FSH concentration after *cis*-permethrin treatment. Moreover, *cis*-permethrin exposure significantly diminished the testicular mitochondrial mRNA expression levels of peripheral benzodiazepine receptor (PBR), steroidogenic acute regulatory protein (StAR), and cytochrome P450 side-chain cleavage (P450scc) and enzyme and protein expression levels of StAR and P450scc. At the electron microscopic level, mitochondrial membrane damage was found in Leydig cells of the exposed mouse testis. Our results suggest that the insecticide permethrin may cause mitochondrial membrane impairment in Leydig cells and disrupt testosterone biosynthesis by diminishing the delivery of cholesterol into the mitochondria and decreasing the conversion of cholesterol to pregnenolone in the cells, thus reducing subsequent testosterone production. (*Endocrinology* 148: 3941–3949, 2007)

PERMETHRIN (Chemical Abstracts Service No. 52645-53-1) [3-phenoxybenzyl (1RS)-*cis*,*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclo-propanecarboxylate] is a synthetic pyrethroid insecticide. It is almost insoluble in water, soluble in organic solvents, stable to light and heat, but unstable in alkaline media (1). Since it was marketed in 1977, permethrin has been used worldwide to control noxious insects in agriculture, forestry, households, horticulture, and the public health. Annual 2004 sales of permethrin worldwide were about 50 million U.S. dollars (2), and annual 2004 production of the permethrin active ingredient in Japan was 11.6 tons (3).

Like other synthetic pyrethroid insecticides, permethrin's neurotoxicity acts on the axons of the peripheral and central nervous systems due to its interaction with sodium channels in mammals and/or insects (1). The insecticide has been found to be mildly irritating to the eyes and to cause slight skin irritation (4). In addition, some laboratory animal experiments suggested that permethrin may adversely affect the liver, brain, and immune system (5–7). It also proved to be mutagenic in human and hamster cell cultures (8–10).

However, in recent years, exposure to some insecticides including pyrethroids has been reported to have potentially adverse effects on male reproduction. Fenvalerate exposure in rats was reported (11) to cause significantly reduced weight of testes, epididymal sperm counts, and sperm motility along with a decrease in testicular enzymes for testosterone biosynthesis, 17β-hydroxysteroid dehydrogenase (17β-HSD), and glucose-6-phosphate dehydrogenase, which might be due to interference with testicular testosterone synthesis. Serum testosterone concentration also reportedly decreased significantly in a high-dose exposure group. Except for pyrethroids, organophosphorus insecticides such as dimethoate have also been found to disrupt male reproductive function. Dimethoate inhibited steroidogenesis in mouse

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Abbreviations: GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase; 17β-HSD, 17-hydroxysteroid dehydrogenase; LDL, low density lipoprotein; LDL-R, LDL receptor; 8-OHdG, 8-hydroxy-2-deoxyguanosine; PBR, peripheral benzodiazepine receptor; P450 17α, cytochrome P450 17α-hydroxylase/C_{17-20} lyase; P450scc, cytochrome P450 side-chain cleavage enzyme; SR-B1, scavenger receptor class B type 1; StAR, steroidogenic acute regulatory protein.

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MA-10 Leydig tumor cells primarily by blocking transcription of the steroidogenic acute regulatory (StAR) gene (12).

However, the testicular toxicity of permethrin exposure, particularly in relation to testosterone synthesis, remains poorly understood. Testosterone is a primary male steroid hormone, and the disruption of its production may impair male reproductive health. Therefore, it is important to evaluate whether permethrin exposure interferes with the male reproductive system. Additionally, the toxicity of permethrin is aggravated by factors such as the *cis*:*trans* ratio that significantly affects the LD_{50} value of permethrin. It is also well known that the (+)-*cis*-permethrin is more toxic than the *trans*-isomer (13). Thus, in this study we administered *cis*-permethrin to adult male ICR mice to investigate its effects on their reproductive system.

Materials and Methods

Animals

This study was conducted according to the Guidelines for Animal Experiments of the Nagoya University Animal Center. Adult male ICR mice (8 wk old) were purchased from CLEA Japan Inc. (Tokyo, Japan) and were housed in the animal center with a 12-h light, 12-h dark cycle under a controlled temperature of 23–25 C and 57–60% relative humidity for 1 wk to accommodate them to the new surroundings. They had free access to food and tap water.

Treatment

Mice were randomly divided into three groups (n = 8), and standard *cis*-permethrin (99.2% pure) (Wako, Osaka, Japan) was orally administrated at doses of 0, 35, or 70 mg/kg·d for 6 wk (42 consecutive days). Mice of the control group ingested a vehicle (corn oil) only. Sixteen hours after the last administration, all mice were weighed and killed by decapitation. The blood was collected into the heparinized tube. The following organs were dissected out and weighed as quickly as possible: epididymis, testes, liver, seminal vesicle, and prostate.

Epididymal sperm motility analysis

The left cauda epididymis was weighed, placed in a dish containing 2 ml Hank's solution at 37 C, and then cut into small pieces. The sperm was gently filtered through gauze under a microscope on a warming plate (Tokai Hit Co., Fujinomiya, Japan) while maintaining the temperature at 37 C. Filtrates (spermatozoa) were used to manually count motile sperm.

Epididymal sperm morphology analysis

A drop of sperm suspension was uniformly smeared on three glass slides per sample. After being air-dried, the smeared slides were fixed in methanol and then stained according to Bryan's method (14). Based on the method of Mori *et al.* (15), abnormalities in sperm morphology were examined under the microscope.

Epididymal sperm count

The sperm suspension was diluted with saline containing 0.5% formalin. The number of sperm was counted using a Neubauer-type hemocytometer (Erma, Tokyo, Japan). Epididymal sperm counts were expressed as the number of sperm per gram of cauda epididymis.

Hormone assay

Plasma was separated by centrifugation at 4 C and stored at −80 C until assay for testosterone, LH, and FSH. Concentrations of LH and FSH in plasma were measured using National Institute of Diabetes and Digestive and Kidney Diseases rat RIA kits (Torrance, CA) for rat LH and FSH. As for the antisera, antirat LH-S-11 and antirat FSH-S-11 were used, whereas for the iodinated preparation, rat LH-I-7 and FSH-I-7 were used. For measurements of testicular testosterone levels, a part of the left testis was homogenized in 0.6 ml 10 mM PBS (pH 7.4). After centrifugation, testis testosterone was then extracted from aliquots of the supernatant (10 μl) with 2 ml hexane-ethylether mixture (3:2). The plasma testosterone was directly extracted from 10 μl plasma with the same mixture (3:2). Concentrations of testosterone in the testis and plasma were measured by a testosterone EIA kit (Cayman, Ann Arbor, MI).

Real-time quantitative PCR analysis

We measured mRNA levels of several important enzymes, carrier proteins, or receptors involved in the testicular biosynthetic pathway of testosterone. Total RNA was extracted using the RNeasy Mini kit (QIAGEN, Tokyo, Japan) from the left testis of mice exposed to *cis*-permethrin or vehicle only. One microgram of total RNA was reverse transcribed into cDNA in a 20-μl reaction solution containing oligo$(dT)_{20}$ primers and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The primers (Table 1) were designed using Primer Express 1.0 software (Applied Biosystems, Singapore). Real-time quantitative PCR was performed in a 96-well plate using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Reactions were carried out in a 25-μl volume containing 1× SYBR Green Master Mix (Applied Biosystems) and 100 nM of each forward and reverse primer. Reactions were run for 50 cycles (denaturation at 95 C for 15 sec, annealing and extension at 60 C for 1 min) after an initial 2-min step at 50 C for enzyme activation and a 10-min incubation at 95 C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for data analysis. All genes of mRNA expression levels were normalized to GAPDH expression levels.

Western blot analysis

A part of the testis from six mice of the control group and the higher-dose group was homogenized with a 3-fold volume of 10 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose. The protein concentration of each sample was measured with a Protein Assay Kit (Bio-Rad, Tokyo, Japan). After electrophoresis in 10% SDS-PAGE, proteins were transferred onto a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The membrane was blocked with 3% nonfat milk for 1 h at room temperature and then reacted with the primary antibodies against StAR (rabbit polyclonal IgG, 1:1000; kindly provided by Dr. Dale Buchanan Hales) (16, 17), P450scc (rabbit polyclonal IgG, 1:1000; Chemicon International Inc., Temecula, CA), and P450 17α (goat polyclonal IgG, 1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), respectively, for 12 h at 4 C followed by reaction with the secondary goat

TABLE 1. Primers used for real-time PCR to quantify mRNA expression of biomolecules involved in testicular steroidogenesis

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
GAPDH	AGAACATCATC CCTGCATCCA	CCGTTCAGCTCT GGGATGAC
HMG-CoA synthase	TGTGGCACCGG ATGTCTTT	GACCAGATACCA CGTTCCTTCAA
HMG-CoA reductase	TGTGGTTTGTG AAGCCGTCAT	CGTCAACCATAG CTTCCGTAGTT
SR-B1	CCCTTCGTGCA TTTTCTCAAC	CATCCCAACAAA CAGGCCA
LDL-R	GGAAAATGCAT CGCTAGCAAGT	ATTGGACTGACA GGTGACAGACA
PBR	AGTTCGTGGCA CTGCATAAGC	GCTGCCCATTCT CTCCTCCTA
StAR	AAGGAAAGCCA GCAGGAGAAC	TCCATGCGGTCC ACAAGTT
P450scc	CCATCAGATGC AGAGTTTCCAA	TGAGAAGAGTAT CGACGCATCCT
3β-HSD	GGAGGCCTGTG TTCAAGCAA	GGCCCTGCAACA TCAACTG
P450 17α	CCATCCCGAAG GACACACAT	CTGGCTGGTCCC ATTCATTT
17β-HSD	CAACGATTCCT CCTGACACGAT	GCTGATGTTGCG TTTGAGGTAA

TABLE 2. Effects of *cis*-permethrin exposure on body and absolute organ weights in mice

Treatment	Dose (mg/kg·d)	n	Body weight (g)	Epididymis (g)	Testes (g)	Prostate + seminal vesicle (g)
Control	Corn oil	8	45.5 ± 2.0	0.108 ± 0.014	0.283 ± 0.041	0.374 ± 0.073
cis-Permethrin	35	8	45.9 ± 3.0	0.107 ± 0.009	0.285 ± 0.033	0.365 ± 0.040
cis-Permethrin	70	8	44.7 ± 2.8	0.106 ± 0.011	0.275 ± 0.040	0.350 ± 0.051

Data represent mean ± SD.

antirabbit IgG (1:1000; Cell Signaling Technology Inc., Beverly, MA) or rabbit-antigoat IgG (1:1000; Dako, Glostrup, Denmark) horseradish peroxidase-conjugated antibody for 1 h at room temperature. The protein bands were visualized using Western blot chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA). The membrane was incubated with stripping solution (Nacalai Tesque Inc., Kyoto, Japan), the blots were reprobed with primary goat polyclonal antibody for GAPDH (1:1000; Santa Cruz Biotechnology), and the secondary rabbit-antigoat IgG horseradish peroxidase-conjugated antibody was used (1:1000; Dako). The immunoblots were detected by LumiVision PRO 400EX (Aisin Seiki, Aichi, Japan), and the densitometry analysis of the immunoreactive protein bands was performed using Lumi Vision Analyzer 2.0 software (Aisin Seiki).

Histopathological evaluation of testes

Ultrastructural study (transmission electron microscopy). Small pieces of the right testis were immediately fixed in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 C for 3 d and then fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) at 4 C for 90 min at room temperature. After dehydration in graded ethanol, the specimens were embedded in Quetol 812 mixture. Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined under a transmission electron microscope (Hitachi 7100).

Light microscopic study. Right testes were first fixed in Bouin's fluid and then placed in 10% formalin solution. Tissue samples were embedded in paraffin and cut at 5 μm thickness, stained with periodic acid Schiff's reagent, counterstained with hematoxylin, and then examined with a light microscope.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Paraffin-embedded sections of testis were used to determine whether *cis*-permethrin exposure induces apoptotic testicular cells. Apoptosis *in situ* Detection Kit was used as instructed by the manufacturer (Wako). This kit is based on the TUNEL procedure, which is the addition of fluorescein-dUTP to 3′-terminals of apoptotically fragmented DNA with terminal deoxynucleotidyl transferase followed by immunochemical detection using anti-fluorescein antibody conjugated with horseradish peroxidase and diaminobenzidine as a substrate.

Analysis of cis-permethrin residue levels in testis. A part of the left testis was homogenized with a 3-fold volume of 10 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose. The level of *cis*-permethrin was measured by the method of van der Hoff *et al.* (18).

Statistical analysis

Data are expressed as mean ± SD. Multiple comparisons were made between the exposure groups and the control group using Dunnett's test after one-way ANOVA. Because variables of LH values were not normally distributed, log transformation was performed before the statistical analyses. Linear regression analysis was performed with *cis*-permethrin testicular residues for log-transformed testicular testosterone levels. Statistical analysis was conducted the JMP version 5.0 (SAS Institute Inc., Cary, NC). P values < 0.05 were considered statistically significant.

Results

Body and reproductive organs weights

During the oral exposure to *cis*-permethrin for 6 wk, no sign of poisoning was found in the mice. Their body and reproductive organs weights are shown in Tables 2 and 3. Neither absolute nor relative weight of reproductive organs in the lower-dose group was significantly different from those of controls. The weights of the higher-dose group showed a trend toward reduction but not to a statistically significant degree.

Epididymal sperm count, motility, and morphology

Figure 1, A and B, shows the effects of *cis*-permethrin on sperm count and motility in the caudal epididymis of mice. Compared with the control group, *cis*-permethrin exposure reduced caudal epididymal sperm count and motility in a dose-dependent manner. However, no significant change was found in sperm morphology compared with the control.

Testicular testosterone production and plasma hormone levels

Intratesticular testosterone production and plasma testosterone concentrations were significantly reduced after *cis*-permethrin exposure in a dose-dependent manner (Fig. 1, C and D). Plasma LH levels increased dose-dependently in *cis*-permethrin-exposed mice (Fig. 1E). However, there was no significant effect of *cis*-permethrin on plasma FSH levels (Fig. 1F).

Real-time quantitative PCR analysis

Using real-time quantitative PCR, we examined the mRNA levels of expression for several genes associated with testicular cholesterol synthesis, transport, and testosterone biosynthesis.

Cholesterol synthesis. In testes, cholesterol is an essential substrate for testosterone production. Both the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase and HMG-CoA reductase involved in testicular cholesterol biosynthesis were measured. The expression of HMG-CoA syn-

TABLE 3. Effects of *cis*-permethrin exposure on relative organ weights in mice

Treatment	Dose (mg/kg·d)	n	Epididymis (g/body weight × 100)	Testes (g/body weight × 100)	Prostate + seminal vesicle (g/body weight × 100)
Control	Corn oil	8	0.250 ± 0.019	0.621 ± 0.088	0.870 ± 0.075
cis-Permethrin	35	8	0.246 ± 0.033	0.626 ± 0.084	0.803 ± 0.096
cis-Permethrin	70	8	0.245 ± 0.033	0.598 ± 0.097	0.805 ± 0.116

Data represent mean ± SD.

thase mRNA was reduced significantly by *cis*-permethrin exposure at a dose of 70 mg/kg (Fig. 2A), and *cis*-permethrin also down-regulated the HMG-CoA reductase mRNA expression level (Fig. 2B).

Cholesterol transport. With the exception of biosynthesis, cholesterol can also be provided by the uptake of both high-density lipoprotein and low-density lipoprotein (LDL) from blood. Scavenger receptor class B type 1 (SR-B1) and LDL receptor (LDL-R) are the high-density lipoprotein and LDL receptors, respectively. In *cis*-permethrin-exposed groups, SR-B1 and LDL-R mRNA levels tended to decrease but not to any statistically significant degree (Fig. 2, C and D). Peripheral benzodiazepine receptor (PBR) and StAR play a key regulatory role in cholesterol transport from the outer to the inner mitochondrial membrane. In the testes of mice exposed to high-dose *cis*-permethrin, PBR mRNA expression levels were markedly inhibited (Fig. 2E). Furthermore, mRNA levels for StAR were severely diminished in both doses of *cis*-permethrin-exposed groups (Fig. 2F).

Testosterone biosynthesis. Cytochrome P450 side-chain cleavage enzyme (P450scc) is responsible for the conversion of cholesterol to pregnenolone. We found that *cis*-permethrin treatment significantly suppressed P450scc mRNA expression in the testes (Fig. 2G). However, mRNA expression levels for other testicular enzymes involved in the testosterone biosynthesis pathway, including 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD), cytochrome P450 17α-hydroxylase/C_{17-20} lyase (P450 17α), and 17β-HSD, were not significantly altered by *cis*-permethrin exposure (Fig. 2, H, I, and J).

Western blot analysis

Protein expression levels of StAR, P450scc, PBR, P450 17α, and 17β-HSD were determined by Western blot analysis. The former three proteins, as representative, were selected because their mRNA expression levels were altered significantly, and the latter two proteins were selected representatively because alteration in mRNA expression levels was not significant.

The higher dose of *cis*-permethrin exposure significantly suppressed the protein expression levels of StAR and P450scc when compared with control (Fig 3). P450 17α protein levels were not significantly altered after the exposure (Fig 3). However, accurate quantification by Western blot analysis of PBR and 17β-HSD could not be obtained.

According to these results, it suggested that results of mRNA expression levels by real-time quantitative PCR analysis were concordant with the results of protein expression levels of these genes by Western blot, and this concordance may be applicable to all the investigated genes.

Histopathological findings of testis

Ultrastructural characteristics of mitochondria in Leydig cells. High magnification profiles of mitochondria in Leydig cells at *cis*-permethrin doses of 0, 35, and 70 mg/kg·d are shown in Fig. 4. Compared with the control mice (Fig. 4A), marked damage was observed in the high-dose-exposed animals (Fig. 4C). The inner membrane was disrupted and the cristae were replaced by the emergence of a denser matrix instead. Moreover, the outer

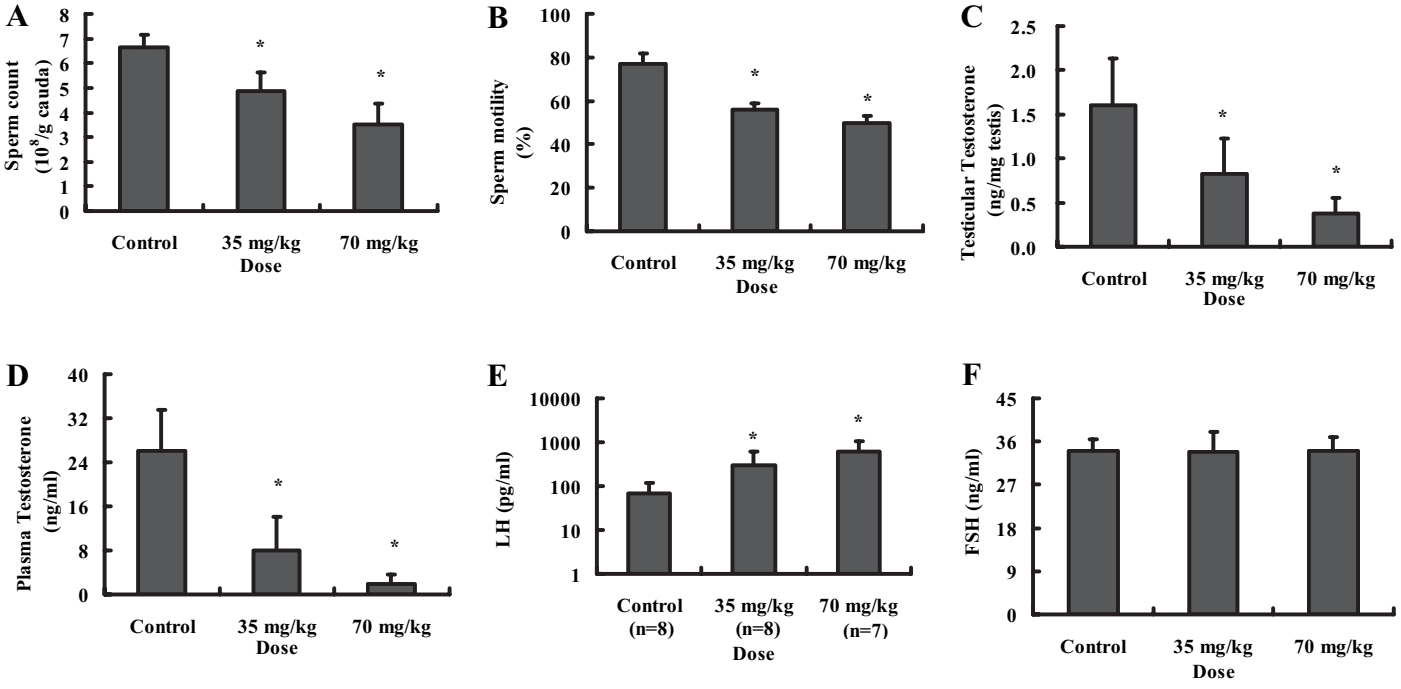

FIG. 1. Effects of oral *cis*-permethrin exposure on mice. A, Sperm count; B, sperm motility; C, testicular testosterone production; D, plasma testosterone concentrations; E, plasma LH concentrations; F, plasma FSH concentrations. The sample size was eight per group (n = 8) except pattern E. In pattern E, only in the 70-mg/kg group, one outlier was discarded based on Grubbs-Smirnov's test, and the sample size was seven and the mean and SD were calculated as n = 7. Values are expressed as mean ± SD. *, Significantly different from control group by Dunnett's test ($P < 0.05$).

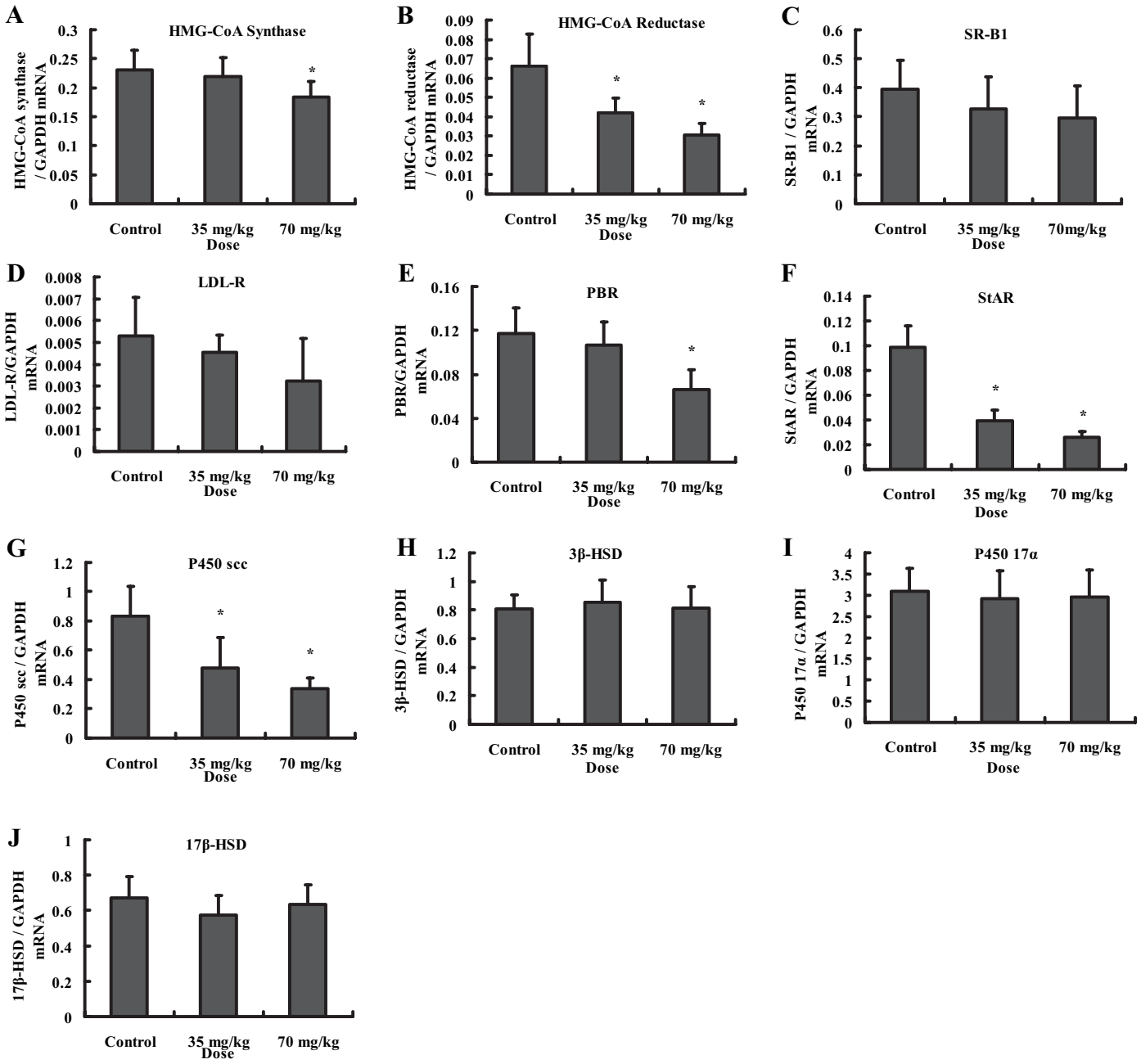

FIG. 2. Real-time quantitative PCR analysis of testicular mRNA levels for testosterone biosynthesis genes from control and *cis*-permethrin-exposed mice. A, HMG-CoA synthase; B, HMG-CoA reductase; C, SR-B1; D, LDL-R; E, PBR; F, StAR; G, P450scc; H, 3β-HSD; I, P450 17α; J, 17β-HSD. Values are expressed as mean ± SD. *, Significantly different from control group by Dunnett's test ($P < 0.05$).

membrane was very thin and sometimes fused with the inner membrane. In the 35-mg/kg·d-dose group, a part with mitochondria damage was observed (Fig. 4B).

Light microscopic. A few abnormal seminiferous tubules with vacuoles or lack of germ cells were found in exposed mice (Fig. 5B), but there were none in any of the controls. The control male mouse showed normal seminiferous tubule morphology with normal spermatogenesis (Fig. 5A).

TUNEL assay. No apoptotic testicular cells were observed in exposed groups and control group (data not shown). Hence, *cis*-permethrin exposure may not induce testicular cells apoptosis.

Relationship between testosterone levels and cis-permethrin residues in individual mice testes

The levels of *cis*-permethrin residues (Fig. 6A) in the testes were increased significantly and dose dependently, suggesting that the chemical dose-dependently induced testis damages in terms of testosterone production, sperm counts, and testis morphology. Regression analysis revealed that in individual mice testes, *cis*-permethrin residues were a signif-

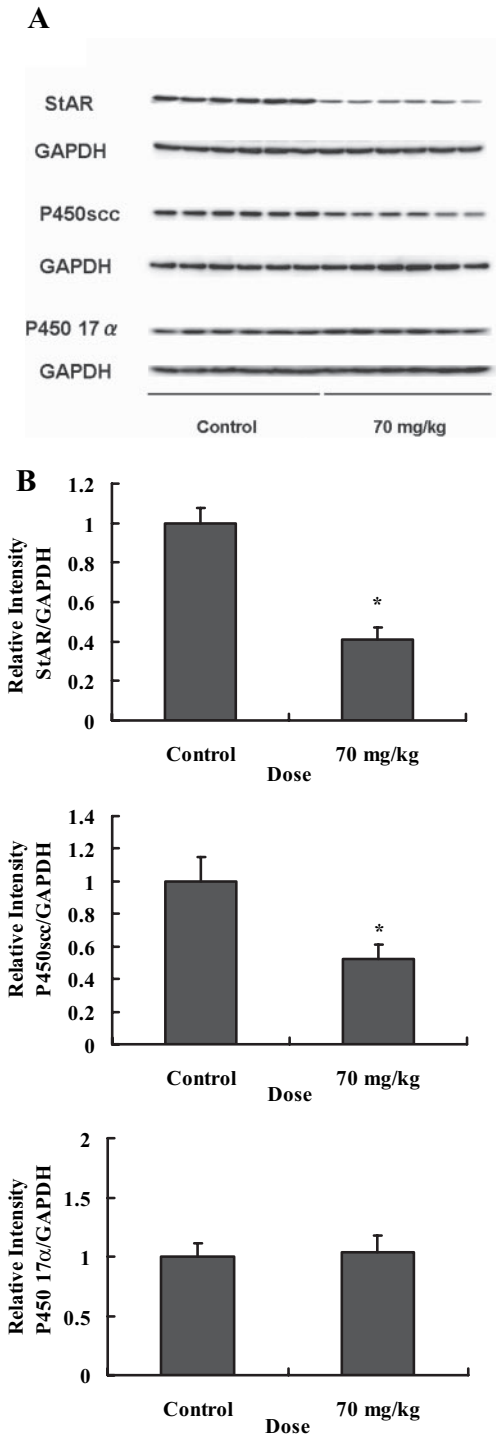

FIG. 3. Western blot analysis of StAR, P450scc, and P450 17α protein expression levels in the testes of control mice and higher-dose *cis*-permethrin-exposed mice. A, Testis lysates from six mice of control and 70-mg/kg·d exposure groups were prepared, and proteins were separated by 10% SDS-PAGE and identified by immunoblot detection of StAR, P450scc, and P450 17α, respectively. Blots were reprobed for GAPDH as an internal loading control. Each band obtained by immunoblot analysis was quantified by densitometric analysis. B, Histogram showing the relative densitometric ratio of StAR, P450scc, and P450 17α to GAPDH, respectively. Results shown are means ± SD of six independent mice. *, Significantly different from control group by Dunnett's test ($P < 0.05$).

icant determinant of testosterone levels ($R^2 = 0.8811$), and it indicated that testicular testosterone levels were decreased with an increase in *cis*-permethrin residues in testes (Fig. 6B).

Discussion

Our present study demonstrated that 6-wk oral administration of *cis*-permethrin to adult male ICR mice had adverse effects on their reproductive system. The *cis*-permethrin exposure significantly reduced epididymal sperm counts, motility, testicular testosterone production, and plasma testosterone levels in a dose-dependent manner. However, plasma LH levels of exposed mice elevated dose dependently, suggesting that secretion of LH from the pituitary was increased in response to decreased testosterone by the normal negative feedback of the hypothalamus-pituitary axis. Additionally, a significant linear regression was observed between testosterone levels and *cis*-permethrin residues in individual mice testes. The reduced testosterone might be responsible for the decreased sperm counts and motility and also morphological abnormality of testis in *cis*-permethrin-treated mice. Similar results were reported in male rats exposed to other synthetic pyrethroids such as cypermethrin (19) and fenvalerate (11). The molecular mechanism below, by which testicular testosterone biosynthesis was reduced by *cis*-permethrin, has been suggested.

Upon LH stimulation, steroidogenesis in the testis begins with the transfer of free cholesterol from intracellular stores into mitochondria (20). PBR and StAR are likely to play a critical role in cholesterol transport (20, 21). PBR is primarily localized in the outer mitochondrial membrane and is particularly abundant in steroidogenic cells (20, 22). Papadopoulos *et al.* (23) demonstrated that a targeted disruption of the PBR gene in R2C Leydig tumor cells (PBR-null cell line) arrested cholesterol transport into mitochondria and dramatically reduced steroid production by 95% compared with wild-type R2C cells, whereas the reintroduction of PBR into these cells rescued steroidogenesis. They also found that mitochondria from PBR-negative cells failed to produce pregnenolone even when supplied with exogenous cholesterol. These results all suggest the important role of PBR in steroid production. In our study, the higher dose of *cis*-permethrin suppressed PBR mRNA expression significantly. In the lower-dose group, PBR mRNA expression levels appeared to be lower than in the control group but not to a statistically significant degree.

StAR is expressed in steroidogenic tissues (21) and acts exclusively on the outer mitochondrial membrane, working in concert with PBR to regulate cholesterol transport (24). StAR-null mice fail to grow normally and die from adrenocortical insufficiency soon after birth. Histologically, the testes of StAR knockout embryos contain lipid deposits denoting impaired cholesterol delivery into the mitochondria, suggesting the essential role of this protein in regulating steroid biosynthesis (25). We observed a marked decrease in mRNA expression of StAR after *cis*-permethrin exposure.

Once cholesterol is transferred to the inner mitochondrial membrane by PBR and StAR, it is converted to pregnenolone, which is catalyzed by P450scc present in the inner mitochondrial membrane (26, 27). In the testis, it is found in the Leydig

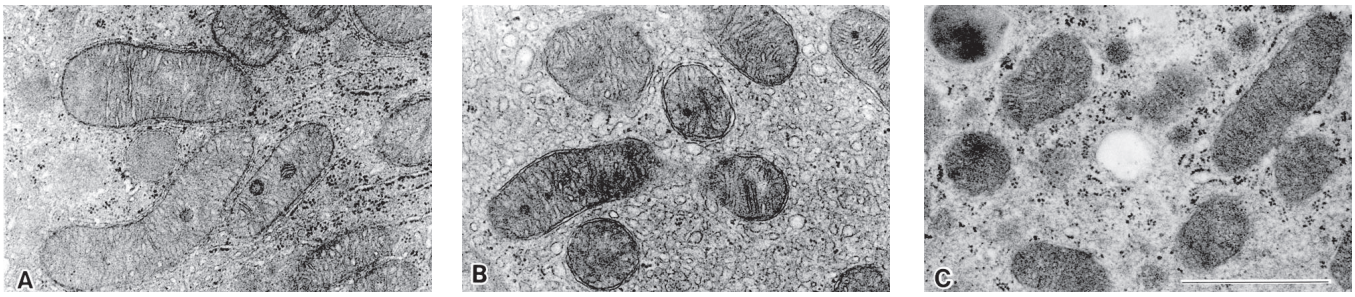

FIG. 4. Electron micrographs of Leydig cells in mouse right testis (×40,000). A, Control mouse; B, 35 mg/kg·d *cis*-permethrin-treated mouse; C, 70 mg/kg·d *cis*-permethrin-treated mouse showing mitochondrial damage with inner membrane disruption and loss of cristae. Three figures are at same magnification. *Bar*, 1 μm.

cells (28), where P450scc initiates the first enzymatic step in steroidogenesis. Our results indicated that the testicular mRNA expression of P450scc was significantly decreased in both *cis*-permethrin-exposed groups. Furthermore, the P450scc protein expression levels were also suppressed significantly by *cis*-permethrin exposure.

Pregnenolone leaves the mitochondrion for the smooth endoplasmic reticulum where it is converted to progesterone by 3β-HSD. Pregnenolone is catalyzed by P450 17α to produce 17-hydroxyprogesterone and androstenedione, with the latter then being converted to testosterone by 17β-HSD (29). However, *cis*-permethrin did not affect these processes.

Because PBR, StAR, and P450scc are in mitochondria, we evaluated the structure of mitochondria in the Leydig cells of testis. In accordance with the expression of these genes, the electron microscopy pictures indicated that exposure to the higher dose of *cis*-permethrin induced mitochondrial membrane impairment and the lower dose slightly induced the impairment. Taken together, these findings suggest that damage of mitochondrial membrane by *cis*-permethrin exposure might disrupt mRNA expression levels of PBR, StAR, and P450scc and protein expression levels of StAR and P450scc, resulting in reduction of cholesterol transport and conversion. In line with our results, the peroxisome proliferator perfluorodecanoic acid (PFDA) was found to inhibit steroidogenesis in the Leydig cells by affecting PBR mRNA stability and the resulting suppression of PBR expression, reducing cholesterol transport into the mitochondria as well as subsequent steroid formation (30). It was reported that an herbicide (Roundup) significantly reduced steroidogenesis by disrupting StAR protein expression, indicating that StAR might be another important target for environmental pollutants disrupting steroidogenesis and impairing the reproduction function (31). Moreover, 2,3,7,8-tetracholorodibenzo-*p*-dioxin (TCDD) was observed to suppress human chorionic gonadotropin-induced progesterone and testosterone secretions in rat Leydig cells due to the reduced expression of P450scc mRNA (32). Exposure to di(*n*-butyl) phthalate (DBP) *in utero* altered steroidogenesis with a resultant reduction in testosterone production in fetal rat testis by decreasing the expression of some genes including StAR and P450scc (33).

Aside from the testosterone biosynthesis pathway, we searched for other possible factors involved in the decreased testosterone production. Leydig cells have the capacity to synthesize testosterone from free cholesterol. Cholesterol is a necessary precursor to steroidogenesis. However, our results showed that *cis*-permethrin seemed not to affect the SR-B1- and LDL-R-related uptake of lipoprotein-derived cholesterol esters for the purpose of testosterone production synthesis in the testes.

Additionally, cholesterol could also be synthesized *de novo* for use in steroid biosynthesis. HMG-CoA synthase mRNA expression levels were decreased at a higher dose of *cis*-permethrin. Because it resides in cytosol, *cis*-permethrin might affect not only the mitochondrial membranes but also the cytosol. Although the effect of *cis*-permethrin on cytosol could not be negated, it was likely not to impact testosterone biosynthesis greatly. HMG-CoA reductase mRNA expression levels were significantly reduced after exposure. It was located in the Leydig cells of testis but was absent from Sertoli cells (34). Thus, we deduced that *cis*-permethrin might partly reduce cholesterol in Leydig cells. However, the impact of decreased HMG-CoA synthase and HMG-CoA reductase on testicular cholesterol synthesis is limited, because mRNA expression levels of SR-B1 and LDL-R, which take up cholesterol esters from blood into the cholesterol pool in Leydig cells, were not markedly reduced. In clinical studies, Travia *et al.* (35) indicated that sustained therapy with simvastatin and pravastatin, HMG-CoA reductase inhibitors,

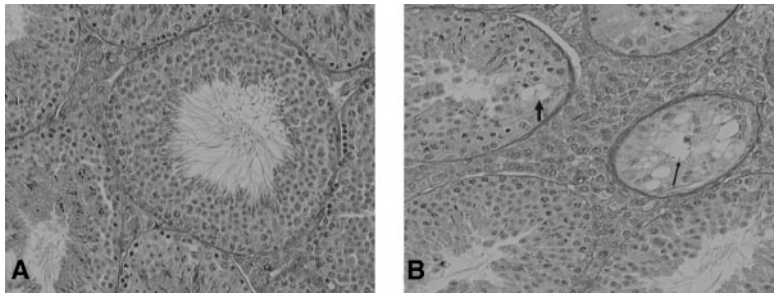

FIG. 5. Light microscopic findings of testis (×200 magnification). A, Control, with no abnormality found; B, 70-mg/kg·d exposure group. *Thicker arrow* shows vacuoles, and *thinner arrow* indicates the lack of germ cells in seminiferous tubule.

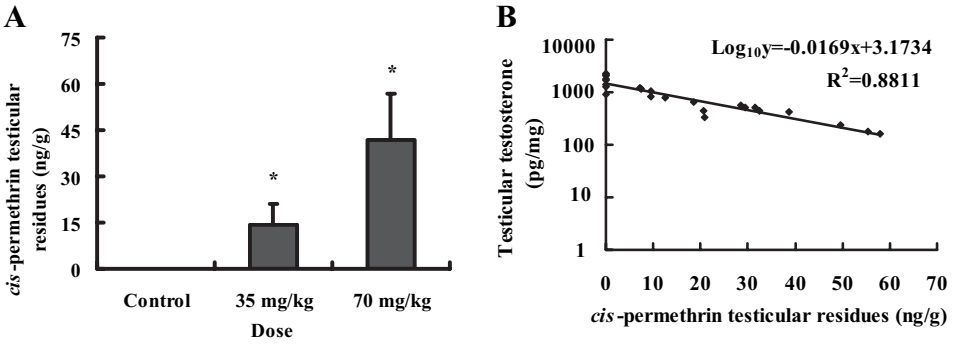

FIG. 6. Relationship between testosterone levels and *cis*-permethrin residues in individual mice testes (n = 24). A, Levels of *cis*-permethrin testicular residues. Values are expressed as mean ± SD. *, Significantly different from control group by Dunnett's test ($P < 0.05$). B, regression analysis of *cis*-permethrin residues for testosterone levels in individual mice testes.

had no negative impact even at maximum therapeutic doses on adrenocortical and testicular steroidogenesis in male patients with hypercholesterolemia, although their serum cholesterol was lowered.

Taken together, PBR, StAR, and P450scc were considered to be the major factors involved in the testosterone biosynthesis pathway. Therefore, our results suggest that decreased mRNA expression levels of PBR, StAR, and P450scc and protein expression levels of StAR and P450scc, in the absence of protein expression levels for PBR because we could not obtain accurate quantification by Western blot analysis of PBR, are the main factors causing steroidogenesis disruption and testosterone reduction, although HMG-CoA synthase and HMG-CoA reductase mRNA expression levels were suppressed.

Additionally, by TUNEL assay, *cis*-permethrin may not induce testicular cell apoptosis in line with the result of Abu-Qare and Abou-Donia (36). They reported that DEET (*N*,*N*-diethyl-*m*-toluamide) caused significant increase in the rats' urinary excretion of 8-hydroxy-2-deoxyguanosine (8-OHdG) when applied alone or in combination with permethrin; however, permethrin did not cause significant increase in the amount of 8-OHdG in the urine. Induction of 8-OHdG is a biomarker of apoptosis (37). Abou-Donia *et al.* (38) found that administration of a combination of three chemicals, pyridostigmine bromide, DEET, and permethrin, induced rat germ-cell apoptosis, whereas in our experiment, mice were exposed to permethrin only, and no apoptosis was found in testis. We think the combination of chemicals may have synergistic and/or additive effects on animals, but the single exposure to permethrin may not evoke testicular cells apoptosis.

To our knowledge, this is the first report to demonstrate that *cis*-permethrin exposure mainly induced Leydig cell mitochondrial membrane damage and disrupted the limiting steps of the steroidogenic process, suppressing mRNA expression levels of PBR, StAR, and P450scc and protein expression levels of StAR and P450scc so as to reduce adult mouse testicular testosterone biosynthesis. And the effect of *cis*-permethrin on Leydig cell gene expression and steroidogenesis may be indirect due to the direct effect of mitochondrial damage. It does so by inhibiting the delivery of cholesterol into the mitochondria and diminishing the subsequent conversion of cholesterol to pregnenolone. The resultant decrease in testosterone leads to adverse effects on the development of mouse reproductive organs and to a reduction in epididymal sperm count and motility and morphological abnormality of testis. The present study did not obtain the no-observed-adverse-effect level of *cis*-permethrin. The dose of human occupational exposure was roughly estimated to be 0.12 mg/kg (unpublished data), about 100 times lower levels than the exposed mice on a weight basis, and hence nonoccupationally exposed human population exposures would be even much lower. In conclusion, it should be emphasized that the insecticide permethrin might induce testicular toxicity as well as the better-known neurotoxicity.

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