Experimental and Toxicologic Pathology 68 (2016) 463–469

Contents lists available at ScienceDirect

Experimental and Toxicologic Pathology

journal homepage: www.elsevier.de/etp

Impact of co-exposure with butachlor and triadimefon on thyroid endocrine system in larval zebrafish

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ARTICLE INFO

Article history:
Received 12 May 2016
Accepted 25 July 2016

Keywords:
Co-exposure
Thyroid disruption
Herbicides
Fungicides
Zebrafish

ABSTRACT

Introduction: Butachlor (BTL) and triadimefon (TDF), the widely used herbicide and fungicide, are unavoidable enter into the aquatic environment. However, there were limited study regarding to the joint toxicity of these two pesticides on fish at present.

Aim: To evaluate the potential thyroid-disrupting toxicity and exposed to different concentrations of BTL mixed with TDF.

Materials and methods: Zebrafish embryo (n = 3) were exposed to 0.01 and 0.05 fold of LC_{50} from the acute joint toxicity test, of which 0.32 mg/L (BTL) and 9.41 mg/L (TDF) for single or mixture agents (BTL: 0.0064 mg/L, 0.032 mg/L; TDF: 0.1882 mg/L, 0.9410 mg/L; co-exposure: 0.0032 mg/L BTL + 0.0941 mg/L TDF, 0.016 mg/l BTL + 0.4705 mg/L TDF) after 10-day post-fertilization. Hatching, malformation, survival rates and thyroid hormones (THs), genes expression involved in HPT-axis of embryos were measured and detected in control and separately/co-exposure treatments. THs contents were evaluated by ELISA kit and the expression levels of genes were determined by RT-PCR.

Results: Hatching, malformation and survival rates of embryos exposed to single BTL exhibited no statistically significant difference from the control besides decreased of high concentration in survival rates. Exposure to TDF reduced hatching, survival rate and increased malformation. The combined exposure to BTL and TDF resulted in greater adverse effects on embryonic development. BTL exposure significantly increased free T_3 and T_4 contents. Elevated free T_3 content was also observed in the larvae exposed with single BTL. Co-exposure of the two pesticides caused greater enhanced of T_3 and T_4 levels. Furthermore, gene data showed BTL up-regulated the mRNA expression of *tpo, tshβ, tg, ttr, dio2*, TDF up-regulated the mRNA expression of *tpo, trα, ttr, dio2* and down-regulated *trβ* gene. The mixture of the two pesticides caused up-regulation mRNA expression of *trα, trβ, tg, ttr, dio2*.

Conclusion: BTL and TDF resulted in adverse effects on zebrafish embryonic development and caused thyroid endocrine disruption, BTL and TDF have a synergistic effect on development and thyroid endocrine by enhanced level of thyroid hormone.

1. Introduction

For over half a century, substantial evidence has surfaced on the hormone-like effects of environmental chemicals in fish, wildlife and humans (Sonnenschein and Soto, 1998). EPA released a variety of chemicals which have been found to disrupt the endocrine systems, these endocrine disruptor become research hotspots and pesticides take a large part in these endocrine disruptors (U.S. Environmental Protection Agency, 1996; Mansilha et al., 2010). Butachlor (BTL) is a chloroacetamide herbicide, and is one of the top three herbicides extensively applied to control weeds in rice fields in China (Chang et al., 2013). Triadimefon (TDF) is one of the most important group of sterol demethylation inhibiting fungicides (DMIs), which used widely in agriculture and medicine, and its environmental relevant concentrations in water up to 0.922 mg/L (Liu et al., 2014; Watschke et al., 2000). The most widespread pesticides in a variety of agriculture environmental applications belong to herbicide, fungicide and insecticide in China, whatever their using environment, consequently they are evitably enter into the aquatic environment (Kaegi et al., 2008). Thus, the interactions between the toxicants are of particular concern for the environmental risk assessment of organic contaminants.

BTL is a persistent pollutant in agricultural soil and water system (Yu et al., 2003). The practice environmental concentration of BTL in rivers range from 0.01 μg/L to 0.43 μg/L (Tsuda et al., 1997). Previous studies indicated in succession that BTL had some

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http://dx.doi.org/10.1016/j.etp.2016.07.004
0940-2993/

toxic effect on different aquatic species. The 96h-LC50 s of BTL on *Cyprinus carpio*, *Oryzias latipes* and *Misgurnus anguillicaudatus*, were 0.62, 0.41 and 0.24 mg/L, respectively (Park et al., 2009). BTL exposure can damage the structure and electrocardiogram of myocardial cell in *Bufobufo gargarizans* (Shao et al., 2009). In addition, exposure of BTL to goldfish resulted in oxidative stress, disorder of energy metabolism and amino acids metabolism, as well as disturbance of neurotransmitter balance (Xu et al., 2015). In zebrafish embryos, BTL exposure caused developmental toxicity, endocrine disruption and immune toxicity (Tu et al., 2013). A recent study also show that BTL exposure induced the increased plasma thyroxine (T_4) and triiodothyronine (T_3) levels in adult zebrafish (Chang et al., 2013).

TDF has been suggested to disrupt testosterone homeostasis, induce oxidative response genes, activate the nuclear receptor, and affect central nervous system catecholamines and induce a transient syndrome in rat (Goetz et al., 2007; Hester, 2008; Crofton, 1996) as well as increase male-rat locomotor activity and induce stereotyped behavior (Ikaiddi, 1997). In fish, TDF enhanced CYP3A and CYP1A activity, which associated with the tumorigenesis in adult medaka (Li et al., 2009). Moreover, previous study also found TDF can alter gene expression in the hypothalamic-pituitary-thyroid (HPT) axis of zebrafish and the disruption occur at synthesis, regulation, and action of thyroid hormones (Liu et al., 2011). In another study, T_4 levels were significantly decreased, while T_3 concentrations were significantly increased in zebrafish after exposure to triazole fungicides, hexaconazole and tebuconazole, indicating thyroid endocrine disruption (Yu et al., 2013).

Fish are the most important component of aquatic ecology and the highest biological enrichment with locating the top of food chain, so the endocrine disruptor can make serious threat to early development, sex differentiation, reproduce and the stability of population. From these factors, it is important to investigate the impact of pesticide on fish. Zebrafish embryo have some advantages like transparency, low cost, transgenic and morpholino capabilities, conservation of cell signaling, and concordance with mammalian developmental phenotypes, which make it considered as an alternative model for traditional in vivo developmental toxicity screening (Sipes et al., 2011). In fish, hypothalamic–pituitary–thyroid (HPT) axis responsible for regulating thyroid hormone dynamics by coordinating their synthesis, secretion, transport and metabolism and play important roles in development and growth, particularly in the early life stages of fish (Carr and Patiño, 2011; Kawakami et al., 2008). There is evidence that BTL and TDF cause a disturbance in the concentration of circulating THs (Chang et al., 2013; Liu et al., 2011), the impact of co-exposure toxicity of two type pesticide on fish HPT axis as well as the underlying mechanisms remain not to be elucidated. The present study was designed to evaluate the individual and combined toxicity of pesticides to the early development of zebrafish, and limited study has been reported to investigate the mixed toxicity of two different type pesticides. Moreover, the mechanisms of co-exposure of these two pesticides to early development of zebrafish remain poorly understood (Yang et al., 2014).

Therefore, in the present study, zebrafish embryos/larvae were selected as models for evaluating the potential thyroid-disrupting toxicity and exposed to different concentrations of mixed pesticides. The mRNA expression involved in the HPT axis were quantitatively examined by using a HPT-PCR array. Meanwhile, the levels of THs (T_3 and T_4) were also measured by an enzyme-linked immunosorbent assay (ELISA) after chemicals exposure.

2. Materials and methods

2.1. Chemicals

BTL (purity = 96%) and TDF (purity = 96%) was purchased from Hangzhou Qingfeng Agrochemical Co., Ltd. (Zhejiang, China) and Jiangsu Sword Shares Co., Ltd. (Jiangsu, China) respectively. Dimethyl sulfoxide (DMSO) was purchased from Sigma (St. Louis, MO, USA). All other chemicals used in this study were of analytical grade.

2.2. Zebrafish maintenance and embryo exposure

Adult zebrafish (*D. rerio*) were raised in a flow-through system with dechlorinated tap water (pH 7.0–7.4) at a constant temperature ($28 \pm 1\,^{\circ}C$). The light regime was 14-h light, 10-h dark. Fish were fed with freshly hatched brine shrimp (*Artemia nauplii*) twice a day in a quantity that was consumed within 5 min. Males and females were paired in spawning boxes on the afternoon the day before spawning in a ratio of 2:1. Embryos of 6 hpf (hours post fertilization) were transferred into glass beakers containing 500 mL of exposure solution until 10 dpf.

The 96-h acute toxicity of BTL with TDF, was determined according to guidelines provided by the Organization for Economic Co-operation and Development (OECD, 2013). Adult zebrafish randomly selected were assigned to six pesticide treatments and one control. Concentration of BTL was designed 0.18, 0.22, 0.26, 0.32, 0.38, 0.46 mg/L, 5.33, 6.39, 7.67, 9.20, 11.04, 13.25 mg/L for TDF.

The exposure concentrations were set as 0.01 and 0.05 fold of LC_{50} (predicted environmental safety concentration and environmental relevant concentration) from the acute joint toxicity test, of which 0.32 mg/L (BTL) and 9.41 mg/L (TDF) for single or mixture

Table 1
Primers used for the quantification of the mRNA expression by qPCR.

Gene name	Sense primers (5′–3′)	Size/bp	Gene bank accession no.
β-actin	FP 5′-ACCCACACCGTGCCCATCTA-3′ RP 5′-CGGACAATTTCTCTTTCGGCTG-3′	152	AF057040
tg	FP 5′- CCAGCCGAAAGGATAGAGTTG-3′ RP 5′- ATGCTGCCGTGGAATAGGA-3′	175	XM_001335283
tpo	FP 5′- GCGCTTGGAACACAGTATCA-3′ RP 5′- CTTCAGCACCAAACCCAAAT-3′	130	EU267076
ttr	FP 5′- CGGGTGGAGTTTGACACTTT-3′ RP 5′- GCTCAGAAGGAGAGCCAGTA-3′	129	BC081488
trα	FP 5′- CTATGAACAGCACATCCGACAAGAG-3′ RP 5′- CACACCACACACGGCTCATC-3′	85	NM_131396
trβ	FP 5′- TGGGAGATGATACGGGTTGT-3′ RP 5′- ATAGGTGCCGATCCAATGTCNM-3′	110	NM_131340
dio2	FP 5′- GCATAGGCAGTCGCTCATTT-3′ RP 5′-TGTGGTCTCTCATCCAACCANM-3′	103	NM_212789