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Exposure *in utero* to 2,2′,3,3′,4,6′-hexachlorobiphenyl (PCB 132) impairs sperm function and alters testicular apoptosis-related gene expression in rat offspring

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Abstract

Toxicity of the polychlorinated biphenyls (PCBs) depends on their molecular structure. Mechanisms by prenatal exposure to a non-dioxin-like PCB, 2,2′,3,4′,5′,6-hexachlorobiphenyl (PCB 132) that may act on reproductive pathways in male offspring are relatively unknown. The purpose was to determine whether epididymal sperm function and expression of apoptosis-related genes were induced or inhibited by prenatal exposure to PCB 132. Pregnant rats were treated with a single dose of PCB 132 at 1 or 10 mg/kg on gestational day 15. Male offspring were killed and the epididymal sperm counts, motility, velocity, reactive oxygen species (ROS) generation, sperm–oocyte penetration rate (SOPR), testicular histopathology, apoptosis-related gene expression and caspase activation were assessed on postnatal day 84. Prenatal exposure to PCB 132 with a single dose of 1 or 10 mg/kg decreased cauda epididymal weight, epididymal sperm count and motile epididymal sperm count in adult offspring. The spermatozoa of PCB 132-exposed offspring produced significantly higher levels of ROS than the controls; ROS induction and SOPR reduction were dose-related. In the low-dose PCB 132 group, p53 was significantly induced and caspase-3 was inhibited. In the high-dose group, activation of caspase-3 and -9 was significantly increased, while the expressions of *Fas*, *Bax*, *bcl-2*, and *p53* genes were significantly decreased. Gene expression and caspase activation data may provide insight into the mechanisms by which exposure to low-dose or high-dose PCB 132 affects reproduction in male offspring in rats. Because the doses of PCB 132 administered to the dams were approximately 625-fold in low-dose group and 6250-fold higher in high-dose group than the concentration in human tissue levels, the concentrations are not biologically or environmentally relevant. Further studies using environmentally relevant doses are needed for hazard identification.

Keywords: Polychlorinated biphenyls (PCBs); Prenatal exposure; Male reproduction; Sperm dysfunction; Apoptosis-related genes; Caspase activation

Introduction

Even though the commercial production of polychlorinated biphenyls (PCBs) was banned at the end of the 1970s, they continue to be a health problem due to their lipophilic nature, persistence in the environment and demonstrated toxic effects. A deterioration in male fertility has been linked to elevated concentrations of PCBs. Between 1978 and 1979, a tragedy referred to as "Yu-Cheng exposure" occurred in Taiwan, where more than 2000 people ingested rice oil contaminated with PCBs and their pyrolytic product (Hsu et al., 1984). In comparison with the general population, the young men prenatally exposed to PCBs in the Yu-Cheng cohort had a higher proportion of abnormal sperm and reduced sperm capacity to penetrate hamster oocytes (Guo et al., 2000). Moreover, men postnatally exposed to contaminated rice oil had a higher percentage of oligospermia, abnormal sperm morphology and reduced sperm binding

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capability and penetration (Hsu et al., 2003a). Accidental incidences such as this have demonstrated direct links between exposure and adverse health outcomes. PCBs occur in mixtures of multiple congeners that differ in the numbers and positions of chlorines around the biphenyl ring. Different congeners can exert distinct effects (Giesy and Kannan, 1998). The halogenated aromatic hydrocarbons are of particular concern as hormone mimics since they often have similar molecular recognition factors, similar bulk physico-chemical properties controlling uptake and distribution in biological systems, and are relatively more resistant to metabolism and elimination (McKinney and Waller, 1998). Because of the broad range of toxic effects and their persistence in the biota, studies of the effects of PCB congeners and the subsequent upregulation or downregulation of physiological processes at critical stages of development have been discussed (DeRosa et al., 1998). 2,2′,3,4′,5′,6-Hexachlorobiphenyl (PCB 132) is a stable non-dioxin-like chlorinated biphenyls congener. Since its metabolites can be detected in human tissues (Chu et al., 2003; Glausch et al., 1995; Weistrand and Noren, 1997) as well as biota (Riedel et al., 2002), it is important to investigate possible adverse effects of this congener. There is limited evidence that 2,3,6-substituted chlorinated biphenyls (CB) congeners may possess certain toxicities that have not previously been identified. We have demonstrated that postnatal exposure to PCB 132 affects serum triiodothyronine (T_3) levels, and sperm motility, velocity and capability of penetrating oocytes in rats (Hsu et al., 2003b). Although these data are suggestive of probable toxicity to sperm function, it is not clear whether prenatal exposure to PCB 132 affects postnatal testicular and sperm function, and if so, by what mechanisms.

Another study indicated that mice exposed postnatally by a single gavage of 10 or 100 mg/kg of noncoplanar PCBs, 2,2′,4,4′,5-pentachlorobiphenyl (PCB 99) or 2,2′,4,4′5,5′-hexachlorobiphenyl (PCB 153) showed a significant increase in Leydig cell apoptosis (Oskam et al., 2004). However, another study postulated that the coplanar PCB 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) does not directly induce apoptosis in germ cells or Sertoli cells of organ-cultured testes during the neonatal stages (Fukuzawa et al., 2003). Apoptosis is a physiological process that entails the programmed death of the cells. Although apoptosis has a functional role in normal development and tissue homeostasis, aberrant triggering of the process by toxicants may lead to abnormal function or disease. *Bcl-2* was the first member to be identified of a growing family of genes that regulates cell death in either a positive or a negative fashion (Vaux et al., 1988). Members of the Bcl-2 family of proteins play a major role in governing this mitochondria-dependent apoptotic pathway, with proteins such as Bax functioning as inducers, and proteins such as Bcl-2 as suppressors of cell death (Chittendenm et al., 1995). The ability of p53 to serve as a transcription regulator may be associated with its role in maintaining cell cycle checkpoints and causing cell cycle arrest at the G1 and G2 checkpoints or in leading to cell death by inducing the expression of Bax (Miyashita and Reed, 1995). In addition, the Fas system has been implicated as a possible key regulator of germ-cell apoptosis in the mammalian testis (Lee et al., 1997). Fas is a type I transmembrane receptor protein that belongs to the tumor necrosis factor (TNF)/nerve growth factor receptor family, and Fas ligand (Fas-L) is a type II transmembrane protein of the TNF family (Cai et al., 1997). Binding of Fas-L to Fas induces trimerization of Fas receptors, which recruit Fas-associated death domain (FADD) protein through shared death domains. FADD protein also contains a "death effector" domain (DED) in its N-terminal region. The Fas/FADD complex then binds to the initiator caspase-8 or -10, through interactions between the DED of the FADD and these caspase molecules. Finally, the activated caspases cleave various cellular substances, such as actin, fordin, lamin, polymerase, and DNA-dependent protein kinase, resulting in apoptosis (Sinha Hikim et al., 1998).

Although a growing body of evidence has indicated that dioxin-like PCBs can significantly contribute to sperm dysfunction *in vivo* and *in vitro*, little information is available on detailed molecular mechanisms of apoptosis underlying testicular toxic effects mediated by non-coplanar PCBs. Therefore, the aim of the present study was to determine whether prenatal exposure to the non-dioxin-like congeners PCB 132 affects epididymal sperm function and the expression of apoptosis-related genes including *Fas*, *Bax*, *bcl-2* and *p53* as well as activities of caspase-3, -8, and -9 in the testis. Molecular mechanisms of apoptosis induced or inhibited by low-dose or high-dose exposure to PCB 132 are discussed.

Materials and methods

Overview of study design. Sprague-Dawley rats were purchased from the Animal Center of National Cheng Kung University Medical Center (Tainan, Taiwan) and were housed in standard plastic cages maintained at controlled room temperature (22±1 °C), relative humidity (55±5%) and light–dark cycle (light from 08:00 to 20:00 h). Laboratory Rodent Diet 5001 (LabDiet, Richmond, IN, USA) and water were available *ad libitum*. The day of vaginal plug was considered to be gestational day (GD) 0. Pregnant rats (12 per group) were treated by intraperitoneal injection with a single dose of PCB 132 at 1 or 10 mg/kg on GD 15. The control pregnant rats received the vehicle, corn oil, as 10 ml/kg BW on the same day. On postnatal day (PND) 22, offspring were weaned and 3 male offspring were randomly selected and housed on a litter basis. At puberty (PND 84), male offspring were killed and the testes and cauda epididymides were immediately removed and weighed. Epididymal sperm counts, motility and velocity were measured and an epididymal sperm suspension was prepared for assay of reactive oxygen species (ROS). Sperm were incubated with ova harvested from non-PCB-exposed female rats, then sperm–oocyte penetration rate (SOPR) was assessed after 24 h of co-culture. The right testis was fixed in Bouin's solution for histopathology assay. The left testis was snap frozen in liquid nitrogen and maintained at −80 °C until investigation for apoptosis-related gene expression and caspase activation.

Chemicals. PCB 132 was obtained from AccuStandard (New Haven, CT, USA; guaranteed >99% pure). Phosphate-buffered saline (PBS) and human tubule fluid (HTF) medium consisting of 105.6 mM NaCl, 5.06 mM KCl, 0.73 mM $CaCl_2$, 1.17 mM KH_2PO_4, 1.01 mM $MgSO_4{\cdot}7H_2O$, 25.3 mM $NaHCO_3$, 0.27 mM sodium pyruvate, 21.6 mM sodium lactate, 5.56 mM glucose, 1000 IU/ml penicillin and 50 μg/ml streptomycin were from Gibco Life Technologies (Grand Island, NY, USA). The medium had a pH of 7.4, and an osmolarity of 330 mosM/l when gassed with 5% CO_2 and air, and prewarmed to a temperature of 34 °C or 37 °C. Corn oil was obtained from Merck (Darmstadt, Germany). 5-Amino-2,3-dihydro-1,4-phthalhydrazide (luminol), ferrous sulfate ($FeSO_4$), dimethyl sulfoxide (DMSO), mineral oil, bovine albumin, postmenopausal serum gonadotropin (PMSG), human chorionic gonadotropin (hCG) and hyaluronidase were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Dose calculation. The doses of PCB 132 administered to the dams were approximately 625-fold and 6250-fold higher than the concentration (1.6 ng/g wet weight) in human liver tissue reported from the Belgian general population (Chu et al., 2003). This was achieved by administering a single dose of 1 or 10 mg PCB 132/kg to the pregnant female rats. The decision to test a dose 625-fold or 6250-fold higher was based on risk assessment approaches, which widely use a margin of safety of from 1000- to 10,000-fold.

Sperm count, motility and velocity. Male offspring aged 12 weeks were killed. The right cauda epididymis at termination was dissected from each male and transported to the laboratory in 1 ml 34 °C HTF buffer supplemented with 5 mg/ml bovine albumin. The cauda epididymis was removed from the transport buffer, slashed, and placed in 1 ml HTF-albumin buffer, overlaid with mineral oil. A 1:10 dilution of spermatozoa was prepared and an epididymal sperm count done with a hemocytometer. The motile epididymal sperm count was obtained from multiplying the epididymal sperm count by percent motile spermatozoa. Computer-assisted sperm analysis (CASA) with a Hamilton Thorn Research motility analyzer (version HTM-IVOS Specification, Beverly, MA, USA) at a temperature of 37 °C determined sperm motility parameters: curvilinear velocity (VCL, μm/s), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), amplitude of lateral head displacement (ALH, μm), and beat-cross frequency (BCF, Hz).

Sperm ROS assay. The generation of ROS by rat epididymal sperm was determined with luminol as the chemiluminescence probe. A mixture of 300 μl PBS-washed rat epididymal sperm suspension ($40–45\times10^6$ cells/ml) with 100 μl of 30 mM $FeSO_4$ was treated with 50 μl luminol stored as a 1 mM stock solution in DMSO. The chemiluminescent signal was measured immediately on aluminometer (Model LB 953, Berthold Technologies, Bad Wildbad, Germany), the counts being integrated over a 60-s period. The consistency of the luminometer's photomultiplier response was assessed by making a number of independent assessments of the chemiluminescent signal generated when luminol was chemically oxidized. The sperm ROS levels were measured with chemiluminescence counts/10^6 cells per second.

Sperm–oocyte penetration assay. Control female Sprague-Dawley rats were superovulated at age 63–70 days, weighing 330–380 g. On the morning of day 1, they were injected with 25 IU of PMSG. On day 3, 52 h later, they were injected with 25 IU of hCG. About 20 h later, they were killed and the cumuli were dissected from the oviducts, collected in HTF-albumin buffer, and dissolved with 10 mg/ml hyaluronidase. After adjustment to 10×10^6 sperm/ml with HTF-albumin buffer, 10 ml of epididymal sperm suspension was added to 100 ml of HTF-albumin buffer containing 10–15 zona-intact rat ova. Each culture well of the sperm-ova preparation was overlaid with mineral oil and incubated at 37 °C in 95% air/5% CO_2. After 24 h of co-culture, the number of oocytes penetrated by spermatozoa was determined by phase-contrast microscopy at 400 × magnification. SOPR was used to evaluate the sperm–oocyte penetration capacity as described in the following equation:

$$\text{SOPR}(\%) = [1 - (\text{No. unpenetrated oocytes}/\text{Total no. oocytes})] \times 100.$$

RNA extraction and analysis of expression for apoptosis-related genes by real-time RT–PCR. Total RNA was extracted from each frozen testicular tissue sample with REzol reagent (PROtech Technology, Tainan, Taiwan) following the protocol, except that nuclease-free DNase I digestion (TaKaRa Bio Inc., Otsu, Shiga, Japan) was performed to eliminate genomic DNA contamination. Purified RNA was reverse-transcribed with oligo-deoxythymidine as a primer. RNA quality was checked by agarose electrophoresis. Gene-specific cDNA was amplified from first-strand cDNA by using a pair of gene-specific primers and the LightCycler-FastStart DNA Master SYBR Green I system (Roche Diagnostics GmbH, Mannheim, Germany) as described previously (Li et al., 2004). The housekeeping gene beta-glucuronidase (GUS; GenBank accession M13962) was used as a control to normalize expression of the genes concerned (Aerts et al., 2004). For quantitative analysis, GUS was co-amplified as an internal control. All PCR reactions were performed for 45–55 cycles in 4 mM $MgCl_2$. Gene-specific primer pairs were designed with Probe Design software (Roche). The primer sequences are listed in Table 1. The PCR product of each primer pair was verified by sequencing.

Table 1
Primers for apoptosis and housekeeping genes in real-time RT–PCR

Gene	Forward primer	Reverse primer
Fas	5′TGTCAACCGTGTCAGC 3′	5′GGTCACAGAGAGAAGC 3′
Bax	5′ATGATTGCTGACGTGG 3′	5′CCACAAAGATGGTCACT 3′
Bcl-2	5′TGGACAACATCGCTCT 3′	5′ACTGCTTTAGTGAACCT 3′
p53	5′CCGTATGCTGAGTATCT 3′	5′ACAAACACGAACCTCAA 3′
GUS	5′CTAAAGCTACGACTACCT 3′	5′CCTTAGCCGGTAACCA 3′

Caspase activation. Testis monocellular suspensions were prepared, collected and washed with PBS and suspended in 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiolthione, 2 mM phenylmethanesulfonyl fluoride, 10 μg/mL pepstatin A and 10 μg/mL leupeptin. Cells were clarified by centrifugation at 12,000×*g* for 20 min at 4 °C. The caspase activity in the supernatant was determined by a fluorogenic assay (Promega's CaspACE Assay System, Madison, WI, USA). Briefly, 50 μg total protein, as determined by Bio-Rad protein assay (Bio-Rad Laboratories), was incubated with 50 μM of one of the following fluorogenic substrates: Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) (caspase-3-specific substrate); Ac-Ile-Glu-Thr-Asp-AMC (Ac-IETD-AMC) (caspase-8-specific substrate); Ac-Leu-Glu-His-Asp-AMC (Ac-LEHD-AMC) (caspase-9-specific substrate) at 30 °C for 1 h. The release of methylcoumaryl-7-amine (AMC) was measured by excitation at 360 nm and emission at 460 nm on a fluorescence spectrophotometer (Varian, Palo Alto, CA, USA).

Histopathology of testis. The right testis was removed immediately after death, fixed in Bouin's solution for 48 h, and then embedded in paraffin. Sections from each testis (thickness 3–4 μm) were stained with hematoxylin and eosin for evaluation of damage to seminiferous tubules. Sections were observed under a light microscope (400×) (Zeiss, Axioskop2, Germany) interfaced with an image analyzer (CoolSNAP-pro, Germany).

Statistics. All values are presented as mean±SD. For the analysis of male offspring, the litter was considered the experimental unit. Comparisons between PCB 132-exposed and control groups were made by one-way analysis of variance (ANOVA), followed by the Tukey–Kramer Honestly Significant Difference (HSD) test with the JMP statistical package (SAS Institute, Inc., Cary, NC, USA).

Results

Body weights and tissue weights

A significant decrease in average cauda epididymis weight was found in 1 mg/kg group (Table 2). However, there were no statistical differences in body weight, right and left testis weights, right and left epididymis weights, and right and left cauda epididymidis weights between control and treated groups (data not shown).

Sperm quality

Significant ($P<0.05$) decreases in sperm count and total motile sperm count were found in the male offspring of 1 and 10 mg/kg PCB 132-treated dams (Table 2). Sperm counts were about 29–38% lower in both 1 mg/kg and 10 mg/kg groups than in controls. Total motile sperm counts were about 26–37% lower in both 1 mg/kg and 10 mg/kg groups than in controls (Table 2). However, there were no significant differences in

Table 2
Average cauda epididymal weight and sperm quality of rats prenatally exposed to PCB 132 (1 mg/kg or 10 mg/kg administered to the dam) and of unexposed controls

Parameters	Control	Treatment with PCB 132	
		1 mg/kg	10 mg/kg
Average cauda epididymal weight (mg)	232.2±28.6	201.7±15.9*	214.6±17.0
Epididymal sperm count (10^6/mg right cauda epididymis)	0.45±0.11	0.32±0.11*	0.28±0.05*
Motility (%)	64.6±16.8	60.2±11.1	60.9±18.1
Motile epididymal sperm count (10^6/ml) [a]	0.27±0.10	0.20±0.08	0.17±0.06*
VCL (μm/s) [b]	103.6±30.2	92.9±15.4	99.0±32.8
VAP (μm/s) [c]	69.5±22.0	56.0±10.0	61.2±20.5
VSL (μm/s) [d]	50.1±20.4	39.1±6.3	43.1±16.1
ALH (μm) [e]	3.5±2.1	3.6±1.0	3.9±2.0
BCF (Hz) [f]	13.3±9.5	11.9±1.8	12.8±5.7

[a] Motile epididymal sperm count (10^6/ml)=[epididymal sperm count (10^6/mg)×motility (%)]/100.
[b] VCL: curvilinear velocity.
[c] VAP: average path velocity.
[d] VSL: straight-line velocity.
[e] ALH: amplitude of lateral displacement.
[f] BCF: beat frequency.
* $P<0.05$ as compared with control group.

sperm VCL, VAP, VSL, ALH or BCF between the control and treated groups (Table 2).

ROS generation and sperm–oocyte penetration rate

There were dose-related increases in sperm ROS generation and decreases in SOPR, with these effects being significant ($P<0.05$) in offspring after 10 mg/kg PCB 132 treatment of the dam (Table 3).

Apoptosis-related gene expression

To determine whether apoptosis in rat testis is induced or inhibited by prenatal exposure to PCB 132, apoptosis-related gene expression was examined in frozen testicular tissue. In offspring whose dam received 1 mg/kg PCB 132, the gene expression of *p53* was significantly lower than in the control and 10 mg/kg groups (Table 4). In contrast, 10 mg/kg PCB 132 in the dam caused significant down-regulation of Fas, bax, bcl-2 and p53 as compared with control and 1 mg/kg groups (Table 4).

Table 3
Reactive oxygen species (ROS) generation and sperm–oocyte penetration rate (SOPR) of rats prenatally exposed to PCB 132 (1 mg/kg or 10 mg/kg administered to the dam) and of unexposed controls

Parameter	Control	Treatment with PCB 132	
		1 mg/kg	10 mg/kg
Chemiluminescence (counts/10^6 cells per s)	11.0±2.0	14.9±4.2	23.1±5.4*,**
SOPR (%) after 24 h of co-culture	35.4±17.4	24.1±14.6	19.1±11.6*

* $P<0.05$ as compared with control group.
** $P<0.05$ as compared with 1 mg/kg group.

Table 4
Apoptosis-related gene expression between rats prenatally exposed to PCB 132 (1 mg/kg or 10 mg/kg administered to the dam) and unexposed controls

Parameters	Control	Treatment with PCB 132	
		1 mg/kg	10 mg/kg
Fas/GUS	1.13±0.06	1.14±0.05	0.91±0.06*,**
Bax/GUS	1.07±0.08	1.17±0.08	0.65±0.06*,**
Bcl-2/GUS	1.21±0.09	1.28±0.06	0.88±0.05*,**
p53/GUS	1.02±0.05	1.19±0.04*	0.52±0.05*,**

Results were expressed relative to the amount of GUS transcripts used as internal control.
* $P<0.05$ as compared with control group.
** $P<0.05$ as compared with 1 mg/kg group.

Caspase activation

Maternal exposure to the higher dose of PCB 132 induced an approximately 1.9-fold and 1.8-fold increase in caspase-3 and -9 activity in the testis of the offspring, respectively, as compared with controls (Fig. 1). In contrast, a significant decrease in caspase-3 was observed in offspring in the low-dose group. There were no statistical differences in activation of caspase-8 between control and treated groups (Fig. 1).

Histopathology of testis

There were no significant differences in spermatogenesis among the three groups (Fig. 2); in all groups, most of the seminiferous tubules revealed normal spermatogenesis. The presence of loose epithelium in the seminiferous tubules was rare and mostly associated with the method of histological preparation.

Discussion

PCB 132 is a stable non-dioxin-like PCB congener and its metabolites can be detected in human tissues (Chu et al., 2003; Glausch et al., 1995; Weistrand and Noren, 1997). However, possible effects of prenatal exposure to PCB 132 on testicular

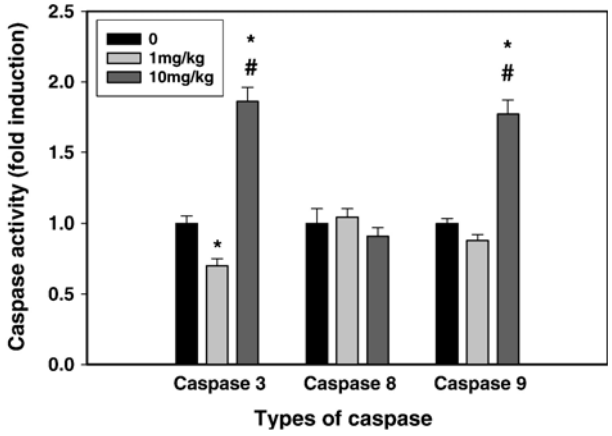

Fig. 1. Activity of caspase-3, -8, and -9 in rats prenatally exposed to PCB 132. *$P<0.05$ as compared with control group; #$P<0.05$ as compared with 1 mg/kg group.

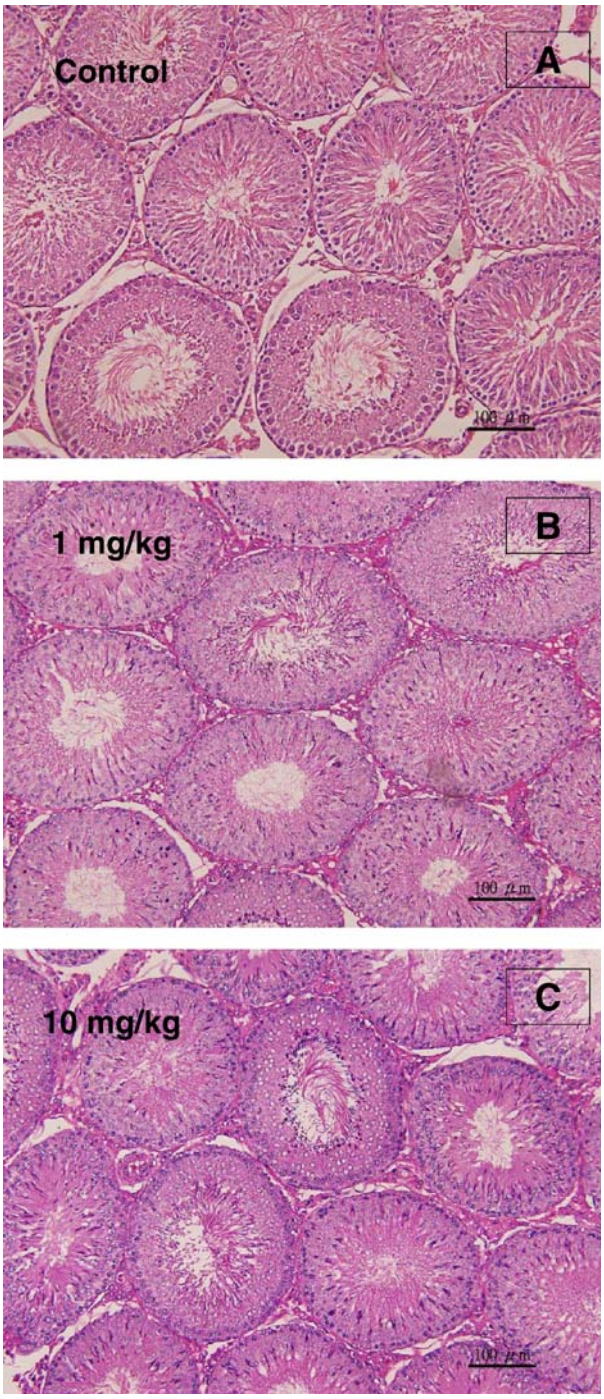

Fig. 2. Photomicrographs of H and E stained sections of rat offspring testis showing normal testicular histology with evidence of spermatogenesis in all three groups: (A) controls; (B) 1 mg/kg PCB 132-treated group; and (C) 10 mg/kg PCB 132-treated group. Scale bar: 100 μm.

and sperm functions are not well documented in animal and human studies. In our previous study, a single dose of 9.6 mg/kg of PCB 132 at PND 21 in male rats decreased serum total T_3 level and SOPR at PND 112; a single dose of 96 mg/kg significantly decreased sperm motility, velocity, SOPR, and serum-free T_3 (Hsu et al., 2003b). In the present study, prenatal exposure to PCB 132 with a single dose of 1 or 10 mg/kg decreased average cauda epididymal weight, epididymal sperm count, and motile epididymal sperm count in adult offspring. Kuriyama and Chahoud (2004) have demonstrated that adult male rat offspring exposed pre- and postnatally to a low dose of a mono-ortho PCB congener, PCB 118, showed significant changes in reproductive organ weights and decreases in spermatid and sperm number. Recent epidemiological studies have suggested that low-level exposure to PCBs, such as may be experienced by the general population, have been associated with effects on sperm motility, sperm concentration, and total sperm count (Hauser et al., 2003, 2005; Richthoff et al., 2003). Using PCB 132, we have demonstrated that sperm count and total motile sperm count in rats were decreased in a way similar to that reported in humans. In view of the strong evidence for adverse effects of PCBs on human male fertility, further investigations using environmentally relevant doses are important for hazard identification and risk assessment. In addition, we investigated that exposure of dams to PCB 132 led in the offspring to dose-related increases in ROS and decreases in SOPR in the spermatozoa. A potential mechanism whereby PCBs may produce oxidative stress is through hydroxylated PCB metabolites, which are found in human serum in relatively high concentrations (Sjodin et al., 2000). These metabolites can be oxidized to form semiquinones and quinones, which are reactive electrophiles that may induce free-radical-mediated oxidative DNA damage (Li and Trush, 1993). Mammalian sperm are particularly susceptible to oxidative stress because they contain an extremely high concentration of polyunsaturated fatty acids, exhibit no capacity for membrane repair, and have a significant ability to generate ROS, chiefly superoxide anion and hydrogen peroxide (Jones et al., 1979). In men with fertility problems, the negative association between ROS generation and sperm capability to fuse with the oocyte strongly suggests a significant role of peroxidative damage to the sperm plasma membrane. The possibility that oxidative stress to the sperm plasma membrane might be involved in those cases of infertility characterized by a failure to exhibit sperm–oocyte fusion was suggested by a study indicating an association between the appearance of defects and the hyperactive generation of ROS by sperm (Aitken and Clarkson, 1987).

In the present investigation, the relationships of testicular gene expression, enzymatic activity of caspases and epididymal sperm functions with PCB 132 treatment are summarized in Fig. 3. In the low-dose PCB 132 group, p53 was significantly induced and caspase-3 was inhibited (Fig. 3A). Under stress, such as DNA damage and hypoxia, increased levels of p53 cause cell cycle arrest at the G1 and G2 checkpoints in dividing cells (Dulic et al., 1994; Miyashita and Reed, 1995) or induce apoptosis (Graeber et al., 1996). It has been hypothesized that p53 arrests cell cycle progression and DNA replication in order to allow time for DNA exchange and repair of damaged DNA. p53 has been shown to regulate the activity of *GADD45* (Kastan et al., 1992) and *p21WAF1/CIP1* (Harper et al., 1993) genes associated with growth suppression and DNA repair (Smith et al., 1994). In cases where DNA damage is severe, p53 is suspected of inducing apoptosis. In addition, p53 mediates spontaneous testicular germ cell damage, and failure to remove defective germ cells by this mechanism results in

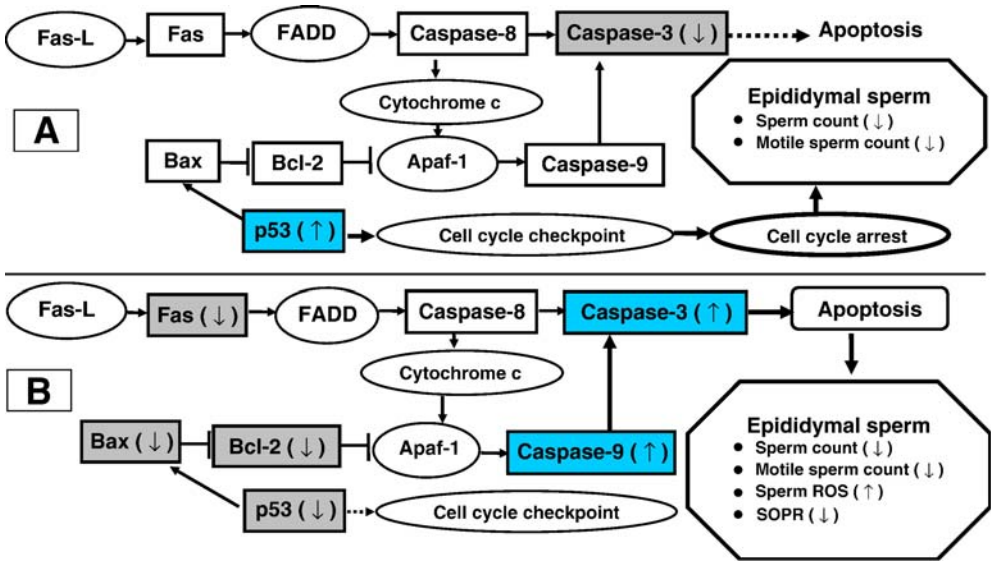

Fig. 3. Key components of gene expression and caspase activation on the pathway of apoptosis induced or inhibited by low-dose (A) or high-dose (B) exposure to PCB 132. Prenatal exposure to non-dioxin-like congeners, PCB 132, affected epididymal sperm function by altering expression of apoptosis-related genes including Fas, Bax, bcl-2, and p53 as well as activation of caspase-3, -8, and -9 in testis. Gene expression and caspase activation (analyzed with real-time RT–PCR and fluorogenic assay, respectively): blue boxes, increased; gray boxes, decreased. In mammalian cells, the first pathway of apoptosis involves release of cytochrome *c* from mitochondria into the cytosol where it binds to Apaf-1, resulting in the activation of caspase-9 and the subsequent activation of the executioner caspase-3, -6, and -7. The second pathway involves ligation of Fas to Fas-L, resulting in the activation of a different set of initiator caspases, namely caspases-8 and -10, through interactions between death domains and death effector domains of an adopter molecule such as FADD and these caspases. The bcl-2 family of proteins usually governs the first pathway for apoptosis. In addition, the ability of p53 to serve as a transcriptional regulator may be associated with its functional role in maintaining cell cycle checkpoints and causing cell cycle arrest at the G1 and G2 checkpoints or leading to cell death by inducing the expression of Bax. The third pathway involves the ER and caspase-12 (not shown in this figure). All these pathways eventually converge on caspase-3 and other executioner caspases that drive the terminal events of programmed cell death. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increased percentages of abnormal sperm and hence in reduced fertility. This study showed that, after exposure to low-dose PCB 132, spermatogenesis might be impaired by the pathway of p53-mediated cell cycle arrest but not by the p53–Bax pathway.

The balance between germ cell proliferation, differentiation, and apoptosis is critical to the maintenance of spermatogenesis. During spermatogenesis, Fas has been localized to germ cells, and Fas-L to Sertoli cells, within the rat testis (Lee et al., 1997). The *Fas* and *Fas-L* genes and their protein products have been shown to be up-regulated in rats exposed to Sertoli cell toxicants that induce apoptosis of the germ cells (Lee et al., 1997). There are some clues for understanding the underlying molecular mechanisms governing germ cell death in the testis. For example, bcl-2 transgenic mice, in which a human *bcl-2* transgene, an antiapoptotic gene, is overexpressed in spermatogonia, have overpopulated spermatogonia and a decreased incidence of germ cell apoptosis (Furuchi et al., 1996). Targeted gene disruption of *Bax*, a proapoptotic gene, in mice revealed hyperplasia of spermatogonia as well as massive death of early spermatocytes, suggesting *Bax*-dependent and -independent apoptosis pathways (Knudson et al., 1995). In addition to physiologic germ cell apoptosis, massive increases in germ cell apoptosis are observed after exposure of laboratory animals to various toxicants (Uzumcu et al., 2004), raised testicular temperature (Yin et al., 1998), or radiation (Lee et al., 2002). Apoptosis can be raised through different pathways, the main intracellular effectors being the caspases, a family of cysteine proteases. These enzymes exist in cells as inactive zymogens and become activated through proteolysis when cells receive apoptotic signals. In apoptosis, caspases function both in cell disassembly and in initiating this disassembly. The first or the intrinsic pathway for apoptosis involves the release of cytochrome *c* into the cytosol where it binds to apoptotic protease-activating factor-1 (Apaf-1). Once activated by the cytochrome *c*, Apaf-1 then binds to procaspase-9 via the caspase recruitment domain (CARD) at the amino terminus in the presence of dATP, resulting in activation of the initiator caspase-9 and the subsequent proteolytic activation of the executioner caspase-3, -6, and -7. The active executioners are then involved in the cleavage of a set of proteins, including poly (ADP) ribose polymerase (PARP), lamin, actin, and gelsolin, and this causes morphological changes to the cell and nucleus typical of apoptosis. Members of the bcl-2 family of proteins play a major role in governing this mitochondria-dependent apoptotic pathway, with proteins such as Bax functioning as inducers, and proteins such as bcl-2 as suppressors, of cell death (Hengartner, 2000; Reed, 2000). All these pathways converge on caspase-3 and other executioner caspases and nucleases that drive the terminal events of programmed cell death.

In this study, activation of caspase-3 and -9 was significantly increased. In contrast, the gene expressions of *Fas*, *Bax*, *bcl-2*, and *p53* were significantly decreased after prenatal exposure to a high dose (10 mg/kg) of PCB 132. Kijima et al. (2004) found that di(2-ethylhexyl) phthalate suppressed the expression of the genes related with atrophy and connective tissue growth factor,

as well as the collagen alpha 2 type V gene, cell adhesion kinase gene and the testosterone-repressed prostatic-message-2 (*TRPM-2*) gene. Those results indicated that the suppression of gene expressions might be caused by the atrophic toxicity of the endocrine disruptor with long-term exposure. In the present study, treatment of fetal rats with PCB 132 did not cause toxic effects in the morphology of the seminiferous epithelium. However, there was up-regulation of p53 in the low-dose PCB 132 group and down-regulation of Fas, Bax, bcl-2, and p53 in the high-dose group. Hence, there were PCB 132-related molecular alterations in gene expression and enzyme activity, although there were no changes in testis histology. Several experiments have demonstrated a dose–response relationship characterized by low-dose stimulation and high-dose inhibition and inverted-U dose–response curves at dose ranges close to those of human exposure (Calabrese and Baldwin, 2003; vom Saal et al., 1997). This indicates the need for further discussion of low-dose and high-dose risk assessment. In conclusion, this study demonstrated the adverse effects of prenatal exposure to PCB 132 on subsequent epididymal sperm function. Although the data on gene expression and caspase activity may provide insights into the mechanisms by which PCB 132 affects reproduction in the male offspring in rats, the PCB 132 concentrations are not biologically or environmentally relevant. Further efforts using environmentally relevant doses are required for a more complete understanding of the underlying molecular mechanisms governing the pathway of apoptosis in different cells of the testis, and the role of endocrine disruptors in male reproductive disorders.

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References

Aerts, J.L., Gonzales, M.I., Topalian, S.L., 2004. Selection of appropriate control genes to assess expression of tumor antigens using real-time RT–PCR. BioTechniques 36, 84–91.

Aitken, R.J., Clarkson, J.S., 1987. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. J. Reprod. Fertil. 81, 459–469.

Cai, L., Hales, B.F., Robaire, B., 1997. Induction of apoptosis in the germ cells of adult male rats after exposure to cyclophosphamide. Biol. Reprod. 56, 1490–1497.

Calabrese, E.J., Baldwin, L.A., 2003. Hormeosis: the dose–response revolution. Annu. Rev. Pharmacol. Toxicol. 43, 175–197.

Chittendenm, T., Harrington, E.A., O'Connor, R., Flemington, C., Lutz, R.J., Evan, G.I., Guild, B.C., 1995. Induction of apoptosis by the Bcl-2 homologue Bak. Nature 374, 733–736.

Chu, S., Covaci, A., Schepens, P., 2003. Levels and chiral signatures of persistent organochlorine pollutants in human tissues from Belgium. Environ. Res. 93, 167–176.

DeRosa, C., Richter, P., Pohl, H., Jones, D.E., 1998. Environmental exposures that affect the endocrine system: public health implications. J. Toxicol. Environ. Health, B. Crit. Rev. 1, 3–26.

Dulic, V., Kaufmann, W.K., Wilson, S.J., Tisty, T.D., Lees, E., Harper, J.W., Elledge, S.J., Reed, S.I., 1994. p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell 76, 1013–1023.

Fukuzawa, N.H., Ohsako, S., Nagano, R., Sakaue, M., Baba, T., Aoki, Y., Tohyama, C., 2003. Effects of 3,3′,4,4′,5-pentachlorobiphenyl, a coplanar polychlorinated biphenyl congener, on cultured neonatal mouse testis. Toxicol. In Vitro 17, 259–269.

Furuchi, T., Masuko, K., Nishimune, Y., Obinata, M., Matsui, Y., 1996. Inhibition of testicular germ cell apoptosis and differentiation in mice misexpressing Bcl-2 in spermatogonia. Development 122, 1703–1709.

Giesy, J.P., Kannan, K., 1998. Dioxin-like and non-dioxin-like toxic effects of polychlorinated biphenyls (PCBs): implications for risk assessment. Crit. Rev. Toxicol. 28, 511–569.

Glausch, A., Hahn, J., Schurig, V., 1995. Enantioselective determination of chiral 2,2′,3,3′,4,6′-hexachlorobiphenyl (PCB 132) in human milk samples by multidimensional gas chromatography–electron capture detection and by mass spectrometry. Chemosphere 30, 2079–2085.

Graeber, T.G., Osmanian, C., Jacks, T., Housman, D.E., Koch, C.J., Lowe, S.W., Giaccia, A.J., 1996. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 379, 88–90.

Guo, Y.L., Hsu, P.C., Hsu, C.C., Lambert, G.H., 2000. Semen quality after prenatal exposure to polychlorinated biphenyls and dibenzofurans. Lancet 356, 1240–1241.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., Elledge, S.J., 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75, 805–816.

Hauser, R., Chen, Z., Pothier, L., Ryan, L., Altshul, L., 2003. The relationship between human semen parameters and environmental exposure to polychlorinated biphenyls and *p,p′*-DDE. Environ. Health Perspect. 111, 1505–1511.

Hauser, R., Williams, P., Altshul, L., Calafat, A.M., 2005. Evidence of interaction between polychlorinated biphenyls and phthalates in relation to human sperm motility. Environ. Health Perspect. 113, 425–430.

Hengartner, M.O., 2000. The biochemistry of apoptosis. Nature 407, 770–776.

Hsu, S.T., Ma, C.I., Hsu, S.K., Wu, S.S., Hsu, N.H., Yeh, C.C., 1984. Discovery and epidemiology of PCB poisoning in Taiwan. Prog. Clin. Biol. Res. 137, 71–79.

Hsu, P.C., Huang, W., Yao, W.J., Wu, M.H., Guo, Y.L., Lambert, G.H., 2003a. Sperm changes in men exposed to polychlorinated biphenyls and dibenzofurans. JAMA 289, 2943–2944.

Hsu, P.C., Li, M.H., Guo, Y.L., 2003b. Postnatal exposure of 2,2′,3,3′,4,6′-hexachlorobiphenyl and 2,2′,3,4′,5′,6-hexachlorobiphenyl on sperm function and hormone levels in adult rats. Toxicology 187, 117–126.

Jones, R., Mann, T., Sherins, R., 1979. Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides and protective action of seminal plasma. Fertil. Steril. 31, 531–537.

Kastan, M.B., Zhan, Q., El-Deiry, W.S., Carrier, F., Jacks, T., Walsh, W.V., Plunkett, B.S., Vogelstein, B., Fornace Jr., A.J., 1992. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia–telangiectasia. Cell 71, 587–597.

Kijima, K., Toyosawa, K., Yasuba, M., Matsuoka, N., Adachi, T., Komiyama, M., Mori, C., 2004. Gene expression analysis of the rat testis after treatment with di(2-ethylhexyl) phthalate using cDNA microarray and real-time RT–PCR. Toxicol. Appl. Pharmacol. 200, 103–110.

Knudson, C.M., Tung, K.S.K., Tourtellotte, W.G., Brown, G.A.J., Korsmeyer, S.J., 1995. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science 270, 96–99.

Kuriyama, S.N., Chahoud, I., 2004. In utero exposure to low-dose 2,3′,4,4′,5-pentachlorobiphenyl (PCB 118) impairs male fertility and alters neurobehavior in rat offspring. Toxicology 202, 185–197.

Lee, J., Richburg, J.H., Younkin, S.C., Boekelheide, K., 1997. The Fas system is a key regulator of germ cell apoptosis in the testis. Endocrinology 138, 2081–2088.

Lee, K., Park, J.S., Kim, Y.J., Lee, Y.S., Hwang, T.S., Kim, D.J., Park, E.M., Park, Y.M., 2002. Differential expression of Prx I and II in mouse testis and their up-regulation by radiation. Biochem. Biophy. Res. Commun. 296, 337–342.

Li, Y., Trush, M.A., 1993. DNA damage resulting from the oxidation of

hydroquinone by copper: role for a Cu(II)/Cu(I) redox cycle and reactive oxygen generation. Carcinogenesis 14, 1303–1311.

Li, L.A., Wang, P.W., Chang, L.W., 2004. Polychlorinated biphenyl 126 stimulates basal and inducible aldosterone biosynthesis of human adrenocortical H295R cells. Toxicol. Appl. Pharmacol. 195, 92–102.

McKinney, J.D., Waller, C.L., 1998. Molecular determinants of hormone mimicry: halogenated aromatic hydrocarbon environmental agents. J. Toxicol. Environ. Health, B. Crit. Rev. 1, 27–58.

Miyashita, T., Reed, J.C., 1995. Tumor suppressor p53 is a direct transcriptional activator of the human *bax* gene. Cell 80, 293–299.

Oskam, I.C., Ropstad, E., Smith, A.J., Skaare, J.U., Tverdal, A., Berg, K.A., Wiger, R., 2004. Effects of PCB99 and PCB153 exposure on spermatogenesis in young adult C57BL6 mice. Reprod. Toxicol. 19, 169–180.

Reed, J.C., 2000. Mechanisms of apoptosis. Am. J. Pathol. 157, 1415–1430.

Richthoff, J., Rylander, L., Jonsson, B.A., Akesson, H., Hagmar, L., Nilsson-Ehle, P., Stridsberg, M., Giwercman, A., 2003. Serum levels of 2,2,4,4,5,5-hexachlorobiphenyl (CB-153) in relation to markers of reproductive function in young males from the general Swedish population. Environ. Health Perspect. 111, 409–413.

Riedel, R., Schlenk, D., Frank, D., Costa-Pierce, B., 2002. Analysis of organic and inorganic contaminants in Salton sea fish. Marine Pollut. Bull. 44, 403–411.

Sinha Hikim, A.P., Wang, C., Lue, Y., Johnson, L., Wang, X.H., Swerdloff, R.S., 1998. Spontaneous germ cell apoptosis in humans: evidence for ethnic differences in the susceptibility of germ cells to programmed cell death. J. Clin. Endocr. Metab. 83, 152–156.

Sjodin, A., Hagmar, L., Klasson-Wehler, E., Bjork, J., Bergman, A., 2000. Influence of the consumption of fatty Baltic Sea fish on plasma levels of halogenated environmental contaminants in Latvian and Swedish men. Environ. Health Perspect. 108, 1035–1041.

Smith, M.L., Chen, I.T., Zhan, Q., Bae, I., Chen, C.Y., Gilmer, T.M., Kastan, M.B., O'Connor, P.M., Fornace Jr., A.J., 1994. Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science 266, 1376–1380.

Uzumcu, M., Suzuki, H., Skinner, M.K., 2004. Effect of the anti-androgenic endocrine disruptor vinclozolin on embryonic testis cord formation and postnatal testis development and function. Reprod. Toxicol. 18, 765–774.

Vaux, D.L., Cory, S., Adams, T.M., 1988. Bcl-2 promotes the survival of haemopoietic cells and co-operates with c-*myc* to immortalize pre-B cells. Nature 335, 440–442.

vom Saal, F.S., Timms, B.G., Montano, M.M., Palanza, P., Thayer, K.A., Nagel, S.C., Dhar, M.D., Ganjam, V.K., Parmigiani, S., Welshons, W.V., 1997. Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. Proc. Natl. Acad. Sci. U.S.A. 94, 2056–2061.

Weistrand, C., Noren, K., 1997. Methylsulfonyl metabolites of PCBs and DDE in human tissue. Environ. Health Perspect. 105, 644–649.

Yin, Y., DeWolf, W.C., Morgentaler, A., 1998. Experimental cryptorchidism induces testicular germ cell apoptosis by p53-dependent and -independent pathways in mice. Biol. Reprod. 58, 492–496.