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Effects of triclosan (TCS) on hormonal balance and genes of hypothalamus-pituitary- gonad axis of juvenile male Yellow River carp (*Cyprinus carpio*)

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HIGHLIGHTS

- TCS exposure enhanced aromatase mRNA, the enzyme increase E_2 to induce Vtg.
- Exposure to TCS changed GnRH and GtH-β expression and secretion to increase E_2.
- TCS treatment led to the decrease of Ar to active Vtg synthesis.

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ABSTRACT

Triclosan (TCS) is a broad spectrum antimicrobial agent which has been widely dispersed and determinated in the aquatic environment. However, the effects of TCS on reproductive endocrine in male fish are poorly understood. In this study, male Yellow River carp (*Cyprinus carpio*) were exposed to 0, 1/5, 1/10 and 1/20 LC_{50} (96 h LC_{50} of TCS to carp) TCS under semi-static conditions for 42 d. Vitellogenin (Vtg), 17β-estradiol (E_2), testosterone(T), gonadotropin (GtH), and gonadotropin-releasing hormone (GnRH) levels were measured by enzyme-linked immunosorbent assay (ELISA). Meanwhile, we also examined the mRNA expressions of aromatase, GtHs-β, GnRH, estrogen receptor (Er), and androgen receptor (Ar) by quantitative Real-time Polymerase Chain Reaction (qRT-PCR). TCS induced Vtg levels of hepatopancreas, E_2 levels of serum, and inhibited Ar and Er mRNA levels, suggesting that the induction of Vtg production by TCS was indirectly caused by non-Er pathways. TCS-induced Vtg levels by interfering with the reproductive axis at plenty of latent loci of male carps: (a) TCS exposure increased the aromatase mRNA expression of hypothalamus and gonad aromatase, consequently increasing serum concentrations of E_2 to induce Vtg in hepatopancreas; (b) TCS treatment changed GtH-β and GnRH mRNA expression and secretion, causing the disturbance of reproductive endocrine; (c) TCS exposure decreased Ar mRNA levels, indicating potential Ar-mediated antiandrogen action. These mechanisms showed that TCS may induce Vtg production in male carp by non-Er-mediated pathways.

1. Introduction

Triclosan (TCS, 2, 4,4′-trichloro-2′- hydroxydiphenylether), one of pharmaceutical and personal care products (PPCPs), is a broad spectrum antimicrobial agent found in consumer products, including toothpaste, soaps, detergents, deodorants, antiseptics, toys, mattresses, and surgical cleaning treatments. Based on the wide use of TCS, the majority of consumer products containing TCS are finally flushed down drains and discharged with wastewater effluent (Gao et al., 2014; Axelstad et al., 2013: Sabaliunas et al., 2003; Peng et al., 2017). The incomplete removal of TCS during wastewater treatment processes results in the continual exposure of aquatic organisms to it.

TCS is characterized by accumulation, degradability, and transportation through food chains in the aquatic environment, and has a variety of toxic effects on aquatic organisms (Orvos et al., 2002; Martins et al., 2017). It is typically transformed into metabolites as soon as it enters the environment, including wastewater

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treatment plants. The major metabolite found in wild fishes is methyl triclosan (MTCS) (Rüdel et al., 2013; Leiker et al., 2009) and high levels of this metabolite have been previously reported in common carp near a wastewater treatment plant (Patino et al., 2015). MTCS is more persistent and toxic, found more commonly in fish than TCS (Rüdel et al., 2013). Because TCS metabolites may not be readily available, most laboratory studies have relied on the parent compound.

There is strong evidence that some hydrobiological species such as algae, invertebrates and certain types of fish are much more sensitive to TCS than mammals. Moreover, it has become evident that TCS acts as a hormone disrupting chemical in fish in recent years (Raut and Angus, 2010; Orvos et al., 2002; Wilson et al., 2003; Ishibashi et al., 2004; Tatarazako et al., 2004; Axelstad et al., 2013).

Modified morphology and hyperplasia in the thyroid tissue and a significant upregulation of the sodium–iodide symporter (NIS) and thyroid-stimulating hormone (TSH) in zebrafish exposed to TCS suggest that a reduction in circulating thyroid hormones probably occurred (Pinto et al., 2013). TCS could interfere with thyroid hormone (TH)-mediated gene expression, accelerate TH-induced metamorphosis and/or alter larval growth on amphibian (Veldhoen et al., 2006; Fort et al., 2011; Helbing et al., 2010). TH-disrupting effects observed on amphibian for TCS could also be mediated through its metabolite (Hinther et al., 2011). These results showed that TCS and its metabolite disrupt TH action.

Changes in fin length and non-significant trends in the sex ratio of medaka (*Oryzias latipes*) exposed to TCS indicated that TCS was a potentially weakly androgenic contaminant (Foran et al., 2000). However, in another study on male South African clawed frogs (*Xenopus laevis*), exposure to TCS lowered the plasma vitellogenin (Vtg) and testosterone (T) levels, indicating antiestrogenic effects (Matsumura et al., 2005). Moreover, exposure to TCS significantly induced hepatic protein secretion of Vtg in male medaka (Ishibashi et al., 2004) and mosquito fish (*Gambusia affinis*), suggesting that TCS has significant estrogenic properties (Raut and Angus, 2010). In addition, TCS decreased the sperm counts in mosquito fish (Raut and Angus, 2010), changed sperm morphology (Halden, 2014), induced abnormal development of sea urchin embryos through the concomitant suppression of a number of genes that are necessary for embryonic differentiation in the blastula stage (Hwang et al., 2017), suggesting that TCS affects reproduction and embryo differentiation.

Previous studies results show TCS has reproductive endocrine disrupting effects. Moreover, most of the studies supported the opinion that TCS has estrogenic effects (Wang and Tian, 2015).

Vtg production in male or juvenile fish has become one of the important and useful biomarkers for detecting estrogenic contamination in aquatic environments (Ishibashi et al., 2004). Endocrine-disrupting chemicals (EDCs) could induce Vtg in males by interfering with the hypothalamic–pituitary–gonadal (HPG) axis through some pathways (Ankley et al., 2009). Gonadotropin-releasing hormones (GnRHs) are mainly secreted from the hypothalamus. It is well known that GnRHs regulate pituitary gonadotropins (GtHs). Two forms of GnRH have been identified in common carps: sGnRH-1 and cGnRH-ii-2 (Xu et al., 2011). GtHs include follicle stimulating hormone (FSH) and luteinizing hormone (LH). They are composed of a α subunit (GP-α) and a β subunit (FSH-β or LH-β), and are pivotal to regulate gametogenesis and steroidogenesis in teleosts (Nyuji et al., 2013).

Aromatase of teleost may affect the balance of androgen and estrogen (Tian et al., 2010; Han et al., 2011). Cyp19a and cyp19b are two of key aromatases. The cyp19b is known to be controlled via a positive auto-regulatory feedback loop which is driven by 17β-estradiol (E_2) in the brain of fish (Callard et al., 2001; Ji et al., 2013a). The cyp19a plays a pivotal role for gonadal sex differentiation and sex change in fish by catalyzation of the last step in the transition of androgen to estrogen (Guiguen et al., 2010; Urbatzka et al., 2012; Ji et al., 2013b). Sex steroids may bind with receptors in the HPG axis to produce a feedback system.

TCS is structurally similar to anthropogenic estrogenic and androgenic EDCs (e. g., diethylstilbestrol, bisphenol A, 2, 3, 7, 8 tetrachloro-p- dibenzo-dioxin) (Jacobs et al., 2005). It is well known that xenoestrogens mimic E_2 to bind with estrogen receptors (Ers) to activate estrogen response element which include Vtg. Moreover, estrogen levels generated via some non-Er pathways can also induced Vtg (Tian et al., 2010). Previous study showed TCS probably does not act as an environmental estrogen acting via estrogen receptor alpha (ERα) (Sébillot et al., 2014).

The Yellow River carp (*Cyprinus carpio*) is an iconic fish in the lower Yellow River of China, and a suitable ecological indicator of river health impacts from flow regulation. Previous study showed the majority of mass inventories of TCS are stored into sediment of Yellow River in China, which could be a potential pollution source for Yellow River water (Zhao et al., 2013). To better characterize the effects of TCS on the ecological balance of water in the Yellow River of China and TCS hormone effect mechanisms, we investigated the effects of TCS on sex steroids and Vtg level in Yellow River carps. Meanwhile, we also determined the expression of aromatase, sex hormone receptor, and the synthesis and secretion of GtHs and the regulation of GnRHs to examine potential underlying mechanisms.

2. Material and methods

2.1. Chemicals

TCS (>98.0% purity) was purchased from Wako Pure Chemical Industries Ltd., Tokyo, Japan. It was dissolved in dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries), and the stock solution (2, 000 mgL^{-1}) was stored in the dark at 4 °C, and used to prepare the test solutions by serially diluting to the test concentrations. The test solution contained 0.1% DMSO. MS-222 (3- aminobenzoic acid ethyl ester, methanesulfonate salt) was abstained from Sigma (St. Louis, MO, USA). All other chemicals used were analytical grade.

2.2. Fish

The use of all fish was approved by the institutional animal protection board of the Chinese Academy of Sciences, and subsequent procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Chinese Animal Welfare Committee. Juvenile Yellow River carps at 7 weeks old were purchased from the Yellow River carp breeding field in Henan Province of China. The mean body weights and lengths were 5.50 ± 0.38 g and 6.27 ± 0.42 cm. All carps were put in aerated tap water (20 ± 2 °C; pH 7.0 ± 0.1) in the laboratory for 10 d prior to TCS exposure. During this period, fish were fed daily with freshly hatched brine shrimp twice a day, and one fourth of the water was replaced every day. The carps were starved for 3 d before acute toxicity tests.

2.3. Acute exposure experiment

Based on the results of our preliminary experiments, the concentrations of TCS, which are between the safe dose and the total lethal dose of TCS to the carp in 48 h, were used for the acute toxicity tests. Ten randomly selected carps were placed in the glass aquaria of 10 L of TCS test solutions. Exposure tests were done with 3 tank replicates per treatment. Half of test solution in each glass aquaria was daily replaced with the fresh test solution. The other experiment conditions were the same as those of the acclimation