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p,p′-DDE induces testicular apoptosis in prepubertal rats via the Fas/FasL pathway

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ABSTRACT

1,1-Dichloro-2,2 bis(p-chlorophenyl) ethylene (p,p′-DDE), the major metabolite of 2,2-bis(4-chlorophenyl)-1,1,1-trichloroethane (DDT), is a known persistent organic pollutant and male reproductive toxicant. It has antiandrogenic effect. However, the mechanism by which p,p′-DDE exposure causes male reproductive toxicity remains unknown. To elucidate the mechanism underpinning the testicular effects of p,p′-DDE, we sought to investigate Fas/FasL apoptotic pathway in the testis of prepubertal rats, including Fas, FasL, caspase-8, -3, and NF-κB. Animals were administered with different doses of p,p′-DDE (0, 20, 60, 100 mg/kg b.wt) every other day by intraperitoneal injection for 10 days. The results indicated that p,p′-DDE exposure at over 20 mg/kg b.wt showed the induction of apoptotic cell death. p,p′-DDE could induce increase in the MDA level, and decrease in SOD and GSH-Px activity. Significant elevations in the mRNA levels of Fas along with an increase in FasL, caspase-3, -8 were observed in 100 mg/kg b.wt group. In protein level, p,p′-DDE could induce increase of FasL and reduction of procaspase-8. NF-κB p65 was activated by p,p′-DDE treatment in rat testis. In addition, the activities of caspase-3, -8 were increased in 100 mg/kg b.wt group. Taken together, these results lead us to speculate that in vivo exposure to p,p′-DDE might induce testicular apoptosis in prepubertal rats through the Fas/FasL pathway.

1. Introduction

A large variety of synthetic organic chemicals such as organochlorine pesticides (OCPs), have been released into the environment over the last few decades (Valeron et al., 2009). As widespread environmental pollutants, OCPs are highly lipophilic and chemically stable compounds that persist in the environment and accumulate in the food chain and in human tissues (Alvarez-Pedrerol et al., 2008). 2,2-Bis(4-chlorophenyl)-1,1,1-trichloroethane (DDT), the first widely used synthetic organochlorine pesticide, was given credit for having helped one billion people live free from malaria. However, its bioaccumulation, long-range transport, persistence in the environment and antiandrogenic properties raise the concern about its possible long-term adverse effects. Though having being banned or restricted for three decades, DDT is still being used for the control of vectors in public health in some developing countries (Aulakh et al., 2007; LopezCarrillo et al., 1996; RiveroRodriguez et al., 1997).

1,1-Dichloro-2,2 bis(p-chlorophenyl) ethylene (p,p′-DDE), DDT's major metabolite with the highest persistence, is the form usually found in human tissues in the highest concentration (Rogan and Chen, 2005). It persists in the environment and can be detected in the sera of more than 90% of the population in northern American (Daxenberger, 2002). It is a widespread environmental endocrine disrupting chemical. It has been reported that some abnormalities in sexual development in rats and wildlife might be associated with exposure to p,p′-DDE (Gray and Kelce, 1996; Kelce et al., 1995). p,p′-DDE is antiandrogenic and can inhibit androgen binding to the androgen receptor (Kelce et al., 1995; Xu et al., 2006).

Cell death by apoptosis is a part of normal development and maintenance of homeostasis (Tebourbi et al., 1998), but is also involved in pathological situation associated with sterility. In the testis, apoptosis is such a common programmed event that 75% of germ cells are reduced by spontaneous apoptosis (Allan et al., 1992). However, excessive or inadequate apoptosis of testicular cells result in abnormal spermatogenesis or testicular tumors (Lin et al., 1997). In rodents heat and irradiation as well as xenohormones and testis intoxicants are known inducers of germ cell apoptosis (El-Gohary et al., 1999; Shin et al., 1999; Shinoda et al., 1998). Some studies have shown that the level of germ cell apoptosis in male rats peaks during the first spermatogenetic cycle from postnatal days 16 to 32 (Billig et al., 1995; Dalgaard et al., 2001).

The Fas/FasL system is a widely recognized apoptosis signal transduction pathway in which a ligand–receptor interaction triggers the cell death pathway (Feng et al., 2004). Fas is a surface

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receptor that triggers apoptotic cell death when cross-linked by FasL (Nagata, 1997; Nagata and Golstein, 1995). Ligation of FasL to Fas in the cell membrane triggers activation of caspase-8. Once activated, caspase-8 transduces a signal to effector caspases, including caspase-3, -6, and -7, and eventually lead to the hydrolysis of cytosolic and nuclear substrates (DeMaria et al., 1997).

Although there have been some reports concerning p,p′-DDE-induced toxicity in male reproductive system (Makita et al., 2005; O'Connor et al., 1999), few studies investigated testicular apoptosis in prepubertal rats. We have previously examined the effects of p,p′-DDE on Sertoli cells (Xiong et al., 2006). That study demonstrated that p,p′-DDE does affect the expression of several functional marker genes including transferrin (Tf) and androgen-binding protein (ABP). Besides, ROS generation might play a critical role in the initiation of p,p′-DDE-induced apoptosis in rat Sertoli cells through mitochondria-mediated and FasL-dependent pathway (Shi et al., 2009; Song et al., 2008). The aim of the present study was to determine the effects of different doses of p,p′-DDE on testicular apoptosis in prepubertal rats (20-day old), and to investigate Fas/FasL apoptotic pathway. We investigated the importance of lipid peroxidation, expressions of Fas-FasL, activation of NF-κB in the testis of p,p′-DDE-treated rat. Because caspase family members play an important role in spermatogenesis and apoptosis, it is also of interest to determine the regulation of caspase-3 and -8 in the testis of p,p′-DDE-treated rat.

2. Materials and methods

2.1. Animals and treatments

Twenty healthy prepubertal male Sprague–Dawley rats (20-day old, weighing 45–55 g) were purchased from Tongji Medical College Animal Laboratory (Wuhan, China). 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. The rats were randomly divided into four groups, each group containing five rats, given different doses of p,p′-DDE (DR Co., Augsburg, Germany, purity: 98.5%) 0, 20, 60, 100 mg/kg b.wt respectively in corn oil (Sigma–Aldrich, St. Louis, MO, USA) every other day by intraperitoneal injection for 10 days. The doses and time used in the present study were selected from published data (Ashby and Lefevre, 1997; Kelce et al., 1995; Yamasaki et al., 2009; You et al., 1998) and the results of our preliminary experiment. After 10-day treatment, the rats were sacrificed, the testis were removed immediately and weighed. The left testis was immersed in 4% formaldehyde-phosphate buffer solution for terminal deoxynucleotidyl transferase-mediated nick and labeling (TUNEL) analysis. The right testis was stored at −80 °C until analysis.

All procedures on animals followed the Guide for the Care and Use of Laboratory Animals published by Ministry of Health of People's Republic of China.

2.2. Apoptosis assay with TUNEL staining

To detect apoptotic cell death, paraffin-embedded sections were stained by the TUNEL technique using an in situ apoptosis detection kit (Wuhan Boster Biological Technology, Ltd., Wuhan, China) according to the instructions provided by the manufacturer. The sections were deparaffinized with xylene, rehydrated and treated with 200 μg/ml proteinase K for 15 min at room temperature. Endogenous peroxidase was inactivated by covering the sections with 3% H_2O_2 in H_2O for 5 min at room temperature. End-labeling was achieved by catalytically adding residues of digoxygenin-labeled 11-dUTP and dATP to the 3′-hydroxyl ends of DNA with the enzyme TdT. The reaction buffer containing dUTP, dATP, and TdT was applied for 60 min at 37 °C in a humid atmosphere. The digoxygenin was detected immunohistochemically with a digoxygenin-specific peroxidase-conjugated antibody (30 min in a humid atmosphere at room temperature). For the color reaction, metal-enhanced diaminobenzidine was used as substrate. The sections were counterstained with hemato-xylin. Positive and negative control sections were included in each sample.

Apoptotic cells were identified by their brownish staining. The whole area of the section was scanned with the high-power magnification (400×). The "apoptotic index" (AI) was then calculated as follows: AI = (number of apoptotic cells per section/total number of cells per section) × 100%.

2.3. Measurement of glutathione peroxidase (GSH-Px) activity, superoxide dismutase (SOD) activity and malondialdehyde (MDA) level

Testes lysates were prepared in lysis buffer (containing 1 mM Na_2EDTA, 150 mM NaCl, 10 mM PMSF, 10 mM Tris, 1 mM aprotin) to evaluate oxidative stress following the protocol of GSH-Px, SOD and MDA assay kit (Jiancheng Bioengineering Ltd., Nanjing, China).

GSH-Px activities were assayed by quantifying the rate of oxidation of the reduced glutathione to the oxidized glutathione by H_2O_2. One unit of GSH-PX was defined as the amount that reduced the level of GSH by 1 μM in 1 min/mg protein. SOD activity in supernatant was determined by determining the reduction of nitro blue tetrazolium (NBT) by O^{2-} produced from the xanthine–xanthineoxiase system. One unit of SOD was defined as the amount protein inhibits the rate of NBT reduction by 50%. Results were defined as U/mg protein. The concentrations of MDA were assessed by measuring thiobarbituric-acid (TBA) reacting substances at 532 nm. The level of MDA was expressed as nmol MDA per milligram protein. Protein content was measured according to Bradford method.

2.4. Real-time quantitative PCR

Real-time quantitative PCR was performed to determine the mRNA levels of Fas, FasL, caspase-3 and -8. The total RNA of testis was extracted with Trizol reagents (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Trizol reagents are ready-to-use, monophasic solutions of phenol and guanidine isothiocyanate suitable for isolating total RNA, DNA, and proteins. During sample homogenization or lysis, Trizol reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. The testis tissue (100 mg) for each sample was homogenized, 1.0 ml Trizol was added, and the contents were then placed in 1.5-ml Eppendorf Micro Test Tube (EP tube) without RNase. They were incubated at 0 °C for 5 min. Then, 0.2 ml chloroform was added and the tubes were shaken for 15 s, and placed on ice for 2–3 min, then they were centrifuged at 12,000 rpm for 15 min at 4 °C. The colorless upper aqueous phase containing the RNA was transferred to a new EP tube without RNase. An equal volume of isopropanol was added, and the RNA was precipitated by centrifugation. The RNA pellet was washed with 75% ethanol and dissolved in water treated with diethylene pyrocarbonate (10–20 μl). RNA purity was tested with eppendorf BioPhotometer (Eppendorf, Germany), which showed an optical density ratio (OD_{260}/OD_{280}) that was between 1.8 and 2.0. Total RNA of 1 μg was reverse transcribed to complementary DNA using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Lithuania).

Real-time RT-PCR was performed with an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, USA) using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, USA). β-Actin was used in parallel for each run as internal control. A 10 μl PCR reaction system was used and included the appropriate cDNA concentration of 2 μl, SuperMix 5 μl, ROX Reference Dye 0.2 μl, 0.2 μl Forward and Reverse primers (10 μM) and 2.4 μl DEPC-treated H_2O. A four-step experimental run protocol was carried out and the amplification conditions were as follows: 50 °C for 2 min (UDG incubation); 95 °C for 10 min (initial denaturation); 40 cycles of 15 s at 95 °C (denaturation) and 1 min at 60 °C (elongation). A melting curve was generated at the end of every run to ensure product uniformity (95 °C for 15 s, 60 °C for 15 s, 95 °C for 15 s). The relative expression of target genes was calculated using $2^{-\Delta\Delta Ct}$.

The primer sequences were designed according to cDNA sequence from GenBank (Table 1). All primers were synthesized by the Bioasia Corp (Shanghai, China).

2.5. Western blotting

Testis lysates were prepared in lysis buffer (10 mM EDTA, 2 mM EGTA, 20 mM Tris–HCl (pH 7.4), 250 mM sucrose, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl chloride, and 100 mM PMSF) to detect Fas, FasL and caspase proteins. Each protein sample was measured by a Bio-Rad DC kit (Bio-Rad, Hercules, CA). Cell extracts were separated in SDS-polyacrylamide gel and transferred electrophoretically onto a PVDF membrane. The membranes were blocked in PBS containing 5% non-fat dry milk (w/v), and then incubated at 4 °C overnight with anti-Fas (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 2:100 dilution, anti-FasL (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 2:100 dilution, anti-procaspase-8 (Wako, Saitama, Japan) at a 1:200 dilution or anti-GAPDH (Protein Tech Group, Inc., Chicago, USA) at 1:1000 dilution. Then membranes were incubated at 37 °C for 2 h with the secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia, Bucking-

Table 1
Description of primers used in the present study.

Primers	Type	Primer sequence	Length (bp)
Fas	Forward	5′-AAATGAAAGCCAACTGCATCGAC-3′	88
	Reverse	5′-ATTGGACCCTCGCTGAGCAC-3′	
FasL	Forward	5′-CACCAACCACAGCCTTAGAGTATCA-3′	171
	Reverse	5′-ACTCCAGAGATCAAAGCAGTTCCA-3′	
Caspase-3	Forward	5′-GCAGCAGCCTCAAATTGTTGACTA-3′	144
	Reverse	5′-TGCTCCGGCTCAAACCATC-3′	
Caspase-8	Forward	5′-TGGTATATCCAGTCACTTTGCCAGA-3′	93
	Reverse	5′-CACATCATAGTTCACGCCAGTCAG-3′	
β-Actin	Forward	5′-GGAGATTACTGCCCTGGCTCCTA-3′	150
	Reverse	5′-GACTCATCGTACTCCTGCTTGCTG-3′	

Table 2
The change of body weights and organ coefficient of testis in male rat treated with p,p′-DDE.

Groups	Body weight (g)	Testis weight (g)	Organ coefficient of testis (%)
Control	102.0 ± 24.1	0.966 ± 0.298	0.938 ± 0.113
p,p′-DDE (20 mg/kg b.wt)	100.8 ± 25.5	0.949 ± 0.190	0.951 ± 0.069
p,p′-DDE (60 mg/kg b.wt)	103.4 ± 14.3	1.014 ± 0.082	0.981 ± 0.070
p,p′-DDE (100 mg/kg b.wt)	110.2 ± 14.9	0.961 ± 0.145	0.872 ± 0.078

hamshire, UK) diluted at 1:5000. And immune-reactive proteins were detected using ECL western blotting detection system (Pierce Biotechnology Inc., Rockford, IL, USA). Densitometric analysis of immunoblots was performed with Gel pro 3.0 software.

For measurement of NF-κB p65, the cytoplasmic protein and the nuclear protein were extracted according to instructions of Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotech Inc., Nantong, China). Equal quantities of protein were subjected to SDS-polyacrylamide gel and transferred electrophoretically onto a PVDF membrane as described above. Blots were incubated with primary antibody for NF-κB p65 (1:200) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4 °C. The following day, blots were washed in PBS and incubated for 1 h at room temperature with the secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia, Buckinghamshire, UK) diluted at 1:5000. Immune-reactive proteins were detected using ECL western blotting detection system (Pierce Biotechnology Inc., Rockford, IL, USA).

2.6. Caspase activity assay

Caspase activities were determined by a colorimetric assay based on the ability of caspase-3, -8 to change acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-*p*NA) and acetyl-Ile-Glu-Thr-Asp *p*-nitroanilide (Ac-IETD-*p*NA) into a yellow formazan product [*p*-nitroaniline (*p*NA)], respectively. Testis lysates were prepared after their respective treatment with various designated treatments. Assays were performed on 96-well microtitre plates. 10 μl testis lysate, 80 μl reaction buffer and 10 μl caspase substrate were added by turns. Then they were incubated at 37 °C for 24 h. Samples were measured with an ELISA Reader (Bio-Rad instrument Group, Hercules, CA, USA) at an absorbance of 405 nm. All the experiments were carried out in triplicates. The detail analysis procedure was described in the manufacturer's protocol (Beyotime Institute of Biotechnology, Nantong, China). Protein content was measured according to Bradford method.

2.7. Statistic analysis

Results are represented statistically as mean ± SD. Significance was assessed by One Way ANOVA following appropriate transformation to normalized data and equalized variance where necessary. Mean values were compared by subsequent Student–Newman–Keuls (SNK) using the SPSS statistical package 12.0 (SPSS Inc., Chicago, IL, USA). A difference at $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of p,p′-DDE on body weights and organ coefficient of testis in rat testis

As seen in Table 2, 10-day treatment with 20, 60, 100 mg/kg b.wt p,p′-DDE did not result in change of body weight and the weights of testis. Moreover, there were no significant differences in the organ coefficient of testis between different doses of p,p′-DDE groups and control group ($P > 0.05$).

3.2. Apoptotic effects of germ cells in rat treated with p,p′-DDE

TUNEL staining was performed to detect programmed cell death in situ. Fig. 1A showed a control testis from a representative rat treated with corn oil. No or few TUNEL-positive cell was noted along the basement of seminiferous tubules. Treatment with 20, 60, 100 mg/kg b.wt p,p′-DDE resulted in selective degeneration of germ cells at the seminiferous tubules. Most germ cells undergoing apoptosis were located along the periphery of the seminiferous tubules. The majority of these apoptotic cells was located in the region usually occupied by primary spermatocytes, although some were located in the region corresponding to spermatogonia (Fig. 1B–D). Compared with the control group, the apoptotic index (AI) increased significantly in different p,p′-DDE-treated groups (Table 3).

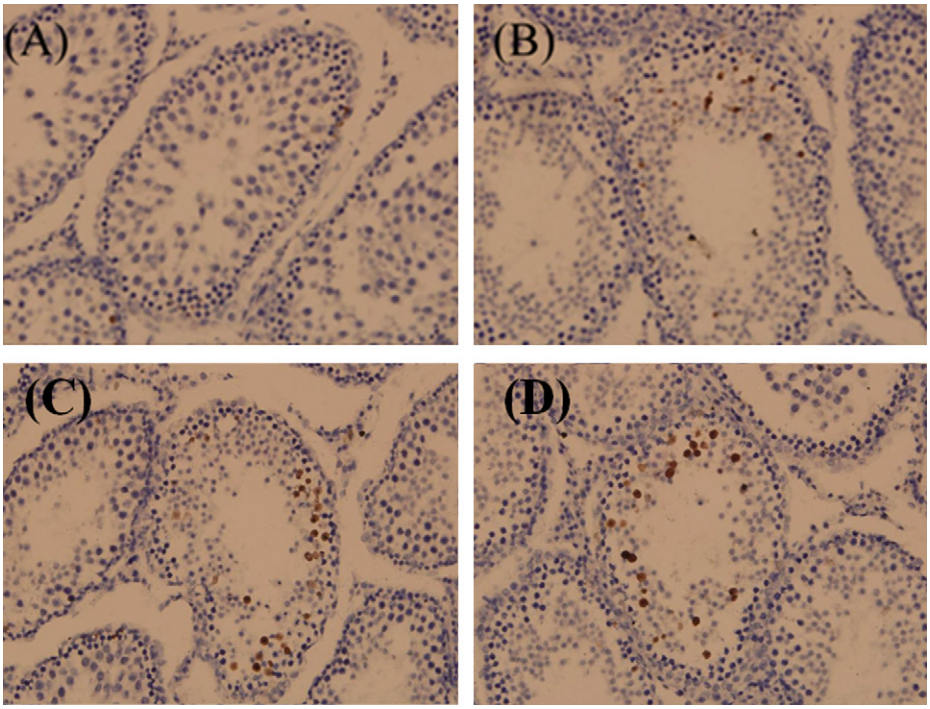

Fig. 1. Effects of p,p′-DDE on TUNEL-positive apoptotic changes in testis of rats. Most cells undergoing apoptosis were located along the basement of seminiferous tubules in groups treated with p,p′-DDE, but few apoptotic cells were found in control group. (A) Control; (B) 20 mg/kg b.wt p,p′-DDE; (C) 60 mg/kg b.wt p,p′-DDE; (D) 100 mg/kg b.wt p,p′-DDE.

Table 3
Apoptotic effects of testis in male rat treated with p,p′-DDE.

Groups	Numbers of rat	Apoptotic index (%)
Control	5	0.5 ± 0.02
p,p′-DDE (20 mg/kg b.wt)	5	5.67 ± 1.44*
p,p′-DDE (60 mg/kg b.wt)	5	5.92 ± 0.83*
p,p′-DDE (100 mg/kg b.wt)	5	7.83 ± 0.57*

* Significant difference: $P < 0.05$, compared with the control group.

3.3. Effect of p,p′-DDE on GSH-Px activity, SOD activity and MDA level in rat testis

To assess the importance of oxidative stress, GSH-Px activity, SOD activity and MDA level were evaluated in rat testis. SOD activity was illustrated in Fig. 2A. It could be observed that 20, 60, 100 mg/kg b.wt p,p′-DDE induced a significant decreasing activities of SOD production ($P < 0.05$). The GSH-Px activity in 100 mg/kg b.wt p,p′-DDE group was significantly lower than that in the control group ($P < 0.05$), and the MDA level in 60, 100 mg/kg b.wt p,p′-DDE groups was remarkably higher than that in control group ($P < 0.05$) (Fig. 2B).

3.4. Effect of p,p′-DDE on Fas, FasL, caspase-3 and -8 mRNA in rat testis

To assess the effect of p,p′-DDE on apoptosis-related gene, the mRNA levels of Fas, FasL, caspase-3 and -8 in rat testis were determined by real-time quantitative PCR. As indicated in Fig. 3C, in 100 mg/kg b.wt p,p′-DDE group, the mRNA levels of Fas, FasL, caspase-3 and -8 were significantly higher than that of the control group, and the differences were statistically significant ($P < 0.05$).

3.5. Effect of p,p′-DDE on Fas, FasL and procaspase-8 protein in rat testis

To assess the effect of p,p′-DDE on Fas/FasL pathway, the levels of Fas, FasL, procaspase-8 protein were evaluated in rat testis. p,p′-DDE treatment was shown to result in an increase of FasL protein in 100 mg/kg b.wt p,p′-DDE group, but Fas protein in all p,p′-DDE groups was no significantly higher than that in the control group ($P > 0.05$) (Fig. 4E). A significant reduction was observed in procaspase-8 in 100 mg/kg b.wt p,p′-DDE group (Fig. 4F), suggesting the caspase activation.

3.6. Effect of p,p′-DDE on NF-κB p65 protein in the nuclear extracts of rat testis

To delineate the role of NF-κB in testicular apoptosis, the levels of NF-κB p65 were evaluated in the nuclear extracts of testis by western blotting analysis. As indicated in Fig. 4E, p,p′-DDE treatment induced an increase of NF-κB p65 in 60, 100 mg/kg b.wt p,p′-DDE groups ($P < 0.05$).

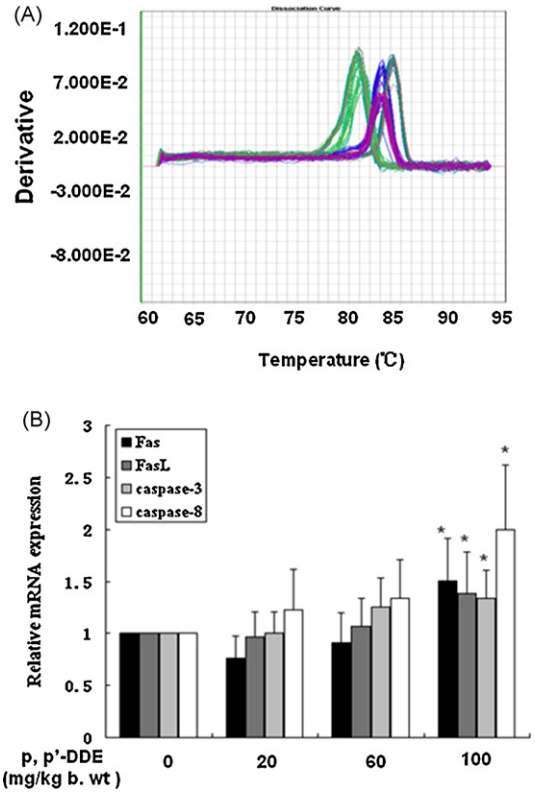

Fig. 3. Effects of p,p′-DDE on the Fas, FasL, caspase-3 and caspase-8 mRNA expression levels in rat testis by real-time quantitative PCR. (A) Dissociation curve of Fas, FasL, caspase-3, caspase-8 and β-actin, the curves featured by a single peak at expected Tm; (B) quantitative analysis of Fas, FasL, caspase-3 and caspase-8 mRNA expression levels of rat testis exposed to different doses of p,p′-DDE. The housekeeping gene β-actin was used as an internal positive control standard for quantitative analysis. The relative expression of target genes was calculated using $2^{-\Delta\Delta Ct}$. Data are indicated as mean ± SD. Significant difference: *$P < 0.05$, compared with the control group.

3.7. Effect of p,p′-DDE on caspase-3 and -8 activities in rat testis

The activities of caspase-3, -8 were determined using the Caspase-3, -8 Activity Kit (Fig. 5). Compared with the control group, the activities of caspase-3, -8 significantly increased in 100 mg/kg b.wt p,p′-DDE group ($P < 0.05$).

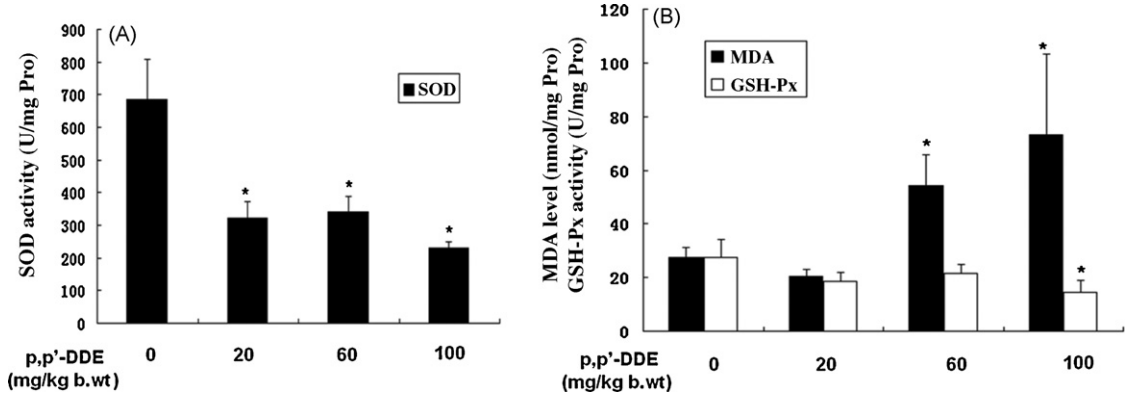

Fig. 2. Effects of p,p′-DDE on SOD activity, MAD level and GSH-Px activity in rat testis. Data are indicated as mean ± SD. Significant difference: *$P < 0.05$, compared with the control group.

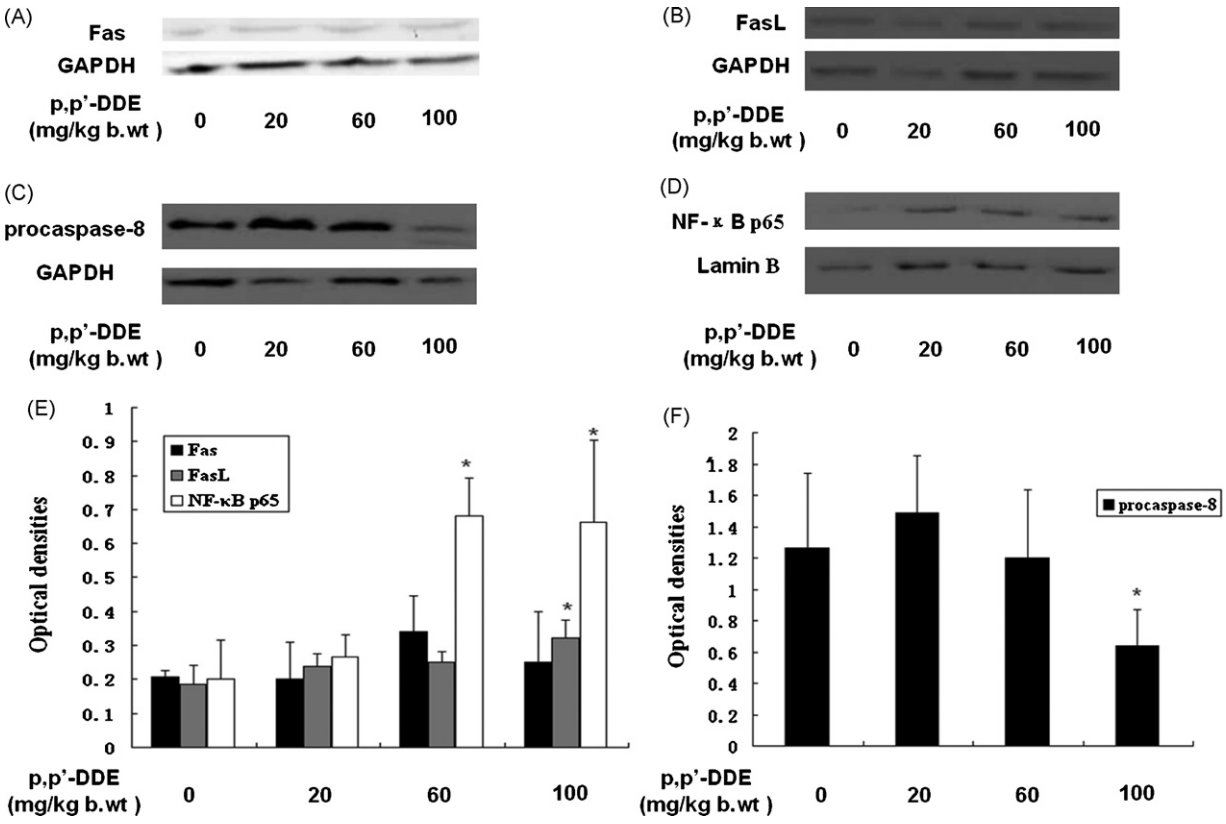

Fig. 4. (A–C) Effects of p,p′-DDE on the Fas, FasL and procaspase-8 protein expression levels in rat testis. Protein from whole cell lysates was used in Western blotting for Fas, FasL and procaspase-8 detection. (D) Effect of p,p′-DDE-induced NF-κB p65 expression in the nuclear extracts. Protein from nuclear extracts was used in Western blotting for NF-κB p65 detection. (E and F) Quantitative analysis of the immunoreactive Fas, FasL, procaspase-8 and NF-κB p65. Data are indicated as mean ± SD.Significant difference: $^{*}P < 0.05$, compared with the control group.

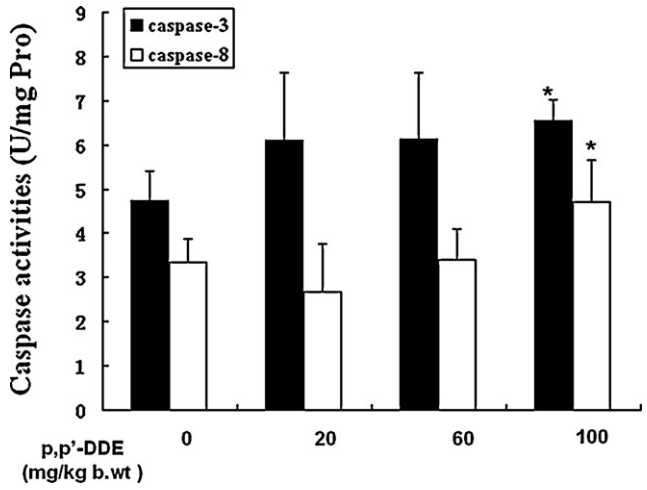

Fig. 5. Effects of p,p′-DDE on caspase-3 and caspase-8 activity in rat testis. Data are indicated as mean ± SD. Significant difference: $^{*}P < 0.05$, compared with the control group.

4. Discussion

In the present study, p,p′-DDE could induce testicular apoptosis in prepubertal rats through the Fas/FasL-dependent pathway including lipid peroxidation, increase of the Fas–FasL expression, and activation of the caspase-8 and -3. Furthermore, NF-κB could promote cell apoptosis through the Fas/FasL pathway in the testis of p,p′-DDE-treated rats.

The impact of organochlorine pesticides (OCPs) on the reproductive function was put forward in 1967 by Ratcliffe (1967), who is the first to report eggshell thinning in some raptorial species. DDT is a principal organochlorine compound used for a long time as an insecticide. It can impair the male reproductive health by possible mechanism of antiandrogen effect. Animal experiment demonstrated that exposure of rats to 50 and 100 mg DDT/kg b.wt during 10 consecutive days induced reproductive toxicology. The relative weight of testes and the number as well as the motility of epididymal spermatozoa were reduced (Ben Rhouma et al., 2001). In our previous study, p,p′-DDE could induce increase in apoptotic rate of Sertoli cells by a mechanism possibly involving FasL-dependent pathway (Shi et al., 2009). The present study demonstrated that p,p′-DDE could induce testicular apoptosis in prepubertal rats through the Fas/FasL pathway.

In FasL-induced spermatogenic cell death, it is generally accepted that FasL from Sertoli cells kill the spermatogenic cells by engaging the Fas receptors present on them (Nair and Shaha, 2003). Recently, it has been demonstrated that Sertoli cells also express Fas and germ cells express FasL (D'Alessio et al., 2001; Ogi et al., 1998). Exposure to perfluorononanoic acid, a synthetic perfluorinated chemical, has been shown to induce cell apoptosis in rat testis and the apoptosis was probably associated with the Fas death receptor-dependent apoptotic pathway (Feng et al., 2009). Animal experiment demonstrated that exposure of rats to 5 mg lindane/kg b.wt induced a significant elevation in the levels of Fas–FasL in the testis of rats (Saradha et al., 2009). In our study, we showed that p,p′-DDE exposure induced an increase in the mRNA expression of Fas–FasL leading to the activation of protein of FasL in the testis of prepubertal rats. This result indicated that Fas–FasL mRNA levels increased and could lead to the enhancement of FasL protein

expression, then activate the Fas system, and eventually lead to testicular apoptosis and abnormality of spermatogenesis, which might be a possible mechanism elucidating male reproductive abnormality caused by p,p′-DDE.

NF-κB is present as a dimer of protein components (p65/p50) in a latent/inactive form, bound to inhibitory protein IκB in the cytoplasm (Shalini and Bansal, 2007). Stimulation by a variety of extracellular signals leads to degradation of the IκB. The liberated NF-κB then rapidly translocates to the nucleus, where it regulates transcription by binding to consensus κB sites in the promoters of the target genes (Wang et al., 2005). In the rat testis, the NF-κB complex of p65 and p50 proteins is found to be constitutively expressed in the nuclei of Sertoli cells at all stages of spermatogenesis (Pentikainen et al., 2002). Interestingly, NF-κB can exert both pro- and anti-apoptotic effects in different cells types (Barkett and Gilmore, 1999). Whether NF-κB promotes or inhibits apoptosis seems to depend on the specific cell type and the type of the inducer. Numerous reports suggest that NF-κB performs a proapoptotic role. NF-κB p65 complex can directly stimulate the expression of apoptosis-inducible genes such as Fas, FasL, and death receptors 4 and 5 (Kucharczak et al., 2003). Furthermore, ligation of Fas-FasL can in turn stimulate NF-κB (Kreuz et al., 2004), while active NF-κB can in turn induce Fas transcription (Malewicz et al., 2003). Our study demonstrated that in vivo exposure to p,p′-DDE could induce the activation of NF-κB and the expression of Fas–FasL in the testis of prepubertal rats. Thus, it could be speculated that NF-κB can induce Fas–FasL expression, while Fas–FasL can also activate NF-κB, then promote cell apoptosis through Fas/FasL pathway in vivo exposure to p,p′-DDE in the testis of prepubertal rats.

There is a general agreement that male reproductive organs are particularly susceptible to the deleterious effects of reactive oxygen species (ROS) and lipid peroxidation, which ultimately lead to impaired fertility (Williams et al., 1998). SOD and GSH-Px as the ROS scavengers are depleted, and MDA as a product of lipid peroxidation is accumulated. ROS was shown to be able to increase FasL expression. Conversely, various antioxidants potently inhibited the FasL expression (Bauer et al., 1998). Hofmann et al. (2001) reported that oxidative stress enhanced NF-κB activation. Our present study demonstrated that in vivo exposure to p,p′-DDE could induce apoptosis via oxidative stress, increase of FasL and activation of NF-κB in the testis of prepubertal rats.

Caspases are a family of cysteine proteases that are a central component of the apoptotic machinery. Caspases are synthesized as inactive precursors (procaspases) that are cleaved at specific aspartate residues to generate the active subunits. Among Fas/FasL pathway apoptosis, caspase-8 plays the most important role in transduction of death signals (Said et al., 2004). Paasch demonstrated that the signal that transmits caspase-8 is also present during spermatogenesis (Paasch et al., 2001). This result is consistent with our study in which in vivo exposure to p,p′-DDE induce an reduction in procaspase-8 protein and an increase in caspase-8 activity in the rat testis. In addition, caspase-3 is one of the pivotal proteinases that initiate cell apoptosis (Khan et al., 2000). Activated caspase-3 can interact with a large number of targets within an affected cell to bring about its destruction by apoptosis (Philchenkov, 2004). Dalgaard et al. (2001) observed an increased activity of caspase-3 in the rat testis, 3 and 12 h after 0.4 mg/g of MEHP-exposure. Our result demonstrated that in vivo exposure to p,p′-DDE could induce an increase in mRNA level and activity of caspase-3 in the rat testis. These results indicated that caspases were activated in the testis of p,p′-DDE-treated rats.

In conclusion, p,p′-DDE could induce testicular apoptosis in prepubertal rats by a mechanism possibly involving Fas/FasL pathway. In vivo exposure to p,p′-DDE can enhance oxidative stress, then induce activation of NF-κB, expression of Fas–FasL in rat testis. Upon engagement of FasL to Fas, an intrinsic programme of apoptotic death is stimulated in a target cell leading to the activation of caspase-8. Finally, testicular apoptosis is mediated by a terminal executioner, caspase-3, thereby disturbing the spermatogenic process. This study might partly elucidate the mechanism of in vivo exposure to p,p′-DDE on the male reproductive toxicity. However, there are three major apoptosis pathways in the mammalian cells: mitochondria, death receptor and endoplasmic reticulum-mediated apoptosis. It is unlikely that a single mechanism regulates apoptosis in the testis, but rather that multiple apoptotic pathways are involved in the complex process of apoptosis. Some studies showed the cell apoptosis through mitochondria and endoplasmic reticulum-mediated pathway in the rat testis (Hughes et al., 2000; Muguruma et al., 2005; Onorato et al., 2008; Saradha et al., 2009). Hence, it is noted that mitochondria and endoplasmic reticulum-mediated apoptosis pathway in vivo exposure to p,p′-DDE in the rat testis should be regarded as priority in the next studies.

Conflict of interest

None declared.

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