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Thyroid endocrine disruption in zebrafish larvae following exposure to hexaconazole and tebuconazole

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ABSTRACT

The widely used triazole fungicides have the potential to disrupt endocrine system, but little is known of such effects or underlying mechanisms of hexaconazole (HEX) and tebuconazole (TEB) in fish. In the present study, zebrafish (*Danio rerio*) embryos were exposed to various concentrations of HEX (0.625, 1.25 and 2.5 mg/L) and TEB (1, 2 and 4 mg/L) from fertilization to 120 h post-fertilization (hpf). The whole body content of thyroid hormone and transcription of genes in the hypothalamic-pituitary-thyroid (HPT) axis were analyzed. The results showed that thyroxine (T4) levels were significantly decreased, while triiodothyronine (T3) concentrations were significantly increased after exposure to HEX and TEB, indicating thyroid endocrine disruption. Exposure to HEX significantly induced the transcription of all the measured genes (i.e., corticotrophin-releasing hormone (CRH), thyroid-stimulating hormone (TSHβ), sodium/iodide symporter (NIS), transthyretin (TTR), uridine diphosphate glucuronosyltransferase (UGT1ab), thyronine deiodinase (Dio1 and Dio2), thyroid hormone receptors (TRα and TRβ)) in the HPT axis, but did not affect the transcription of thyroglobulin (TG). However, TEB exposure resulted in the upregulation of all the measured genes, excepting that TG, Dio1and TRα had not changed significantly. The overall results indicated that exposure to HEX and TEB could alter thyroid hormone levels as well as gene transcription in the HPT axis in zebrafish larvae.

1. Introduction

Endocrine disrupting chemicals (EDCs) are compounds that interfere with endocrine (or hormone system) in both wildlife and humans. EDCs can modulate the endocrine system through multiple mechanisms of action and possibly cause cancerous tumors, birth defects, and other developmental disorders. Animal studies have indicated that many of those adverse effects were often permanent and some EDCs could act as epigenetic modulators which led to potential transgenerational effects (Anway et al., 2005; Waring and Harris, 2011). The adverse effects of EDCs which have been observed in many vertebrate species cause growing concern among researchers and policy makers. 127 pesticides were identified as having endocrine disrupting properties, including the 91 listed by the Pesticide Action Network (Tebourbi et al., 2011). Consequently, endocrine disrupting pesticides (EDPs) are the largest group of EDCs in numbers compared to other chemical groups.

Triazoles are a class of fungicides largely used in agriculture as crop protection products. Their antifungal activity is due to their ability to inhibit the P450 enzyme (CYP51), which blocks the conversion of lanosterol to ergosterol causing disruption of fungal wall (Di Renzo et al., 2007). But the inhibition potency of these triazole fungicides is not limited to fungi, they may also inhibit other P450-mediated activities resulting in various adverse effects (Robinson et al., 2012). Several triazoles have been reported to alter the concentrations or genes transcription involved in steroid homoeostasis and thirteen triazoles have been identified as endocrine disruptors (Goetz and Dix, 2009; Hester and Nesnow, 2008; Liu et al., 2011; Mnif et al., 2011). Hexaconazole (HEX) and tebuconazole (TEB) are both triazole fungicides, which are applied on a number of crops in China such as grapes, rice, fruits, and vegetables because of their broad-spectrum antifungal activity. The two fungicides had been classified by the US EPA as Group C-Possible Human Carcinogen (U.S. EPA, 2006). TEB is persistent in soils and presents moderate mobility (EFSA, 2008). It is classified as toxic to aquatic organisms and may cause

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long-term adverse effects in the aquatic environment (Bayer CropScience Limited, 2005). The presence of TEB in stream water has increased in recent years (Montuelle et al., 2010) and its concentrations detected in surface waters was up to 175–200 μg/L (Elsaesser and Schulz, 2008). Even more, it was found out in human being with the maximal concentrations of 19.2 μg/L and 2.22 ng/kg in urine and hair samples from farm workers, respectively (Fustinoni et al., 2012; Schummer et al., 2012). HEX is highly active and efficiently acts to inhibit sterol synthesis in many fungi, particularly ascomycetes and basidiomycetes (Worthington, 1991). HEX is highly persistent with the field soil degradation DT_{50} of 225 d, and no degradation of HEX is found in river water incubated at 20 °C for 3 weeks (Tsukatani et al., 2008). Accordingly, there was a potential for exposure to TEB and HEX, thus causing endocrine disrupting effects on human beings and wildlife, especially on aquatic species. But there were few studies regarding their endocrine toxicity, especially disruption on the thyroid hormonal homeostasis of fish.

Thyroid hormones (THs) play a crucial role in the regulation of development, growth, immune, metabolism, energy provision, reproduction and behavior in vertebrates (Jugan et al., 2010). Fish thyroid homeostasis is subject to the regulation of hypothalamic-pituitary-thyroid (HPT) axis. Many different groups of chemicals may interfere with thyroid hormonal homeostasis, such as triadimefon (Liu et al., 2011), perfluorooctane sulfonate (PFOS) (Shi et al., 2009), microcystin-leucine-arginine (MCLR) (Yan et al., 2012). Previous studies suggested that the influence of a chemical on HPT axis, including the alterations of gene transcriptions, hormone levels and enzyme activities, could be applied to evaluate the effect of thyroid endocrine disruption (Chen et al., 2012; Picard-Aitken et al., 2007; Yu et al., 2010). In addition, using gene transcription patterns as endpoints could observe toxic effects at toxicant concentrations that did not cause morphological effects (Hermsen et al., 2012; Yang et al., 2007a) and provide further insight into the mechanisms of action of toxicants.

Zebrafish (*Danio rerio*) are widely used as a predominant test model for the assessment of EDCs due to its small size, ease of culture, high reproductive capacity, rapid organogenesis, morphological and physiological similarities to mammals, etc. (Segner, 2009). Some previous studies suggested that zebrafish embryos/larvae were an ideal model fish for investigating endocrine disruption by chemicals in the laboratory (Chen et al., 2012; Kanungo et al., 2012; Liu et al., 2011; Tu et al., 2013). Therefore, zebrafish embryos were employed as a model in our study. In order to investigate the effect of TEB and HEX on thyroid development of zebrafish embryos, and to discuss the possible mechanisms underlying toxic response, gene transcription in the HPT axis were quantitatively examined, meanwhile, the levels of THs (T3 and T4) were also measured in the present study. The information obtained will add new knowledge about the endocrine toxicity mechanisms of triazoles.

2. Materials and methods

2.1. Chemicals

TEB (technical grade AI: 98%) was purchased from Shanghai Yuanji Chemical Co., Ltd. (Shanghai, China). HEX (technical grade AI: 96.3%) was purchased from Zhejiang Jiahua Group Co., Ltd. (Zhejiang, China). Dimethyl sulfoxide (DMSO) and MS-222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt) were 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*) of the wild-type (AB strain) were raised in a flow-through system with dechlorinated tap water (pH 7.0–7.4) at a constant temperature (28 ± 1 °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. Five days before spawning, females were separately housed to optimize egg production. Males and females were paired in spawning boxes on the afternoon the day before spawning in a ratio of 2:2. Spawning was triggered once the light was turned on the following morning and was usually completed within 30 min.

Eggs from different spawning boxes were collected within 1 h of spawning, were pooled and washed in standardized water (Standardization, 1996) (117.6 mg of $CaCl_2{\cdot}2H_2O$, 49.3 mg of $MgSO_4{\cdot}7H_2O$, 25.9 mg $NaHCO_3$ and 2.3 mg KCl in 1 L of deionized water) to remove any coagulated eggs and debris. At 2 h post-fertilization (hpf), embryos were examined under a dissecting microscope, and those embryos that had developed normally and reached the blastula stage were selected for subsequent experiments. Fertilization rate of the batch of eggs used was at least 90%. Standardized water was aerated during at least half an hour before addition of test chemicals.

Stock solutions of the test chemicals were prepared in DMSO in concentrations 1000 times the highest final exposure concentrations and stored at −20 ± 1 °C before use. Stock solutions were serially diluted in DMSO and 500 μL of each of these were mixed with 500 mL of standardized water in beakers to give a total DMSO concentration of 0.1% in each test solution. Normal embryos (approximately 400) were randomly distributed into glass beakers containing 500 mL of HEX and TEB solution at various nominal concentrations (HEX: 0, 0.625, 1.25, 2.5 mg/L; TEB: 0, 1, 2, 4 mg/L) until 120 hpf. The selected exposure concentrations were previously ascertained by performing a range-finding study; this study revealed that after exposure to the lowest concentration of HEX and TEB, the malformation rates showed a trend toward an increase but the tendency was not statistically significant. Both the control and exposure groups with 6 replicates in each exposure concentration received 0.01% (v/v) DMSO. During the experimental period, embryos were kept in an incubator at 28 ± 0.5 °C with a photoperiod of 14 h light:10 h dark. Chorions, dead eggs and larvae were removed and 50% of the exposure solution was renewed daily. After exposure, the larvae were placed in MS-222 (40 mg/L), and then divided into two groups: one group were placed in *RNAlater* (Ambion, Austin, TX) to stabilize RNA and stored at −20 °C for RNA isolation, and the other group were frozen in liquid nitrogen, and stored at −80 °C for hormone analysis.

2.3. Thyroid hormone extraction and measurement

T3 and T4 levels were measured by using enzyme-linked immunosorbent assay (ELISA) as described by Yu et al. (2011). The test kits were purchased from R&D Company (USA). 300 larvae were used for thyroid hormone extraction according to manufacturer's description of ELISA kit (R&D, USA). The extraction efficiencies were determined by addition of 100 μl ^{125}I radioiodinated T4 and T3 (Beijing North Institute of Biotechnology, Beijing, China) to larvae ($n = 4$). The mean recoveries for larval samples were 67.3% T4 (61.6–74.3%) and 65.7% T3 (62.2–73.7%). The ELISA for T3 and T4 was validated for use with zebrafish samples by demonstrating parallelism between a series of diluted and spiked samples in relation to the standard curve attached to the ELISA kit. The detection limits for T3 and T4 were 0.6 ng/mL and 3 ng/mL, respectively.