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# Effects of octylphenol on the expression of *StAR*, *CYP17* and *CYP19* in testis of *Rana chensinensis*

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ABSTRACT

It has been proposed that a decline in sperm quality is associated with exposure to environmental chemicals with estrogenic activity. Seeking possible explanations for this effect, this study investigated the effects of octylphenol (OP) on the synthesis of steroid hormones in amphibian. *Rana chensinensis* were exposed to $10^{-8}$, $10^{-7}$ and $10^{-6}$ mol/L OP after 10, 20, 30 and 40 days. The cDNA fragments of *StAR* (274 bp), *CYP17* (303 bp) and *CYP19* (322 bp) were cloned. *In situ* hybridization and immunohistochemistry revealed that positive signals of *StAR*, *CYP17*, *CYP19* mRNA and proteins mainly in the Leydig cells of testes. Real-time PCR showed that up-regulation of *StAR* and *CYP19*, and down-regulation of *CYP17* after exposure to $10^{-8}$, $10^{-7}$ and $10^{-6}$ mol/L OP. The results suggest that OP can alter transcriptions of *StAR*, *CYP17* and *CYP19*, thus disturb the expressions of StAR, P450c17 and P450arom, thereby adversely affect steroid synthesis.



## 1. Introduction

There is increasing concern about the environmental exposure to chemicals and consequent adverse health effects in humans in both the scientific community and among the general public. Some chemicals have been clearly demonstrated to be carcinogenic and genotoxic in studies on animals, but it is challenging to establish a causal association between prolonged low-level exposure in humans and adverse health effects. Nevertheless using animals as sentinels for environmental hazards to humans can demonstrate biologically plausible etiologies for some conditions in humans. Endocrine disruptors are chemicals that may interfere with the body's endocrine system and produce adverse developmental, reproductive, neurological, metabolic and immune effects in both humans and wildlife (Crump, 2001; Gancarczyk et al., 2004; Hogan et al., 2006; Adler, 2007; Hejmej et al., 2011; Schwendiman and Propper, 2012; Kim et al., 2015).

It has been proposed that a global decline in sperm counts and semen quality is associated with exposure to environmental chemicals which act as endocrine disruptors particularly those with estrogenic activity (Jure, 2013). One such group of chemicals the Alkylphenol polyethoxylates (APEs) are widely used as non-ionic surfactants in the manufacture of several consumer products and in industrial processing. 4-tert-Octylphenol (OP) is one of degradation product of APEs, and represent about 15–20% of the total APEs market, which is thought to be quite stable in the marine environment through factory waste water, and more than 50,000 tons per year of OP is produced and distributed everywhere (Isobe et al., 2001; OSPAR commission, 2009; Othman et al., 2012). OP has been measured in surface waters of the Danube near Budapest at 1.6–178 ng/L (Nagy et al., 2005), the Haihe River in China at 18–20 ng/L (Jin et al., 2004), the Mai Po Marshes Nature Reserve in Hong Kong at 11–348 ng/L (Li et al., 2007), and in Great Lakes sediments in the USA and Canada at 0.002–23.7 ng/g (Bennett and Metcalfe, 1998).

It has been demonstrated that OP is one of the endocrine-disrupting chemicals (EDCs) that can interfere normal development and reproduction of vertebrate *via* disturbance the endocrine activities (Diamanti-Kandarakis et al., 2009; Casals-Casas and Desvergne, 2011). OP could display potent estrogenicity and reproductive toxicity including disruption of spermatogenesis, feminization of males and changes in reproductive behavior (Ball et al., 1989; White et al., 1994; Gronen et al., 1999; Hogan et al., 2006; Porter et al., 2011), which interferes with the homeostasis, reproduction, growth and behaviors of organisms through its influence on steroid hormones synthesis (Susanne and Thomas, 2002; Rhee et al., 2008; Rhee et al., 2009; Bianco et al., 2011; Li et al.,

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2016). Various steroidogenic enzymes participate in the process such as steroid acute regulatory protein (StAR), cytochrome P450 aromatases and cytochrome P450c17 (Carreau et al., 2003, 2006; Miller, 2007; Ksenia et al., 2008; Wu et al., 2010). OP can also affect expression of some key genes involved in steroid hormone synthesis such as *StAR*, *CYP17*, *CYP19* in multiple vertebrate species (Myllymaki et al., 2005; Lee et al., 2006; Yon et al., 2007; Vang et al., 2007; Kim et al., 2015; Wolff et al., 2015). However, the precise molecular mechanisms for how OP affects the regulation of steroidogenic-related genes are still unclear. The gene expression profile can serve as a new endpoint for assessing ecotoxicology (Snell et al., 2003). Therefore, it is necessary to explore the differential gene expression by OP exposure to reveal its molecular mechanism.

In this project, the Chinese brown frog (*Rana Chensinensis*) were used to study the effect of exposure to OP on the expression of some of the key genes involved in steroid hormone synthesis. This species of frog is highly sensitive to chemical pollution in aquatic environments (Lundberg et al., 2007; Li et al., 2014, 2016). The objective of this study was to explore the mechanism of the estrogenic effects of EDCs on the reproduction of male amphibians by the expression of steroidogenic-related genes *StAR*, *CYP17* and *CYP19*, and the localization of related proteins in the testes of *R. chensinensis* exposed to OP. The results contribute to the further understanding of the estrogenicity and reproductive toxicity mechanisms of OP as EDCs.

## 2. Material and methods

### 2.1. Chemicals

4-tert-Octylphenol (OP) (Sigma-Aldrich, St. Louis, MO, USA); primary antibody for StAR (rabbit anti-mouse polyclonal antibody, Santa Cruz), P450c17 and P450arom (rabbit anti-mouse polyclonal antibody, Bioworld); SABC and DAB kits (Boster); total RNA extraction reagent (Bio Flux); reverse transcription kit and gel purification kit products (TakaRa); T4 DNA Ligase, pGEM-T easy Vector and Taq DNA Polymerase (Promega); Alkaline phosphatase-conjugated anti-DIG antibody; SP6/T7 digoxigenin-labeled RNA kit; NBT/BCIP chromogenic reagent (Roche); plasmid extraction kit (Axygen); SYBR® Premix Ex Taq™ II (TakaRa).

### 2.2. Animals and treatments

Male frogs, *Rana chensinensis*, captured from a rivulet in Qinling Mountain, Shaanxi, China, were housed in aquariums in the lab at 20–22 °C with a 12 h/12 h light/darkness cycle, and fed with worms (*Tenebrio molitor*) twice daily. One hundred and eighty healthy frogs were selected as valid experimental material, aged from 3 to 4 years, weighing from 6.95–7.22 g. Frogs were randomly divided into four groups, including one control group and three experimental groups, 45 in each. The experimental groups were raised in water with added OP at concentration of $10^{-8}$, $10^{-7}$, $10^{-6}$ mol/L, respectively. The control group was raised in dechlorinated tap water. In the process of exposure, water renewal containing OP was performed every 24 h.

All frogs were killed under pentobarbital sodium anesthesia after exposure to OP for 10, 20, 30 and 40 days, respectively. Nine samples were collected from each group at a time. The testes were quickly detached from the frogs, and cut into several parts immediately. Some pieces were frozen in liquid nitrogen stored at −80 °C until RT-PCR and real-time PCR analysis could be undertaken, while other parts were fixed in 4% paraformaldehyde for *in situ* hybridization and immunohistochemistry assessment. The experiment was approved by Institutional Animal Care and Use Committee, College of Life Science, Shaanxi Normal University and was conducted in compliance with good laboratory practices. All frogs received humane care according to the criteria outlined in the guide for the care and use of the experimental animal management rules of Ministry of Health, China.

**Table 1**
PCR primers used in RT-PCR.

| Gene | Primer name | Primer Sequence 5′- 3′ | Tm | Product size |
|---|---|---|---|---|
| *StAR* | Forward-1 | TCATACCGACATCTACGC | 52 °C | 274 bp |
| | Reverse-1 | TTCTGCCACAATCTCAAC | | |
| *CYP*17 | Forward-2 | CGCTGTGTATGTTCGGTGAAGG | 53 °C | 303 bp |
| | Reverse-2 | GGTCTCGAGCTGCCACTGACT | | |
| *CYP*19 | Forward-3 | TCCAAAGGCTGAGAAGATGA | 52 °C | 322 bp |
| | Reverse-3 | GAAACCAAATGGCTGAAAGT | | |

**Table 2**
PCR primers used in Real-time PCR.

| Gene | Primer name | Primer Sequence 5′- 3′ | Tm | Product size |
|---|---|---|---|---|
| *β-actin* | Sense-1 | ACTGTGCTGTCTGGAGGTA | 57 °C | 115 bp |
| | Antisense-1 | TCTGGTGGTGCAATAATCT | | |
| *StAR* | Sense-1 | CGTGAACTGGAAAAACTTGC | 58 °C | 153 bp |
| | Antisense-1 | TAATGCCTCCTGTCCCTGT | | |
| *CYP17* | Sense-1 | ATCCGAGCGGTCACTAATGT | 58 °C | 140 bp |
| | Antisense-1 | AGGAAAGATGTCCACCAAGC | | |
| *CYP19* | Sense-1 | CGATGGCTATTATGTGAAGAAAGG | 58 °C | 88 bp |
| | Antisense-1 | TTCATTTGGCTTTGGGAAGA | | |

### 2.3. Gene cloning and sequence analysis

The total RNA was extracted using Brizol RNA extraction reagent (Bio Flux) following the manufacturer's instructions. cDNA synthesis was performed using a reverse transcriptase kit (Prime Script® RT reagent Kit, TakaRa) according to the protocol of manufacturer. The following amplification conditions of PCR were used: an initial denaturation step at 94 °C for 4 min followed by 35 cycles of denaturation, 94 °C for 30 s; annealing 52 °C (*StAR*), 53 °C (*CYP17*) and 52 °C (*CYP19*) for 30 s and an extension for 5 min at 72 °C. The cDNAs obtained were used as a template for amplification of the gene sections of *StAR*, *CYP17* and *CYP19* with *R. chensinensis* specific primers designed in this study (listed in Table 1). The RT-PCR products were separated by electrophoresis on 2% (w/v) agarose gels that stained with ethidium bromide. The sequence of PCR products were analyzed by Shanghai Biological Engineering Co. Ltd. Homology analysis of the nucleotide sequences of these gene segments were performed using BLAST based on the GenBank database.

### 2.4. Real-time PCR

Real-time PCR was used to determine the differences in the level of *StAR*, *CYP17* and *CYP19* gene expression in testes. The forward and reverse primers were designed based on the sequential fragments of *StAR*, *CYP17* and *CYP19* genes cloned previously, while the reference gene specific primers were designed based on the *β-actin* cDNA of *R. catesbeiana* (GenBank Accession No. GI29467601). All primers synthesized are listed in Table 2. The following reagents were used for amplification in 25 μL, including 2.5 μL cDNA solution, 12.5 μL SYBR Premix Ex Taq (10 μmol/L), and 1 μL each of forward and reverse primer. The thermal cycling conditions were first 1 min at 95 °C and consisted of 40 cycles at 95 °C for 10 s and 58 °C for 30 s. Real-time PCR was performed in MyCycler™ Thermal Cycler iQ5 apparatus (Bio-Rad Company, USA). The quantity of each transcript was normalized to the amount of the *β-actin* housekeeping gene.