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Exposure to butachlor causes thyroid endocrine disruption and promotion of metamorphosis in *Xenopus laevis*

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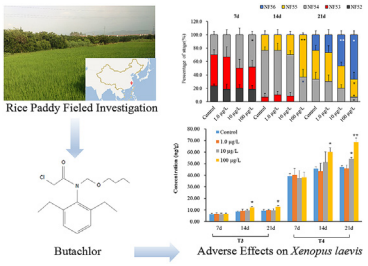
HIGHLIGHTS

- Monitoring of butachlor in a typical rice paddy field.
- Butachlor showed adverse metamorphosis effects in tadpole.
- Butachlor significantly promote the contents of whole-body thyroid hormones (THs, T3 and T4) at higher levels.
- Butachlor increased THs levels and altered the expression of genes involved in the HPT axis of *Xenopus laevis* tadpole.

GRAPHICAL ABSTRACT



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ABSTRACT

Butachlor is extensively applied in rice paddy ecosystem in china, and has been widespread contaminant in the aquatic environment. Here, *Xenopus laevis* was used for the evaluation of teratogenesis developmental toxicity, and disruption of thyroid system when exposure to different concentrations of butachlor by window phase exposure. Acute toxicity investigation shown that 96 h-LC_{50} value of butachlor was 1.424 mg L^{-1} and 0.962 mg L^{-1} for tadpoles (starting from stages 46/47) and embryos (starting from stages 8/9), respectively. Exposure to butachlor caused malformation, including abnormal eye, pericardial edema, enlarged proctodaeum and bent tail. Window phase exposure test indicated that butachlor significantly promote the contents of whole-body thyroid hormones (THs, T_3 and T_4) at higher levels, indicating thyroid endocrine disruption. At 7 days, exposure to butachlor up-regulated the mRNA expression of genes involved in THs synthesis and metabolism (*tshα*, *tg*, *tpo* and *dio*1) and THs receptors (*trα* and *trβ*). At 14 days, up-regulation of the mRNA expression of genes related to THs synthesis and metabolism (*tshα*, *tshβ*, *tg*, *tpo*, *dio1*, *dio2* and *ttr*) and THs receptors (*trβ*) were also observed after the exposure to butachlor. At 21 days, butachlor up-regulated the mRNA expression of *tshα*, *tg*, *tpo* genes and down-regulated the mRNA expression of *tshβ*, *tg*, *dio1*, *ttr* and *trα* genes. These results showed that butachlor could change the mRNA expression of genes involved in the HPT axis and increase whole-body thyroid hormones levels of *X. laevis* tadpoles in a dose- and time-dependent manner, causing thyroid endocrine disruption and developmental toxicity.

1. Introduction

Pesticides residue is one of the most common environmental contaminations worldwide (Brausch and Rand, 2011; Cao et al., 2015; Lan et al., 2014; Toan et al., 2013). In recent years, the

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production and usage of herbicides have increased rapidly (Zhang et al., 2010). Butachlor is an important member of the chloracetamide herbicide group, and is one of the top three herbicides extensively applied in rice paddy fields (Yu et al., 2003). Consequently, butachlor is detectable in surface and underground water, soil and biota worldwide, including China (Shi et al., 2011; Toan et al., 2013; Xue et al., 2014), and the residue concentrations ranged from 0.1 to 1.4 μg L^{-1} in surface water and 29.5–74 μg kg^{-1} in soil, respectively (Mamun et al., 2009; Yang et al., 2010). Butachlor is lipophilic, resistant to degradation in the environment and tend to bioaccumulate in fish, clam, and shrimp (Wang et al., 1992). Therefore, there is increasingly concerns about toxicological effects of butachlor on aquatic organisms.

Toxicological studies have also shown that butachlor is a suspected carcinogen (Ou et al., 2000) and exposure to butachlor has the potential to cause immunotoxicity and cytotoxicity in vitro and *in vivo* (Chang et al., 2012; Tu et al., 2013; Zhu et al., 2014). In addition, several previous studies reported adverse effects of butachlor on aquatic organism. For example, butachlor showed highly acute toxic to fish (the 48 h-LC_{50} values ranged from 240 to 890 μg L^{-1}) (Zhu et al., 2014) and has oxidative toxicity due to depressed glutathione detoxification system in *Clarias gariepinus* (Farombi et al., 2008). Butachlor could adversely affect the normal reproductive success of zebrafish, and disrupt the sex steroid endocrine systems (Chang et al., 2013). Butachlor have potential endocrine disrupting effects as promotion the accumulation of thyroxine (T_4) through inactive deiodinase type 3 in female rare minnow (*Gobiocypris rarus*) (Zhu et al., 2014). Furthermore, negative impacts of butachlor on survival and genotoxicity have been observed in alpine cricket frog tadpoles (*Fejervarya limnocharis*) (Liu et al., 2011).

The embryonic life stages of vertebrates is sensitivity to toxic chemicals exposure, especially for the oviparous organisms, including fish and amphibians (Wu et al., 2009). Most amphibians develop in aquatic environment at the larval stage, and even in their lifetime. *Xenopus laevis* (*X. laevis*) is an ideal model organism for evaluating development toxicity and endocrine-disrupting effects because it undergoes thyroid hormone-dependent metamorphosis with dramatic changes at morphological, biochemical and molecular levels (Morvan-Dubois et al., 2008; Tata, 1999). In amphibians, the hypothalamic-pituitary- thyroid (HPT) axis is responsible for regulating the synthesis, transport and metabolism of the thyroid hormones (THs), thyroxine (T_4) and triiodothyronine (T_3) (Searcy et al., 2012). The biological activities of THs are mediated by thyroid hormone receptors (TRs) (Buchholz et al., 2006). Acetochlor have been suggested to induce teratogenic on embryos by altering thyroid hormone-dependent gene expression and metamorphosis (Crump et al., 2002; Li et al., 2011). Butachlor has the similar chemical structure to acetochlor, it might be inferred that butachlor may share the same toxicity with acetochlor and exhibit a disruption effects on development (Hu et al., 2015). Moreover, effects of butachlor on the growth and development of amphibians have been little known, especially at environmental relevant concentrations. Thus, it is necessary to further clarify this issue to improve the understanding of the effects of butachlor on vertebrate development.

In present study, the concentration of butachlor in a typical rice paddy in East China was monitored (from 2012 to 2013), which regarded as basis for the subsequent research. Development toxicity and metamorphosis assay were evaluated during development stages of 46 and 51, respectively. The effects of butachlor on the expression of genes involved HPT axis and thyroid hormone levels were also assessed to explore the impacts of this compound on thyroid system of *X. laevis*.

2. Materials and methods

2.1. Chemicals and reagents

Butachlor (96%) was obtained from Hangzhou Qingfeng Agrochemical Co., Ltd. Dimethyl sulfoxide (DMSO), ethyl acetate, petroleum ether, n-hexane, acetonitrile, methanol and acetone were purchased from Sinopharm Chemical Reagent Co., Ltd (Beijing, China). 3-aminobenzoic acid ethyl ester, methanesulfonate salt (MS-222) was purchased from Sigma (St. Louis, MO, USA). All other chemicals used in this study were of analytical grade. TRIzol, RNase-free water, PrimeScript RT reagent Kit and SYBR Premix Ex Tap™ II were purchased from Takara (Dalian, China). Stock solutions and serial dilutions of butachlor were prepared in dimethyl sulfoxide (DMSO), and stored in the dark at 4 °C. A series of given amounts of butachlor was dissolved in charcoal filtered tap water (DMSO was used as cosolvent, of which percentage was no more than 0.01%, v/v) to form a set concentrations of exposure solutions.

2.2. Animals and husbandry

X. laevis adults were obtained from Nasco (Fort Atkins, WI, USA). Frogs and tadpoles were maintained in glass tanks containing charcoal filtered tap water at 22 ± 2.0 °C with a 12 h light/12 h dark cycle. The water quality was as follows: pH 7.0 ± 0.5, chlorine concentration <5 μg L^{-1}, the dissolved oxygen concentration >5 mg/L and water hardness ($CaCO_3$) approximately 150 mg L^{-1}. Adult *X. laevis* were fed with chopped pork liver and commercial amphibian diet (HaiHuang, Hangzhou, China) three times a week. The tadpoles were fed with live *Artemia* three times every day. A pair of *X. laevis* was injected (male 300 IU, female 700 IU) by human chorionic gonadotropin (hCG) (Zhejiang, China) to induce breeding. Development stages of tadpoles were determined according to the Nieuwkoop and Faber system (Nieuwkoop and Faber, 1994). All of the experimental procedures were conducted according to Regulations for the Administration of Affairs Concerning Experimental Animals (2013).

2.3. Embryos teratogenesis assay and acute exposure of tadpoles

The teratogenesis of embryos exposed to butachlor was conducted with modified FETAX (Yu et al., 2011). Briefly, *X. laevis* embryos at stage 8/9 were harvested from tank without removing the jelly coats, and then transferred into exposure solutions (20 embryos in 1 L exposure solution per glass tank) with the butachlor concentrations of 0.313, 0.625, 1.25, 2.5 and 5 mg L^{-1}. Control and experimental treatment groups received 0.01% (v/v) DMSO. Three replicate test tanks were used for each treatment group and exposure solutions were renewed every 48 h. After 96 h lasting exposure, the mortalities and malformations of embryos were recorded for the 96 h-LC_{50} and EC_{50} calculation.

Tadpoles were exposed to butachlor from stage 46/47 to carry out the acute exposure. The exposure protocol was just the same as that of teratogenesis assay mentioned above except that malformation of tadpole was not observed.

2.4. Metamorphosis assay

The tadpoles at stage of 51 were exposed to butachlor to perform metamorphosis assay. The three window periods (7, 14 and 21 d) were set as exposure durations and the exposure concentrations were 1.0, 10 and 100 μg L^{-1} (representing the 1/1400, 1/140 and 1/14 of 96 h-LC_{50} of acute toxicity of tadpole), respectively. Each treatment contained two tanks with 40 tadpoles per tank. All tanks with the same size and shape contained 20 L water. At the