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ORGAN TOXICITY AND MECHANISMS

Fenvalerate induces germ cell apoptosis in mouse testes through the Fas/FasL signaling pathway

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Abstract Fenvalerate has a potentially adverse effect on male reproduction and spermatogenesis, whereas the precise mechanism remains obscure. The present study investigated the effects of fenvalerate on germ cell apoptosis in testes. Adult male mice were administered with fenvalerate (15 or 60 mg/kg) by gavage for 28 days. Germ cell apoptosis was determined by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL). The number of TUNEL+ germ cells per tubule and the percentage of tubules with TUNEL+ germ cells were significantly increased in testes of mice treated with fenvalerate in a dose-dependent manner. TUNEL+ germ cells were observed mainly in stages VII–VIII and also stages IX–XII seminiferous tubules in testes. Additional experiments showed that fenvalerate increased the level of active caspase-8 and caspase-3 in testes. In addition, fenvalerate upregulated the expression of Fas and FasL in testes. No significant difference on the expression of Bcl-2 and Bax in testes was observed between fenvalerate-treated mice and controls. Fenvalerate did not affect the leakage of cytochrome c from mitochondria into cytoplasm. In addition, fenvalerate did not cause the activation of caspase-9 in testes. Taken together, these results suggest that fenvalerate induces germ cell apoptosis in testes through the Fas/FasL signaling pathway.

Keywords Fenvalerate · Testis · Germ cells · Apoptosis · Fas/FasL pathway

Introduction

Fenvalerate [(R, S)-α-cyano-3-phenoxybenzyl (R, S)-2-(4-chlorophenyl)-3-methylbutyric ester] is a commonly used synthetic pyrethroid insecticide. Fenvalerate is a developmental toxicant. An earlier study showed that perinatal fenvalerate exposure during the critical periods of male brain sexual differentiation in rat significantly decreased sexual behavior and increased immobility in the open field (Moniz et al. 1999). Our recent study demonstrated that prenatal fenvalerate exposure irreversibly impairs testicular development in male offspring (Zhang et al. 2010a). On the other hand, fenvalerate is also a reproductive toxicant and endocrine disruptor. According to an epidemiological investigation, occupational exposure to fenvalerate was associated with the poor semen quality (Tan et al. 2006). 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, a significant increase in sperm DNA fragmentation was observed among workers exposed to fenvalerate (Bian et al. 2004). Animal experiment demonstrated that a significant reduction in ductus deferens and seminal vesicle weights, and plasma testosterone was observed in male offspring of rats exposed to fenvalerate (Moniz et al. 1999). Recently, we found that lactational and pubertal fenvalerate exposure impaired testicular development and spermatogenesis (Zhang et al. 2009, 2010b). However, little remains known about the mechanism of fenvalerate-induced impairments in spermatogenesis.

Xian-Feng Zhao and Qun Wang contributed equally to this work.

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In the seminiferous tubules, apoptosis is a normal feature of spermatogenic lineages and testicular homeostasis. During various stages of mammalian spermatogenesis, germ cell apoptosis occurs to remove abnormal spermatogenic cells (Print and Loveland 2000; Rodriguez et al. 1997; Russell et al. 2002). On the other hand, some harmful factors, such as hormone deprivation, heat, and radiation exposure, aggravate germ cell apoptosis in testes (Vera et al. 2006; Hikim et al. 2003; Embree-Ku et al. 2002). In addition, some of testicular toxicants and endocrine disruptors have been demonstrated to induce germ cell apoptosis. According to a recent report, mono-(2-ethylhexyl) phthalate, a well-known endocrine disruptor, significantly reduced the number of germ cells by increasing apoptosis without change in testosterone synthesis in human fetal testes (Lambrot et al. 2009). Another study showed that adult diethylstilbestrol exposure induced spermatogenic cell apoptosis in male hamster (Ma et al. 2008). A recent study found that bisphenol A exposure during puberty significantly upregulated the expression of Fas and FasL in testes and increased the incidence of germ cell apoptosis (Li et al. 2009). Excess germ cell apoptosis may disrupt normal testicular development and impair spermatogenesis (Francavilla et al. 2000; Sawhney et al. 2005). Thus, it is especially interesting whether fenvalerate induces germ cell apoptosis in testes. In the present study, we aimed to investigate the effects of adult fenvalerate exposure on germ cell apoptosis in testes. We found that adult fenvalerate exposure induces germ cell apoptosis in testes through the Fas/FasL signaling pathway.

Materials and methods

Chemicals and reagents

Fenvalerate (purity >97%) was purchased from Sigma Chemical Co. (St. Louis, MO). Caspase-3, Fas, FasL, caspase-8, Bcl-2, Bax, cytochrome c, and caspase-9 antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). β-Actin antibody was from Boster Bio-Technology Co. LTD (Wuhan, China). All other reagents were from Sigma or as indicated in the specified methods.

Animals and treatments

Adult male CD-1 mice (8 weeks old, 28–32 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. Mice were administered with different doses of fenvalerate (15 or 60 mg/kg) by gavage for 28 days. The corn oil–treated male mice served as controls. At 24 h after the last treatment with fenvalerate, the testes were excised, dissected, weighted, and then divided into two parts: left one was kept at −80°C for subsequent Western Blot. The other part of the testes was immersed in modified Davidson's fluid (mDF) for 12–24 h for testicular histology and apoptosis analysis (Latendresse et al. 2002). 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.

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) according to the manufacturer's protocols. To assess apoptosis in testicular cells, 200 different seminiferous tubules were observed in predetermined different fields in each section at a magnification of 400×. A histogram of the number of TUNEL-positive germ cells per seminiferous tubule and the percentages of the number of seminiferous tubules containing TUNEL-positive germ cells was analyzed.

Subcellular fractionation and Western blotting

Cytosolic and mitochondrial fractions were prepared as described previously (Vera et al. 2004; Johnson et al. 2008). Briefly, saline-perfused testes were homogenized using a Dounce homogenizer in 3 ml buffer A (0.25 M sucrose, 50 mM Hepes, 10 mM NaCl, 10 mM EDTA, 2 mM dithiothreitol) supplemented with protease inhibitors (Complete Protease Inhibitors; Roche, Indianapolis, IN). The crude homogenates were centrifuged at 1,000×g for 10 min at 4°C and the resultant supernatant centrifuged at 10,000×g for 15 min at 4°C to sediment the low-speed fraction containing mainly mitochondria. The mitochondria were washed two times in buffer A and pelleted. The cytosolic and high-speed fractions were isolated following centrifugation of the 10,000×g supernatant fraction at 100,000×g for 60 min at 4°C. The resulting supernatant was the cytosolic fraction.

Western blotting was performed using testicular lysates and subcellular fractions. In brief, protein extracts 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 10–15% 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 incubated for 2 h with the following antibodies: caspase-3, Fas, FasL, caspase-8, Bcl-2, Bax, cytochrome c, and caspase-9. β-Actin was used as a loading control for total proteins. After washing 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 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).

Testicular histology

Two cross sections from each testis were embedded in parafilm using standard procedures performed by Pathological Lab at Anhui Medical University. Paraffin-embedded tissues were serially sectioned. At least two nonserial sections were stained with hematoxylin and eosin (H&E) using standard procedures for morphological analyses.

Statistical analysis

For western blotting, developed films were scanned and band intensities were analyzed using the public domain NIH Scion Image Program. Fas, FasL, Bcl-2, and Bax 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. At each point, $P < 0.05$ was considered statistically significant. ANOVA and the Student-Newmann–Keuls post hoc test were used to determine the differences between different groups.

Results

Effects of fenvalerate exposure on testis weights

The effects of adult fenvalerate exposure on testis weights were shown in Fig. 1. Results showed that fenvalerate exposure significantly reduced the absolute testis weight (Fig. 1A). In addition, the relative testis weight was significantly decreased in mice treated with 15 and 60 mg/kg of fenvalerate when compared with controls (Fig. 1B).

Effects of fenvalerate exposure on testicular histology

The effects of adult fenvalerate exposure on testicular histology are shown in Fig. 2. The morphology of testes in control mice was normal (Fig. 2A, a). Adult fenvalerate exposure markedly increased the inside diameter of seminiferous tubules and disturbed the array of spermatogenic cells in testicular sections (Fig. 2b, c). Interestingly, pathological damage was observed mainly in stages VII–VIII seminiferous tubules (Fig. 2b, c) and also stages IX–XII seminiferous tubules in testes. No pathological damage was observed in stages I–VI seminiferous tubules of testes in fenvalerate-treated mice (Fig. 2B, C).

Fenvalerate-induced germ cell apoptosis in testes

The effects of fenvalerate exposure on germ cell apoptosis in mouse testes were presented in Fig. 3A–C and Table 1. TUNEL+ germ cells were observed mainly in stages VII–VIII seminiferous tubules and also stages IX–XII seminiferous tubules in testes of mice treated with fenvalerate (Table 1). As shown in Fig. 3D, the number of TUNEL+ germ cells per tubule was significantly increased in a dose-dependent manner. In addition, the percentage of tubules with TUNEL+ germ cells was significantly increased in fenvalerate-treated mice when compared with controls (Fig. 3E). The effects of adult fenvalerate exposure on procaspase-3 and active caspase-3 in mouse testes were presented in Fig. 4. Results showed that the expression of testicular procaspase-3 was significantly upregulated in mice treated with 60 mg/kg of fenvalerate. In addition, fenvalerate exposure significantly increased the level of active caspase-3 in mouse testes.

Effects of fenvalerate exposure on Fas/FasL signaling pathway

The effects of fenvalerate exposure on Fas/FasL pathway in mouse testes were analyzed. Western blot showed that fenvalerate exposure not only upregulated testicular Fas and FasL in a dose-dependent manner (Fig. 5A–C), but also the level of active caspase-8 was significantly increased in testes of mice treated with fenvalerate (Fig. 5D).

Effects of fenvalerate exposure on mitochondrial pathway

Firstly, proapoptotic factors (Bax) and antiapoptotic proteins (Bcl-2) of the Bcl-2 family in testicular tissue were analyzed. Results showed that no significant difference on the expression of testicular Bcl-2 and Bax in whole homogenates was observed between fenvalerate-treated mice and controls (Fig. 6A). In addition, fenvalerate exposure did not affect the distribution of Bax in mitochondria and cytoplasm (Fig. 6B, C). Release of cytochrome c from mitochondria and subsequent activation of caspase-9 represent a key step in the mitochondrion-dependent apoptotic pathway. To investigate whether

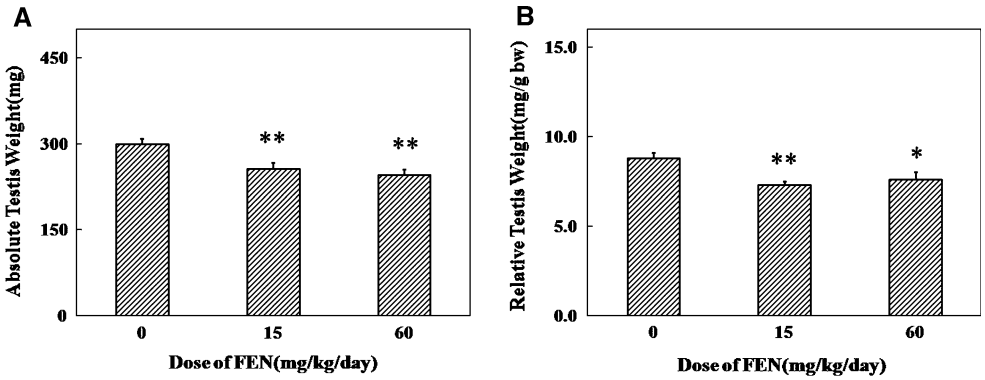

Fig. 1 Effects of fenvalerate exposure on testis weight. Male mice were administered with fenvalerate (15 or 60 mg/kg) by gavage for 28 days. Testes were weighed at 24 h after the last treatment with fenvalerate. **A** Absolute testis weight; **B** relative testis weight. Data were expressed as means ± SEM of 12 mice. $*P < 0.05$, $**P < 0.01$ when compared with controls

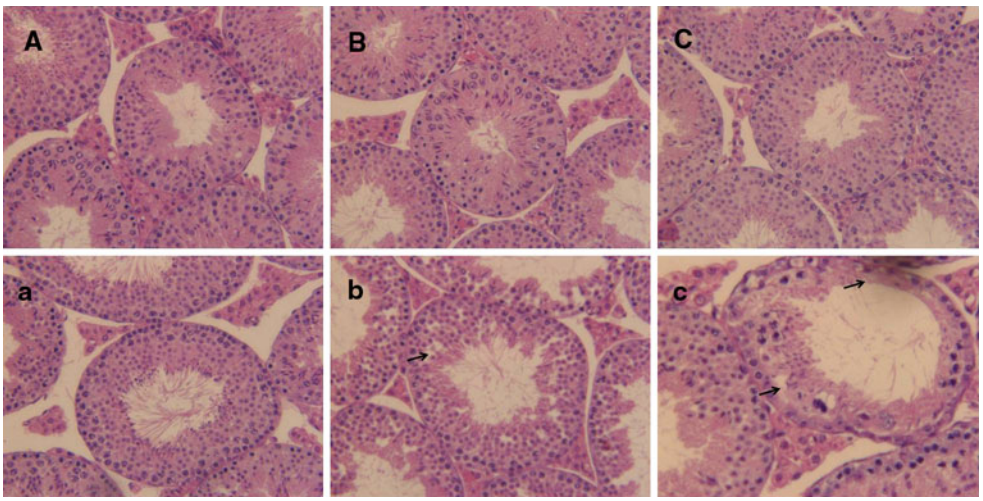

Fig. 2 Effects of fenvalerate exposure on testicular histology. Male mice were administered with fenvalerate (15 or 60 mg/kg) by gavage for 28 days. Testes were collected at 24 h after the last treatment with fenvalerate. Testicular cross sections from controls and fenvalerate-treated mice were stained with H&E at a magnification of ×200 (**A**–**C**), ×400 (**a**–**c**). **A** and **a** Control; **B** and **b** 15 mg/kg of fenvalerate treatment; **C** and **c** 60 mg/kg of fenvalerate treatment. **A**–**C** show stages IX–XII or I–VI seminiferous tubules of testes, **a**–**c** display stages VII–VIII seminiferous tubules of testes.

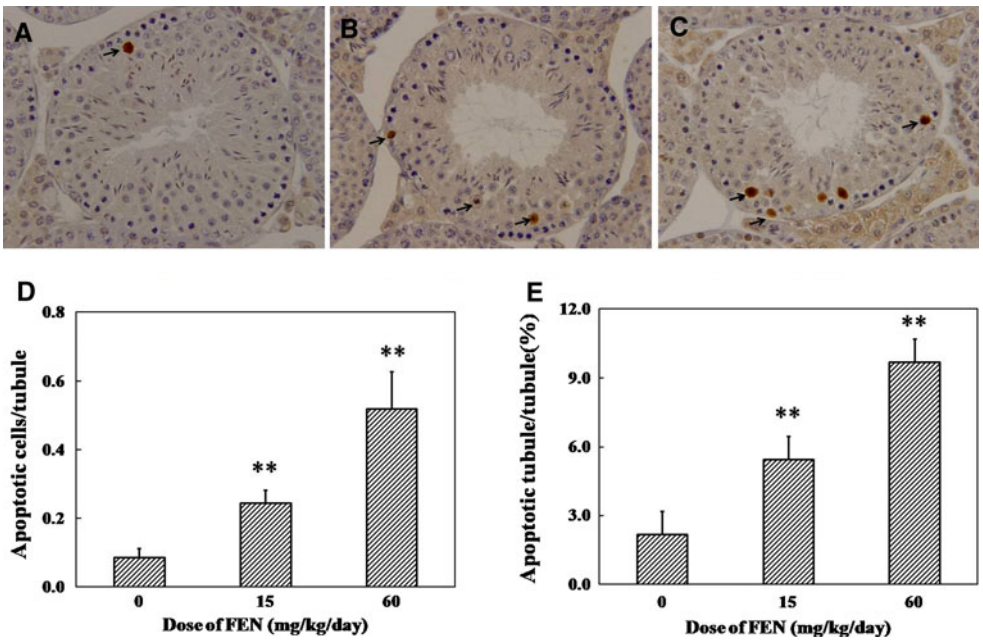

Fig. 3 Effects of fenvalerate exposure on germ cell apoptosis in testes. Male mice were administered with fenvalerate (15 or 60 mg/kg) by gavage for 28 days. Testes were collected at 24 h after the last treatment with fenvalerate. Germ cell apoptosis was detected by TUNEL staining. Testis sections from **A** control, **B** 15 mg/kg fenvalerate, and **C** 60 mg/kg fenvalerate-treated mice. *Arrows* showed TUNEL-positive cells. **D** The number of TUNEL+ germ cells per seminiferous tubule. **E** The percentages of seminiferous tubule with TUNEL+ germ cells. Data were expressed as means ± SEM of 12 samples from 12 male mice. $**P < 0.01$ when compared with controls

Table 1 Distribution of TUNEL+ seminiferous tubules in different stages

Dose (mg/kg/day)	The number of TUNEL+ seminiferous tubules in different stages (means ± SE, $n = 6$)		
	I–VI	VII–VIII	IX–XII
0	2.4 ± 0.6	2.0 ± 1.0	2.0 ± 0.8
15	2.5 ± 1.6	8.0 ± 1.3**	3.5 ± 1.0
60	4.2 ± 0.7	12.4 ± 1.9**	8.0 ± 2.3*

* $P < 0.05$, ** $P < 0.01$ when compared with controls

fenvalerate exposure induces germ cell apoptosis through mitochondrial pathway, mitochondrial and cytosolic cytochrome c abundance and cleaved caspase-9 level were analyzed. As shown in Fig. 6B, C, no significant difference on the level of cytosolic and mitochondrial cytochrome c was observed between fenvalerate-treated mice and controls. In addition, fenvalerate exposure had no effect on the level of cleaved caspase-9 in mouse testes (Fig. 6D).

Discussion

Fenvalerate has a potentially adverse effect on spermatogenesis in human being and rodent animals. Several studies showed that adult fenvalerate exposure markedly reduced the number of spermatozoa in rats (El-Demerdash et al. 2004; Arena et al. 2008). In the present study, we found that the absolute testis weights and the relative testis weights were significantly decreased in mice treated with fenvalerate. In addition, adult fenvalerate exposure markedly decreased the layers of spermatogenic cells, increased the inside diameter of seminiferous tubules, and disturbed the array of spermatogenic cells, whereas no large vacuoles or complete spermatogenic failure were observed in testes of mice treated with fenvalerate. These results were in disagreement with our earlier study, in which abnormal seminiferous tubules with large vacuoles or complete spermatogenic failure were observed in testes of mice whose mothers were exposed to fenvalerate during lactation (Zhang et al. 2009).

According to an earlier report, administration of deltamethrin, another pyrethroid insecticide, to adult rats resulted in the incidence of germ cell apoptosis in testes (El-Gohary et al. 1999). A recent study by our laboratory showed that lactational fenvalerate exposure markedly increased the number of apoptotic cells in testes of pups at weaning (Zhang et al. 2009). In the present study, we investigated the effects of adult fenvalerate exposure on germ cell apoptosis in testes. We found that the number of

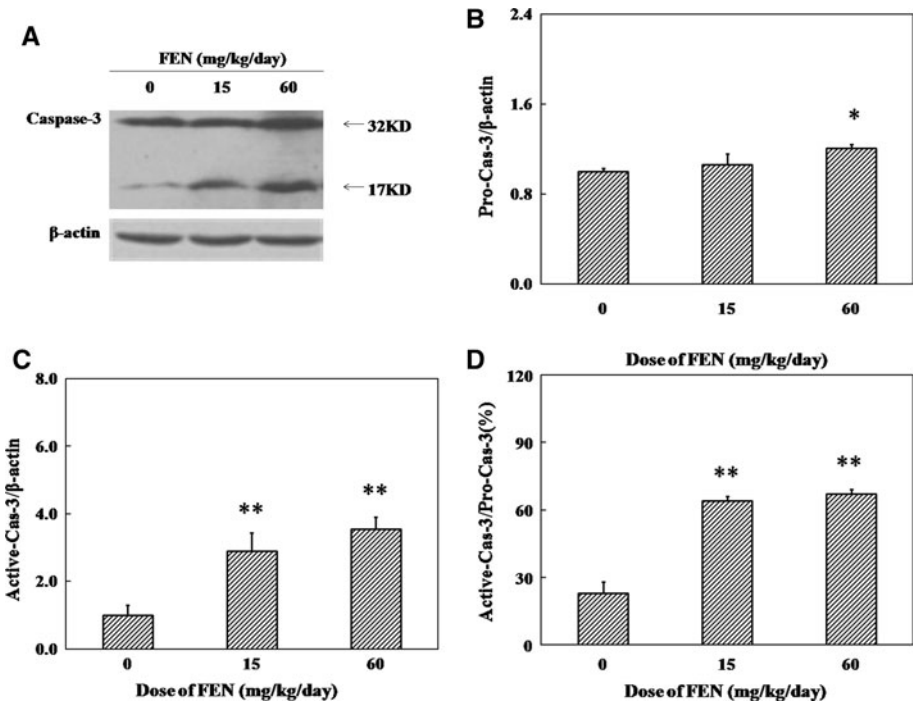

Fig. 4 Effects of fenvalerate exposure on caspase-3 in testes. Male mice were administered with fenvalerate (15 or 60 mg/kg) by gavage for 28 days. Testes were collected at 24 h after the last treatment with fenvalerate. **A** The levels of procaspase-3 and active caspase-3 in testes were measured using Western blot. **B** Quantitative analysis of scanning densitometry was performed. Procaspase-3 was normalized to β-actin level in the same samples. **C** Active caspase-3 was normalized to β-actin level in the same samples. **D** Active caspase-3 was normalized to procaspase-3 level in the same samples. The densitometry unit of the control was assigned as one. All data were expressed as means ± SEM of six samples from six different mice. *$P < 0.05$ when compared with controls

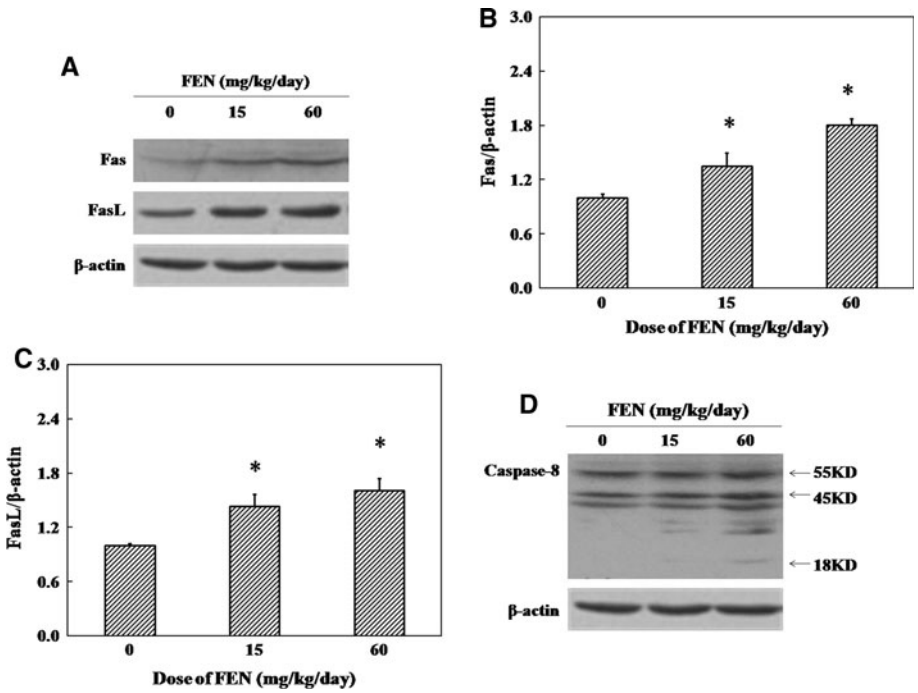

Fig. 5 Effects of fenvalerate exposure on Fas/FasL signaling pathway. Male mice were administered with fenvalerate (15 or 60 mg/kg) by gavage for 28 days. Testes were collected at 24 h after the last treatment with fenvalerate. **A** The protein expression of Fas and FasL in testes was measured using Western blot. Quantitative analysis of scanning densitometry was performed for Fas **B** and FasL **C**. Fas and FasL were normalized to β-actin level in the same samples. The densitometry unit of the control was assigned as one. **D** The level of procaspase-8 and active caspase-8 were measured using Western blot. All data were expressed as means ± SEM of six samples from six different mice. $*P < 0.05$ when compared with controls

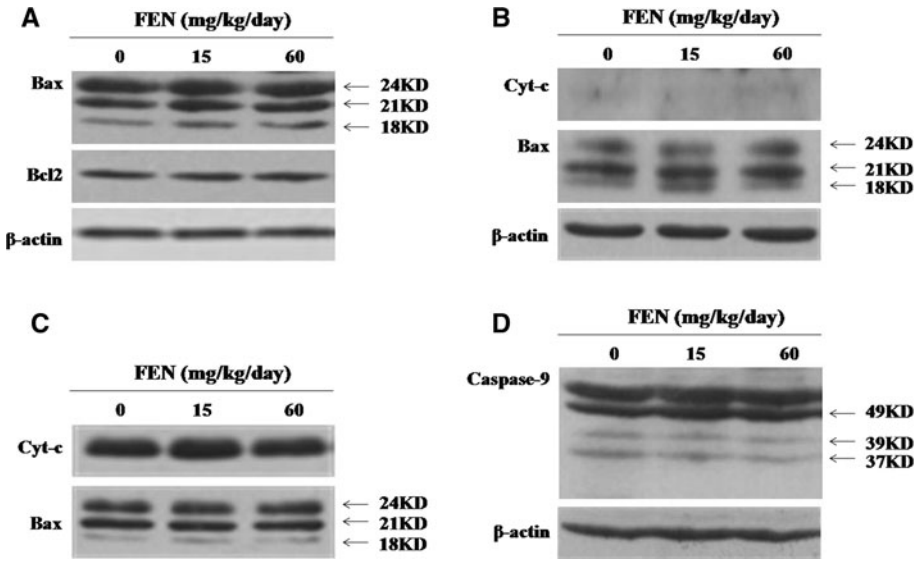

Fig. 6 Effects of fenvalerate exposure on mitochondrial signaling pathway. Male mice were administered with fenvalerate (15 or 60 mg/kg) by gavage for 28 days. Testes were collected at 24 h after the last treatment with fenvalerate. The protein expression of Bcl-2, Bax, cytochrome c, and caspase-9 in testes was measured using Western blot. **A** Bcl-2 and Bax in whole homogenates. **B** Mitochondrial cytochrome c and Bax. **C** Cytosolic cytochrome c and Bax. **D** Procaspase-9 and active caspase-9 in testes

apoptotic germ cells per tubule was significantly increased in a dose-dependent manner. In addition, the percentage of tubules with apoptotic germ cells was significantly increased in fenvalerate-treated mice when compared with controls. Interestingly, apoptotic germ cells were observed mainly in stages VII–VIII and also stages IX–XII seminiferous tubules in testes. These results are in agreement with the pathological observation in testes, in which pathological damage was observed mainly in stages VII–VIII seminiferous tubules and also stages IX–XII seminiferous tubules in testes. These results suggest that fenvalerate impairs spermatogenesis possibly through the mechanism of increased germ cell apoptosis in testes.

The signaling events leading to apoptosis can be divided into two major pathways, either Fas/FasL (extrinsic) or mitochondrial (intrinsic) pathway. Fas/FasL system has been shown to be the major inducer of germ cell apoptosis under particular pathological conditions. Several studies demonstrated that Fas/FasL signaling pathway was involved in the process of germ cell apoptosis following hormone deprivation, heat, and radiation exposure (Pareek et al. 2007; Miura et al. 2002; Embree-Ku et al. 2002). According to several earlier reports, di-ethyl-2-hexyl phthalate (DEHP) and its toxic metabolite mono-(2-ethylhexyl) phthalate (MEHP), the most famous endocrine disruptors, caused Sertoli cell damage and subsequent germ cell apoptosis through Fas/

FasL pathway (Richburg et al. 1999; Ichimura et al. 2003). Recent studies showed that other endocrine disruptors, such as lindane, p,p'-DDE, and bisphenol A, have also been found to upregulate the expression of testicular Fas and FasL and resulted in germ cell apoptosis in testes (Li et al. 2009; Shi et al. 2010; Saradha et al. 2009). To investigate whether Fas/FasL signaling pathway is involved in the process of fenvalerate-induced germ cell apoptosis, the present study measured the expression of Fas and FasL in testes of mice treated with fenvalerate. We found that fenvalerate exposure upregulated the expression of testicular Fas and FasL in a dose-dependent manner. Correspondingly, the level of active caspase-8 was significantly increased in testes of mice treated with fenvalerate. These results suggest that fenvalerate induces germ cell apoptosis possibly through the mechanism of Fas/FasL signaling pathway.

It has been demonstrated that mitochondrial signaling pathway is also involved in the process of germ cell apoptosis in testes. Several earlier studies found that mitochondrial signaling pathway is the key apoptotic pathway for heat-induced germ cell apoptosis in testes (Hikim et al. 2003; Vera et al. 2004). Recent studies showed that several reproductive toxicants, such as doxorubicin, methoxychlor, and lindane, induced germ cell apoptosis in testes through the mitochondrial signaling pathway (Yeh et al. 2007; Saradha et al. 2009; Yeh et al. 2009; Vaithinathan et al. 2010). Translocation of cytochrome c from mitochondria into cytosol is the primary event in mitochondrial signaling pathway for apoptosis (Kluck et al. 1997). Bcl-2 is the antiapoptotic member of Bcl-2 family that retard cytochrome c release from mitochondria into cytosol, whereas Bax is the proapoptotic members of Bcl-2 family and counteracts the cytoprotective effect of Bcl-2 by promoting cytochrome c release from mitochondria into cytosol (Dejean et al. 2006; Autret and Martin 2009). In the present study, we found that fenvalerate treatment did not result in the translocation of cytochrome c from mitochondria into cytosol in germ cells of testes. Correspondingly, no significant difference on the level of cleaved caspase-9 in testes was observed between fenvalerate-treated mice and controls. Furthermore, we analyzed the expression and distribution of proapoptotic factor Bax and antiapoptotic protein Bcl-2 of the Bcl-2 family in mitochondria and cytosol. Our results showed that fenvalerate had little effect on the expression of Bcl-2 and Bax in testes. In addition, fenvalerate did not alter the distribution of Bax in mitochondria and cytosol. Taken together, these results suggest that fenvalerate-induced germ cell apoptosis in testes is independent of mitochondrial signaling pathway.

In summary, the present study investigated the effects of adult fenvalerate exposure on germ cell apoptosis in testes. Our results indicate that adult fenvalerate exposure induces germ cell apoptosis in testes through the Fas/FasL signaling pathway.

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