Chronic Effects of Xenoestrogen, 4-*tert*-Octylphenol, on Apoptosis of Germ Cells in Rat Testis

Suel Kee Kim, Chang Joo Lee, Yong Dal Yoon
Department of Life Science, College of Natural Sciences, Hanyang University, Seoul, 133-791

Introduction

Xenoestrogens have become an increasingly important issue over the last few years because data suggest that there has been a progressive increase in disorders of male reproductive development and fertility and there is concern as to whether these compounds could have contributed to these adverse changes (Yoon, 1998; Safe, 2000).

Alkylphenols are surfactants used as diluents in pesticides and as components in paints, shampoos, and inks as well as cosmetics.

Previous studies have been demonstrated that exposure of the developing male fetus or neonate to estrogen chemicals could result in reduced testicular size and sperm production in adult life (Sharp, 1993; 1994).

The aims of the present experiments were to evaluate (1) the putative toxicity and estrogenicity on the reproductive system and (2) the impairment of steroidogenesis and spermatogenesis in the male rat which were exposed at the prepubertal stages. The present study was also extended to (3) identify the changes of the gene expression of bcl-2, bcl-x, and bax in order to clarify the mechanism(s) for the impairment of reproductive tissue.

Materials and Methods

Male Fischer 344 rats (body wt. 35.6±8.3g, 4 wk old) were used. Rats were injected s.c. with 0.2 ml olive oil alone or containing 20 mg, 40 mg, 80 mg OP, or 0.8 μg EV. EV was used at 1/1000 of the 80-mg dose of OP. Injection were given thrice weekly for 1 month. These animals were then killed on 3 days after the last injection, at 8 weeks old. Blood serum was collected by cardiac puncture and testosterone concentration were measured by radioimmunoassay. For morphological assessments, the left testes were placed in 10% neutral buffered formalin (NBF). Transverse sections, 4 μm thick, stained with hematoxylin and eosin. Labelling of DNA fragmentation in each testis section was performed using *in situ* apoptosis detection kit (Apoptaq; Oncor). Changes of the gene expression of bcl-2, bcl-x, and bax were identified by RT-PCR. Total RNA was extracted from right testis and hypothalamus using RNAzol^{TM}B. Each oligonucleotide primer pairs were designed based on the sequences as follows (Table 1).

	Primer sequences	References
β-actin	5'-CACTGCCGCATCCTCTTCCT-3' 5'-AGCCACCAATCCACACAGAG-3'	Yoshikazu et al, 2000.
Bcl-2	5'-TGGTGGACAACATCGCTCTG-3' 5'-AGGTCTGCTGACCTCACTTGT-3'	Dena B et al, 1999
Bcl-x	5'-AGGCTGGCGATGAGTTTGAA-3' 5'-CGGCTCTCGGCTGCTGCATT-3'	Dena B et al, 1999
Bax	5'-TCCACCAAGAAGCTGAGCGAGT-3' 5'-CACAAAGATGGTCACTGTCTGCC-3'	Masayuki et al, 1999.

Table 1. The primer sequences and conditions for RT-PCR of β-actin, bcl-2, bcl-x, and bax genes used in the present study

Results and Discussion

Present study clearly demonstrate that exposure of a prepubertal male rat to OP can seriously alter the weights of testes and other reproductive organ in their adult life. The most notable changes that occurred were extensive shrinkages of the testis, epididymis, and seminal vesicle (Table 2).

One of the most critical estrogenic influences that OP may have on the reproductive system is to induce a decrease in testosterone secretion. Administration of all three doses of OP and 0.8 μg of EV caused a marked decrease in serum testosterone concentrations (Figure 1). Treatment of OP is known to reduce serum gonadotropin levels (Charles et al., 1997).

Table 2. Changes of rat gonad index and testis volume after the treatment.

	Olive oil	EV (0.8 μg)	OP (20 mg)	OP (40 mg)	OP (80 mg)
Gonad Index (%)	399 ± 6	50 ± 3*	170 ± 22.*	94 ± 6*	71 ± 9*
Testis volume (μl)	2180 ± 117	199 ± 37*	611 ± 74*	313 ± 52*	287 ± 65*

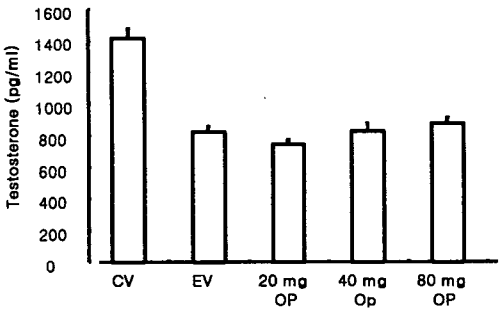

Figure 1. Changes of serum testosterone concentration of rat after the treatment.

Serum testosterone concentrations may be reduced because of insufficient LH in serum to stimulate Leydig cells. It is also known that OP can act directly on Leydig cells to inhibit testosterone synthesis. Treatment of pregnant rats with 4-octylphenol or diethystilbesterol did not affect Leydig cell numbers in fetuses but inhibited fetal Leydig cell 17α-hydroxylase activity, which is needed for testosterone synthesis (Majdic et al., 1996).

By histochemical observation, there was a marked reduction in seminiferous tubule size the and disruption of spermatogenic cell was mainly seen in pachytene primary spermatocytes, round spermatids, elongating spermatids (Table 3). A marked degeneration of theses cell types would likely result in a marked decline in sperm number.

Results from TUNEL immunohistochemistry showed that apoptotic cells of testis markedly increased in rats treated with EV and all three doses of OP (Figure 2). The predominat cell types undergoing apoptosis were spermatocytes and round spermatids located toward the lumen of the seminiferous tubule although apoptotic germ cells sloughed from the epithelium were also observed occasionally.

Table 3. Seminiferous tubule diameter of rats after the treatment.

	Olive oil	EV (0.8 μg)	OP (20 mg)	OP (40 mg)	OP (80 mg)
SD (μm)	239 ± 8	124 ± 4*	173.1 ± 4*	137.7 ± 6*	124.1 ± 5*

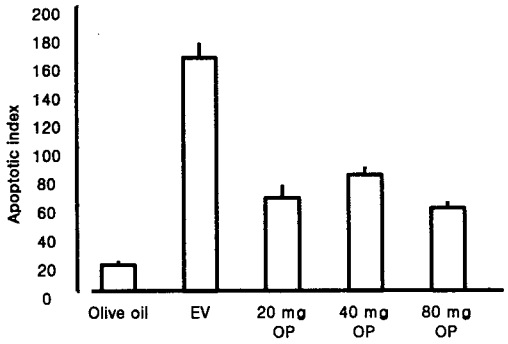

Figure 2. Apoptotic index of testicular cells on rat testis after the treatment.

The expressions of Bcl-2 and Bax mRNA in testis of rats treated with 20, 40, 80 mg of OP were slightly elevated compare to that of olive oil-injected control. In contrast, the expression of Bcl-x (L&S) mRNA were not significantly changed (Figure 3). Since Bax is a pro-apoptotic gene, it is possible that Bax is induced or that its production is increased as a result of the induction of apoptosis, thus explaining its up-regulation after OP treatment. A rise in Bcl-2 mRNA levels could be explained by an increase in Bcl-2 production by the remaining germ cells. Perhaps physiologically, Bcl-2 is up-regulated in these cells as part of a survival mechanism.

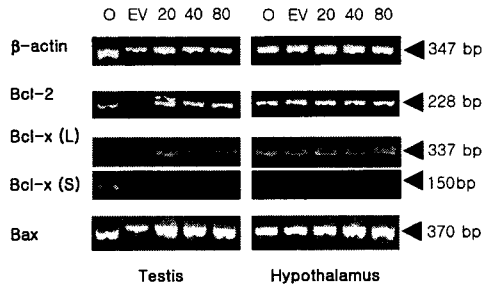

Figure 3. Electrophoretogram of RT-PCR products for -actin, bcl-2, bcl-x and bax mRNA in rat after the treatment.

In summary, this study clearly demonstrated that OP when administered to prepubertal rats

severly disrupts the functions of male reproductive organs. These effects can be explained on the basis of a disruption of the endocrine system and a induction of apoptosis of spermatogenic cell. Although OP likely acts like estrogen to cause such disruption, the additional direct, non-estrogenic toxic influences on reproductive tissue or cells cannot be ruled out. It remains to be determined whether OP in the environment is absorbed in sufficient amounts and for sufficient lengths of time to pose a threat to wildlife and humans.

References

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