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Differences in reproductive toxicity of TBBPA and TCBPA exposure in male *Rana nigromaculata*☆

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ABSTRACT

Tetrabromobisphenol A (TBBPA) and tetrachlorobisphenol A (TCBPA) are persistent toxic environmental pollutants that cause severe reproductive toxicity in animals. The goal of this study was to compare the reproductive toxic effects of TBBPA and TCBPA on male *Rana nigromaculata* and to expound on the mechanisms leading to these effects. Healthy adult frogs were exposed to 0, 0.001, 0.01, 0.1, and 1 mg/L of TBBPA and TCBPA for 14 days. Sperm numbers were counted by erythrometry. Sperm mobility and deformities were observed under a light microscope (400 ×). We used commercial ELISA kits to determine the serum content of testosterone (T), estradiol (E2), luteinizing hormone (LH) and follicle stimulating hormone (FSH). Expression of androgen receptor (AR) mRNA was detected using real-time qPCR. Sperm numbers and sperm mobility were significantly decreased and sperm deformity was significantly increased in a concentration dependent manner following exposure to TBBPA and TCBPA. Sperm deformity was significantly greater in the 1 mg/L TCBPA (0.549) treatment group than in the 1 mg/L TBBPA (0.397) treatment group. Serum T content was significantly greater in the 0.01, 0.1 and 1 mg/L TBBPA and TCBPA experimental groups compared with controls, while E2 content was significantly greater in only the 1 mg/L TBBPA and TCBPA experimental groups. Expression levels of LH and FSH significantly decreased in the 1 mg/L TBBPA and TCBPA treatment groups. AR mRNA expression decreased markedly in all the treated groups. Our results indicated that TBBPA and TCBPA induced reproductive toxicity in a dose-dependent manner, with TCBPA having greater toxicity than TBBPA. Furthermore, changes in T, E2, LH, and FSH levels induced by TBBPA and TCBPA exposure, which led to endocrine disorders, also caused disturbance of spermatogenesis through abnormal gene expressions of AR in the testes.

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1. Introduction

In recent decades, with the rapid development of technology and industry, a variety of chemical products has been developed. However, widespread use of chemical products with persistent toxic pollutants may lead to many environmental problems. Tetrabromobisphenol A (TBBPA) and tetrachlorobisphenol A (TCBPA) are two types of persistent toxic environmental pollutants, primarily used in flame retardants, that have become common in the environment around the world (de Wit et al., 2010; Saint-Louis and Pelletier, 2004; Berger et al., 2004; Morris et al., 2004). TBBPA has been found in drinking water stored in polyethylene-carbonate containers (Peterman et al., 2000), and up to 300 ng/g TBBPA has been detected in the estuarine sediment of the Saint Lawrence River in Quebec, Canada. In addition, high levels of TBBPA have been found in the sludge sediment of a sewage treatment plant in Switzerland and in marine sediment near the Osaka Bay Estuarine in Japan (Letcher and Chu, 2010). TBBPA can enter the atmosphere through various channels and can exist as an in atmospheric particulates (Zweidinger et al., 1979). The average concentration of

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TBBPA in the workshop air was 0.20 ng/m^3, and the average concentration of TBBPA in the computer maintenance plant was 0.035 ng/m^3 (Tollbäck et al., 2006). TCBPA also has been detected in river sediments (Letcher and Chu, 2010). One study found that the concentration of TCBPA in a river sediment sample was 542.6 ng/g (Yuan et al., 2010). TCBPA has also been found in human breast milk (Bastos et al., 2008) and in urine (Rodríguez-Gómez et al., 2014). Therefore, it is essential to further investigate the potentially harmful impacts of TBBPA and TCBPA on both wildlife and humans.

Reproductive toxicity effects of TBBPA and TCBPA have been reported in some organisms (Chapin et al., 2008). Studies have shown that TBBPA has weak estrogenic activity and reproductive toxicity (Thomsen et al., 2001). In one study, TBBPA exposure resulted in reduced sperm counts in Sterlet as well as an increase in the amount of abnormal sperm (Linhartova et al., 2015). Moreover, TBBPA may induce testicular cell apoptosis (Kuiper et al., 2007; Zatecka et al., 2013) and reductions in sperm quality (Ogunbayo et al., 2008). Linhartova et al. (2015) suggested that TBBPA exposure caused epididymal sperm DNA damage and protein distribution anomalies. TCBPA has been shown to alter animal behavior that may lead to potential developmental toxicity and/or endocrine disorders (Zatecka et al., 2014). However, despite research on the effects of these toxins, it is imperative to compare differences in reproductive toxicity and to clarify the reproductive toxicity mechanism of TBBPA and TCBPA. One study has compared the developmental toxicity and estrogenic activity of TBBPA and TCBPA in zebrafish (Song et al., 2014). In this study, the half lethal concentration values of the chemicals suggests that TCBPA has greater toxicity than TBBPA, and this result is particularly beneficial in determining the water quality guidelines for test chemicals in river and lakes. Further comparison of the reproductive toxicity of TBBPA and TCBPA can provide a scientific basis for choosing suitable flame retardants and for setting water quality guidelines for these chemicals in river and lakes.

Sperm count, motility, and deformity are the basic indicators of sperm quality in male reproductive toxicology; these parameters can be used to evaluate the fertility of a species (Ankley et al., 1998). The male sex steroid testosterone (T) acts as a significant factor in spermatogenesis and ensures the normal phenotype of male reproductive system (Scott et al., 2009). One study showed that TBBPA interfered with the secretion of T (Dankers et al., 2013). Estradiol (E2) is also essential for the formation of sperm. Studies had reported that TBBPA has some properties similar to estrogen (Kitamura et al., 2002; Samuelsen et al., 2001) and confirmed that TBBPA exhibits anti-estrogenic activity (Kitamura et al., 2005). However, the estrogenic activity of TCBPA may be higher than BPA (Vandenberg et al., 2009). Luteinizing hormone (LH) and follicle stimulating hormone (FSH) influenced sperm formation, sperm maturation, testis development, and other reproductive functions (Shiraishi and Matsuyama, 2017). Furthermore, LH and FSH can affect the development of spermatocyte and regulate androgen receptors (AR) in the testis. AR also affects sperm formation and male fertility (Wang et al., 2009a). Damage to androgen receptors has been shown to cause infertility in male mice (Beck et al., 2016). Thus, the study of T, E2, LH, FSH and the AR gene helps to clarify the mechanisms by which TBBPA and TCBPA affect the sperm index. While numerous studies have reported on the reproductive toxicity of TBBPA and TCBPA in mammals (Szychowski and Wojtowicz, 2016; Zatecka et al., 2014; Zatecka et al., 2013), little work has examined reproductive toxicity of these chemicals in amphibians.

Stuart et al. (2004) found that the number and variety of amphibians around the world has been rapidly decreasing. The attenuation problem of a variety of amphibian species has been the topic of many studies (Houlahan et al., 2000; Kiesecker et al., 2001; Palen and Schindler, 2010). At least 2468 amphibians are experiencing a decline in numbers (Stuart et al., 2004). There are 100 species of amphibians in China (27.3% of all species) that are considered extinct, critically endangered, or endangered (Xie et al., 2007). It was confirmed that environmental pollution was one of the main causes of amphibians declines (Hayes et al., 2002) and that amphibians are vulnerable to the toxicity of these compounds (Kerby et al., 2010). Amphibians can be used as a yardstick to measure the environmental health of a global ecosystem. Among all amphibians, *Rana nigromaculata* has a high sensitivity to pollution and environmental impacts, which can be a good biological indicator of environmental pollution and ecosystem health (Hopkins, 2007). *R. nigromaculata* has been listed as an endangered species by the International Union for the Conservation of Nature (IUCN) since 2004. Therefore, *R. nigromaculata* is a suitable study species to clarify the reproductive toxicity of TBBPA and TCBPA.

Here, we focused on TBBPA and TCBPA-induced reproductive toxicity, with the hypothesis that PNP-induced reproductive endocrine disturbance may be associated with changes in T, E2, LH and FSH content changes and AR gene expression in male *R. nigromaculata*. We also compared the reproductive toxicity of differences between TBBPA and TCBPA. Those results of this study will provide a new scientific basis and theoretical basis for the toxic effects of both TBBPA and TCBPA on amphibian population.

2. Materials and methods

2.1. Chemicals

TBBPA was purchased from Aladdin Industrial Corporation (Feng Xian, Shanghai, China). TCBPA was purchased from TCI Industrial Development Corporation (Shanghai, China). Dimethyl sulfoxide (DMSO) was obtained from Lingfeng Chemical Reagent Corporation (Shanghai, China). FSH, LH, T and estradiol assay ELISA kits were obtained from Nanjing Jiancheng Bioengineering, Inc., China. Chemical information for TBBPA, TCBPA and DMOS is given in Table 1.

2.2. Animals acquisition and treatment

Three year old male *R. nigromaculata* frogs were purchased from the outskirts of Huzhou (Zhejiang, China). Frogs were acclimatized in aquariums (30 cm × 30 cm × 60 cm) for 14 days. Subsequently, robust male frogs were chosen and randomly assigned to twelve groups of 20 frogs each. Treatment groups were subjected to toxin exposure of either TBBPA or TCBPA at concentrations of 0, 0.001, 0.01, 0.1, 1 mg/L. The control group was kept in a 2 L volume of dechlorinated tap water. A 0.5% DMSO treated group served as a reagent blank. TBBPA and TCBPA were dissolved in 0.5% DMSO to get stock solutions. Four groups were exposed to either 0.001, 0.01, 0.1, or 1 mg/L TBBPA and four groups were exposed to either 0.001, 0.01, 0.1, or 1 mg/L TCBPA for 14 days. All control and treatment groups were maintained under standard experimental conditions (temperature range of 18°C–20 °C; pH range of 6.0–7.0; dissolved oxygen range of 6–8 mg/L). A static displacement method was used to change aerated water every day by 6:00 p.m. Frogs were fed *Eisenia fetida* twice a day (8:00 a.m., 6:00 p.m.) and maintained in a natural light/dark cycle.

After the 14 day exposure to either TBBPA or TCBPA, we sacrificed the frogs by pithing. No other individual deaths were observed throughout the experiment. Blood was collected by cardiac puncture and then transferred to centrifuge tubes for serum separation and precipitation (centrifugation at 4000 rpm for 10 min, 4 °C). Serum samples were stored at - 20 °C prior to get ready for T, E2, LH, and FSH assays. After the appurtenant was eliminated, tissue from the testis was cut and weighed. Tissues

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