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

Yu-Qin Shi, Yu-Ping Wang, Yang Song, Hao-Wen Li, Chang-Jiang Liu, Zhi-Gang Wu, Ke-Di Yang

MOE Key Lab of Environment and Health, Department of Occupational and Environmental Health, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, 13 Hangkong Road, Wuhan 430030, Hubei, PR China

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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.

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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 been banned or restricted for antiandrogenic properties raise the concern about its possible long-range transport, persistence 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 been banned or restricted for

(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 (Tebouri 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; Dalgard 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...
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 leads 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 rats.

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 (0, 20, 60, 100 mg/kg b.wt respectively in corn oil). 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 diethyle pyrocatechrol (10−20 μl). RNA purity was tested with eppendorf BioPhotometer (Eppendorf, Germany), which showed an optical density ratio (OD260/OD280) 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 μmol) and 2.4 μl DEPC-treated H2O. A four-step experiment 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 15s 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−ΔΔCt. 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, 0.1% phenylmethylsulfonyl fluoride, and 100 mM PMSF) to detect FasL, Fas, and caspase proteins. Each protein sample was measured by a Bio-Rad DC kit (Bio-Rad, Hercules, CA). Cell extracts were measured 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 1:200 dilution, anti-β-Actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:200 dilution, anti-caspase-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, Buckingham,

Table 1

<table>
<thead>
<tr>
<th>Primers Type</th>
<th>Primer sequence</th>
<th>Length (bp)</th>
</tr>
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<tbody>
<tr>
<td>Fas</td>
<td>Forward 5′-AAATGAAAGCACCAGTCGTACC-3′</td>
<td>88</td>
</tr>
<tr>
<td>FasL</td>
<td>Forward 5′-CACTACACCAGCAGGATACCA-3′</td>
<td>171</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Forward 5′-TGCTCCGGCTCAGAACCAC-3′</td>
<td>144</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>Forward 5′-GGAGATTACTGCCCTGGCTCCTA-3′</td>
<td>93</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward 5′-GGAGATTACTGCCCTGGCTCCTA-3′</td>
<td>150</td>
</tr>
</tbody>
</table>
The change of body weights and organ coefficient of testis in male rat treated with p,p'-DDE.

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

3. Results

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

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.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Numbers of rat</th>
<th>Apoptotic index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>0.5 ± 0.02</td>
</tr>
<tr>
<td>p,p'-DDE (20 mg/kg b.wt)</td>
<td>5</td>
<td>5.67 ± 1.44*</td>
</tr>
<tr>
<td>p,p'-DDE (60 mg/kg b.wt)</td>
<td>5</td>
<td>5.92 ± 0.83*</td>
</tr>
<tr>
<td>p,p'-DDE (100 mg/kg b.wt)</td>
<td>5</td>
<td>7.83 ± 0.57*</td>
</tr>
</tbody>
</table>

* 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).

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. 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.

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-kB 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-kB 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-kB can exert both pro- and anti-apoptotic effects in different cell types (Barkett and Gilmore, 1999). Whether NF-kB promotes or inhibits apoptosis seems to depend on the specific cell type and the type of the inducer. Numerous reports suggest that NF-kB promotes a proapoptotic role. NF-kB 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-kB (Kreuz et al., 2004), while active NF-kB 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-kB and the expression of Fas–Fasl in the testis of prepubertal rats. Thus, it could be speculated that NF-kB can induce Fas–Fasl expression, while Fas–Fasl can also activate NF-kB, then promote cell apoptosis through Fasl/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 antioxidants potently inhibited the FasL expression (Bauer et al., 1998). Hofmann et al. (2001) reported that oxidative stress enhanced NF-kB 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-kB in the testis of prepubertal rats.


