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Histopathological changes and lipid metabolism in the liver of *Bufo gargarizans* tadpoles exposed to Triclosan

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HIGHLIGHTS

- TCS induced marked histological changes in the liver of tadpoles.
- Hepatic ultrastructural alterations were also observed in exposed animals.
- Transcript levels of key genes involved in lipid metabolism were altered by TCS exposure.
- TCS induced alterations of mRNA levels of detoxification and oxidative stress genes.

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ABSTRACT

In the current study, the adverse effects of TCS on liver health of *B. gargarizans* tadpoles were assessed. *B. gargarizans* larvae were exposed to TCS at 0, 10, 30, 60, and 150 μg L^{-1} from Gosner stage 3 until metamorphic climax. The hepatosomatic index (HSI), hepatic histological and ultrastructural features, and transcript levels of genes associated with detoxification and oxidative stress as well as lipid metabolism in the livers were determined. Exposure to 150 μg L^{-1} TCS resulted in increased HSI of tadpoles at metamorphic climax. Histological changes characterized by an increase in the number of melanomacrophage, nucleus pyknosis, and deposition of collagen fibers were observed in liver at 60 and 150 μg L^{-1} TCS. Moreover, marked ultrastructural alterations including high electron dense in mitochondrial matrix and lipid accumulation were also observed. In addition, abundances of transcripts of Cu/Zn superoxide dismutase (*SOD*), phospholipid hydroperoxide glutathione peroxidase (*PHGPx*), and heat shock protein 90 (HSP_{90}) were decreased in larvae exposed to 60 and 150 μg L^{-1} TCS, while transcript level of HSP_{90} was increased at 30 μg L^{-1} TCS. Also, abundances of transcripts of acetyl-CoA carboxylase (*ACC*), carnitine palmitoyltransferase 2 (*CPT2*), peroxisome proliferator-activated receptor alpha (*PPARα*), fatty acid elongase 1 (*FAE*), sterol carrier protein 2 (*SCP*) were significantly lesser in larvae exposed to 60 and 150 μg L^{-1} TCS. Overall, TCS at high levels induced histopathological changes in the liver of *B. gargarizans* tadpoles. This might have been due to the alteration of oxidative stress–related genes and lipid metabolism-related genes expression levels.

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1. Introduction

Triclosan (TCS), also known as 5-chloro-2-(2,4-dichlorophenoxy) phenol, is a synthetic, broad-spectrum antimicrobial agent with antibiotic and antimycotic properties (Levy et al., 1999). The chemical has been used for over 30 years as biocide in personal care and consumer products, specifically in detergents, cosmetics, soap, toothpaste, and shampoo with concentrations up to 0.3% (w/w) (Piccoli et al., 2002). TCS is an emerging contaminant with a ubiquitous presence in aquatic environments across the globe, owing to its extensive use (Chen and Chou, 2016; Peng et al., 2016; Ramaswamy et al., 2011; Singh et al., 2010), and it has been also detected in wild animals (Fair et al., 2009; Valters et al., 2005) and human plasma, breast milk, urine, and amniotic fluid (Adolfsson-Erici et al., 2002; Dann and Hontela, 2011; Shekhar et al., 2017). The transformation of TCS in aquatic environments

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and the toxicity of the intermediates formed to aquatic organisms have been assessed (Gao et al., 2014). It is therefore unsurprising that growing concerns have been raised about the potential impacts on non-target organisms and the environmental implications of increasing TCS level.

TCS exert its antibiotic effects on fatty acid biosynthesis in bacteria by blocking the enzyme enoyl-acyl carrier protein reductase (FabI), which catalyzes an essential step in the bacterial lipid biosynthesis pathway (Heath et al., 1999; Wright and Reynolds, 2007). Recently, several studies have shown that TCS also affected the lipid metabolism of non-target organisms. For instance, exposure to 250 μg L^{-1} TCS was associated with impairment of lipid beta-oxidation in zebrafish embryos, which induced lipid droplet accumulation in the yolksac (Ho et al., 2016). Similarly, Regnault et al. (2016) reported that *sterol regulatory element-binding protein 2 (SREBP2)* and *hydroxymethylglutaryl-CoA reductase (HMGCR)*, two genes involved in cholesterol biosynthesis regulation, were down-regulated and the mRNA level of *fatty acid synthase (FASN)* gene was significantly increased in liver of *Xenopus tropicalis* exposed to TCS.

Oxidative stress is a recognized mode of action of TCS exposure that has been observed in aquatic organisms including fish, snail, and marine invertebrates (Han et al., 2016; Liang et al., 2013; Wang et al., 2014). Organisms have developed antioxidant defense systems consisting of both enzymatic and non-enzymatic antioxidants to scavenge excess ROS and avoid oxidative damage to cells (Lushchak, 2011). Superoxide dismutase (SOD) and phospholipid hydroperoxide glutathione peroxidase (PHGPx) are highly conserved antioxidant enzymes to combat oxidative stress (Bordo et al., 1994; Margis et al., 2008). SOD as the first line of defense against the accumulation of ROS, which can catalyze the conversion of superoxide anion radical ($O_2^{\bullet}$) to produce hydrogen peroxide (H_2O_2) (Lushchak, 2011). PHGPx is a member of the glutathione peroxidase (GPx) family (Toppo et al., 2008). The H_2O_2 formed by SOD activity can be decomposed to H_2O and O_2 by PHGPx in the presence of reduced glutathione (Imai and Nakagawa, 2003). PHGPx also plays unique roles in the protection of plasma and lysosomal membranes from ROS-induced damage by reducing complex membrane bound hydroperoxides and lipid peroxides (Roveri et al., 1994; Schnurr et al., 1996; Ursini et al., 1985). In addition, heat shock proteins (HSPs) are one of the more studied defense system against cellular oxidative damage (Gupta et al., 2010; Lee et al., 2013). Several ecotoxicological studies have demonstrated SOD, PHGPx, and HSPs are important molecular biomarkers of oxidative stress in aquatic organisms in relation to environmental contaminants (Jiang et al., 2012; Mathieu-Denoncourt et al., 2014; Valavanidis et al., 2006; Woolfson and Heikkila, 2009; Yang et al., 2010).

However, most experimental studies have examined the effects of TCS on the oxidative stress and fatty acid metabolism. Thus, there has been little attention paid to liver health after treatment with TCS, especially to histological features of liver. *Bufo gargarizans*, a species of toad native to China, has shown sensitivity to aquatic pollutants (Wang et al., 2015, 2016; Zhao et al., 2013). In this study, liver health of *B. gargarizans* larvae were assessed after treatment with TCS. Histological and ultrastructural features of liver were investigated, and further investigation was conducted: (1) the transcriptional changes of the antioxidant enzyme genes (*SOD* and *PHGPx*) and *HSP90* using RT-qPCR to evaluate the status of oxidative stress; (2) transcript levels of selected lipid metabolism-related genes (*ACC*, *ACOX*, *CPT2*, *PPAR-a*, *FAE*, and *SCP2*) using RT-qPCR to investigate the effect of TCS exposure on lipid metabolism. This study provides a better understanding of the molecular response and the action mechanisms related to the toxicity of TCS on the amphibian.

2. Material and methods

2.1. Experimental animals

In February 2015, three pairs of adult *B. gargarizans* captured from ponds located in Qinling Mountains, Shaanxi Province, China (109º06′52″E, 34º00′56″N). Eggs were collected and hatching in our laboratory. Embryos at Gosner stage (Gs) 3 (Gosner, 1960) were used for experiment.

2.2. Test solutions

Triclosan (TCS, ≥97% purity) was purchased from Sigma-Aldrich Corporation (Sigma, St. Louis, MO, USA). For the chronic exposure, a 10 mg mL^{-1} stock solution was prepared by dissolving TCS in absolute ethanol, and individual treatment solutions were diluted with dechlorinated tap water to an appropriate concentration of TCS (10, 30, 60, or 150 μg L^{-1}). Basic water quality parameters (pH, conductivity, dissolved oxygen, and salinity) were measured with GDYS-201 M multi-parameter water quality analyzer (Little Swan, China) and PC300 waterproof portable meter (Clean, USA).

2.3. Experimental procedure and sampling

Embryos at Gs 3 (Gosner, 1960) were placed randomly into 4 L of test solutions with a specified concentration of TCS in each aquarium (50 cm × 20 cm × 20 cm). One control (dechlorinated tap water) was also included in the test. Exposure and control treatments had three replicates, and there were 80 embryos per replicate. During the exposure, larvae were fed every other day with fresh boiled vegetables. Aquaria were kept at 18 ± 1 °C and test solutions were renewed every 48 h to maintain optimal water quality conditions. Photoperiod was maintained at a 12 h/12 h light/dark cycle.

Tadpole development was monitored daily. When each *B. gargarizans* individual reached metamorphic climax (forelimb emergence at Gs 42) (Gosner, 1960), it was removed from their tanks immediately upon their identification. For morphometry, animals that metamorphosed were instantly fixed using 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Then, measurements of total length, snout-vent length (SVL), and hind limb length (HLL) were taken. Subsequently, individuals and livers were weighed. Morphometric data were used to calculate condition factor (K): (weight/SVL^3) × 100, and hepatosomatic index (HSI): (liver weight/body weight) × 100 for each individual.

For ultrastructural analysis, fresh liver tissues were cut into small pieces (1 mm^3) and fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2). Liver samples were also collected for histological analysis. Following an abdominal incision to facilitate tissue preservation, G42 larvae were fixed in Bouin's solution for 24 h, and then stored in 70% ethanol. For mRNA expression analysis of genes, Gs 42 tadpoles were eviscerated immediately and livers were flash frozen in liquid nitrogen and stored in liquid nitrogen until subsequent processing.

2.4. Histology

Liver of Gs 42 tadpoles were dissected out and processed for standard paraffin embedding. Serial 6 μm sections were stained with hematoxylin and eosin (H&E) for routine histological examination. Masson's trichrome staining was also performed for collagen fibers evaluation. Stained slides were observed under an Olympus BX-53F microscope equipped with a CCD camera (Olympus Corporation, Tokyo, Japan). Representative images were recorded and saved.

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