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Interaction of 17β-estradiol and ketoconazole on endocrine function in goldfish (*Carassius auratus*)

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ABSTRACT

An understanding of the effects of toxic mixtures of endocrine disrupting chemicals (EDCs) on aquatic organisms is challenging as these organisms are exposed to multiple classes of contaminants in their natural habitat. The aim of the present study was to evaluate the interactions of two classes of EDCs, 17β-estradiol (E_2) and ketoconazole (KTC), on endocrine function in male goldfish (*Carassius auratus*), including vitellogenesis, metabolic capability and serum steroid synthesis. Changes in vitellogenin (VTG) concentration, liver 7-ethoxyresorufin-*O*-deethylase (EROD) activity and circulating serum E_2 level were examined. The expression of related genes was also determined using quantitative real-time polymerase chain reaction. Exposure to E_2 caused a significant increase in VTG concentrations which corresponded with the gene expression of VTG and estrogen receptor (ER) in males, which were further elevated after combined exposure to E_2 and KTC, indicative of a synergetic relationship. Exposure to E_2 also resulted in a distinct increase in serum steroid biosynthesis and associated cytochrome P450 (CYP) aromatase expression after 10 days. However, these changes were inhibited by the presence of KTC, which acted as a steroidogenic inhibitor in fish. Moreover, KTC significantly decreased liver EROD activity and increased the related gene expression of CYP1A. However, these KTC-mediated metabolic reactions in goldfish were up-regulated following exposure to KTC in combination with E_2. These findings reveal complex interactions on endocrine functions in male goldfish when exposed to multiple contaminations and may provide a better understanding of the effects of toxic mixtures.

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1. Introduction

Endocrine disrupting chemicals (EDCs) are either natural or synthetic, and can interfere with the endocrine system of vertebrates and humans through a complex array of regulators which are responsible for synthesis, secretion, transport, metabolism, binding action and elimination in organisms (Diamanti-Kandarakis et al., 2009). There is considerable evidence to show that exposure to environmental EDCs can cause a series of adverse effects on homeostasis, reproduction, and development in vertebrates and humans, resulting in a deviation from normal endocrine function (Li et al., 2012). Since the aquatic environment serves as the ultimate sink for most environmental contaminants and aquatic wildlife species are increasingly being exposed to EDCs in their natural habitat, considerable research efforts have focused on endocrine disruption and the associated potential health threat to aquatic organisms, especially fish.

Among these EDCs researches, the most studied, and probably the most common, topic is that of estrogenic substances. These estrogenic substances can interact with endocrine regulatory systems by mimicking and mediating endogenous hormones, leading to disruption of the endocrine function in exposed fish at multiple levels. Evidence has indicated that wild male fish are feminized due to the presence of estrogens in their natural habitat, with marked synthesis of vitellogenin (VTG, an egg-yolk precursor lipophosphoprotein normally produced in female only during oocyte maturation) and the production of early stage eggs in the testes (Li et al., 2009; Vethaak et al., 2005). In addition to feminization, exposure of male fish to estrogens also caused increased synthesis of the serum sex steroid, 17β-estradiol (E_2), resulting in interference of normal endogenous steroid secretion and reproduction *via* stimulation of steroidogenic cytochrome P450 (CYP) enzymes (Lu et al., 2010). Kirby et al. (2007) also reported that E_2 depressed xenobiotic metabolism in the flounder (*Platichthys flesus*) by affecting catabolic enzymes including CYP1A, which can lead to accumulation of xenobiotics in organisms. In addition, these estrogen-induced responses may also adversely impact fish populations and result in a subsequent decline in abundance. It has been demonstrated that exposure to low concentrations of estrogen in

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the fathead minnow (*Pimephales promelas*) led to various disruptions in endocrine function both in males and females, and almost eliminated the entire population of this species from an experimental lake in Canada within two years (Kidd et al., 2007). Among these estrogenic contaminants, natural estrogen E_2 is thought to play a dominant role in the estrogenic activity found in aquatic environments. For example, in our previous study, E_2 was responsible for 50–90% of the total estrogenic activity in Taihu Lake (Yan et al., 2012b).

In addition to estrogenic substances, the occurrence of pharmaceuticals in the aquatic environment and the subsequent risks to fish are also of concern (Lu et al., 2013). Ketoconazole (KTC), a model pharmaceutical representing imidazole and triazole, is used in the clinic, horticulture and agriculture for the treatment of fungal infections through inhibition of CYP51-catalyzed synthesis of ergosterol. This substance has been detected in various aquatic environments and its concentrations range from ng to μg/L (Lindberg et al., 2010; Van De Steene and Lambert, 2008). Similar to other substances in the class, KTC is a relatively non-specific inhibitor of CYPs, which can reduce the activity of a wide-range of CYP enzymes in vertebrates, including catabolic CYP1A and several CYPs involved in steroid hormone biosynthesis (Hegelund et al., 2004; Marty et al., 2001). However, these studies on KTC have mostly concentrated on its endocrine-disrupting effects in mammals, and there is little information on the effects of KTC in fish.

In the aquatic environment, both estrogens and fungicides may be coexistent in some situations and exert their effects through common pathways in vertebrates, including steroidogenic and metabolic enzymes. This co-exposure to estrogens and fungicides may lead to more or fewer adverse effects in organisms as a result of possible interactions, as different classes of EDCs may disturb each other in the common pathways and result in either synergistic or antagonistic effects (Celander, 2011; Wassmur et al., 2013). Nevertheless, in previous studies, the emphasis has been on the impact of a single class of contaminants, disregarding the fact that fish are usually exposed to a mixture of different classes of contaminants (Burki et al., 2013; Sun et al., 2011). Therefore, the purpose of this study was to investigate the effects of exposure to E_2 and KTC (alone or in combination) on vitellogenesis, metabolic capability and serum steroid hormones in male goldfish. Changes in blood and liver VTG concentration, liver CYP1A-mediated 7-ethoxyresorufin-*O*-deethylase (EROD) activity and circulating serum E_2 level and the transcription of genes associated with these reactions were evaluated. This study will improve our current understanding of interactions between multiple pollutants and their effects on components of endocrine system in male goldfish.

2. Materials and methods

2.1. Chemicals and reagents

Standards of E_2 (98% purity) and KTC (99% purity), goat anti-rabbit IgG labeled with alkaline phosphatase (AP) and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Diethanolamine, heparin sodium, and *p*-nitrophenylphosphate were purchased from Nanjing Sunshine Biotechnology Co. Ltd. (Nanjing, China). Rabbit anti-goldfish VTG and purified VTG were obtained from Ocean University of China (Qingdao, China). Coomassie brilliant blue G-250 (ultrapure grade) was obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Ethyl 3-amino benzoate methanesulfonate (MS-222) was obtained from J&K Scientific (Shanghai, China).

Solvent-free stock solutions of E_2 and KTC were prepared using a "shell-coating" technique, as previously described by Skolness et al. (2011). Briefly, the substance was respectively dissolved in methanol in an amber glass carboy and evenly dispersed on the inside wall under a gentle stream of nitrogen to remove the solvent. The test water, which was prepared daily from fresh water, was subsequently added to the carboy and thoroughly mixed using a vortex mixer. The stock solutions were further diluted with test water to a series of concentrations which were used in the exposure experiments.

2.2. Animals and exposure

The same batches of adult male goldfish of similar weight and length (30.4 ± 1.0 g, 15.5 ± 2.2 cm) obtained from the Nanjing Institute of Fishery Science (Nanjing, China) were used in the experiments. All healthy fish were held for an acclimation period of two weeks prior to the test in dechlorinated municipal water at 20 ± 1 °C and fed once each day with OSI freshwater aquarium pellet food (Fenglin Feed Technology Development Co. Ltd., Shantou, China) at the rate of 6% of their body weight. Feces and uneaten food were removed each day by suction.

According to the maximal concentrations of E_2 and KTC detected in the aquatic environment and their biological effects reported in earlier studies (Ankley et al., 2007; Bolong et al., 2009; Pojana et al., 2004, 2007; Van De Steene and Lambert, 2008; Yan et al., 2012a), the fish were finally exposed to 8 concentrations: blank control (test water only, C), E_2 (80 ng/L, E), KTC alone (25, 50, or 100 μg/L, K1–K3) or in combination with 80 ng E_2/L (E + K1–E + K3). Exposure experiments were designed according to previous studies (Ankley et al., 2012; Brian et al., 2005, 2007; Zhang et al., 2010). Randomly assigned fish were kept in 30-L glass test tanks containing 20 L of the different experimental solutions, which were supplied by a continuous-flow exposure system. Three replicate tanks of the same size in each treatment ran simultaneously. After different periods of exposure, the data from replicate studies were then pooled. The analysis of each biological effect was based on data pooled from three independent exposure studies. Water temperature was maintained at 20 ± 1 °C, with pH = 7.0 ± 0.2, and dissolved oxygen of 6.5 ± 0.2 mg/L during the periods of exposure. Animal research followed the standard procedures of Institutional Animal Care and Use Committee.

Following 3, 7, 10 and 14 days of exposure, three fish were collected from each tank (9 fish in total for each treatment) at each sampling time and anaesthetized with buffered MS-222. Blood samples (approximately 1 mL) were collected from the caudal vasculature using a 2 mL heparinized syringe. After immediate centrifugation at $10{,}000 \times g$ for 10 min at 4 °C, the supernatant was stored at −80 °C until determination of serum VTG and E_2 concentrations. Following blood collection, the liver was collected and homogenized in nine volumes of cold buffer (0.15 M KCl, 0.1 M Tris-HCl, pH 7.4), and subsequently centrifuged for 25 min at $10{,}000 \times g$ and 4 °C. The supernatant was collected and stored at −80 °C until analysis of VTG and EROD. Specifically, after 10 days of exposure, the liver collected from fish was sectioned into two pieces of approximately 50 mg each. One piece was placed in RNAstore Reagent (Tangen, China) and stored until gene expression analyses. The remaining piece was homogenized and separated by centrifugation for VTG and EROD determination. Simultaneously, the brain after 10 days of exposure was also removed and snap-frozen in liquid nitrogen and stored for gene expression analyses.

In order to confirm the exposure concentrations, a water sample (500 mL) was collected from each tank at the beginning, middle and end of the exposure period. Concentrations of E_2 and KTC were measured using liquid chromatography tandem mass spectrometry (Agilent Technologies, Palo Alto, CA, USA), as described by Yan et al. (2012b) and Lindberg et al. (2010). No chemicals were detected in the blank control. The mean measured concentrations of E_2 and KTC

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