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Molecular responses in digestive tract of juvenile common carp after chronic exposure to sublethal tributyltin

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ABSTRACT

The effect of long-term exposure to tributyltin (TBT) on the intestine-related biochemical biomarkers in common carp was investigated in this study. Fish were exposed at sub-lethal concentrations of TBT (75 ng/L, 0.75 and 7.5 μg/L) for 60 days. Multiple biomarkers were measured, including digestive enzymes (trypsin, lipase and amylase), antioxidant responses (malondialdehyde (MDA) and total antioxidative capacity (T-AOC)), RNA/DNA ratio and the expression of digestive-related genes (*try, lipc* and *amy*). TBT exposure at 0.75 and 7.5 μg/L led to significantly inhibited activities of all digestive enzymes. At higher concentration of TBT, oxidative stress was apparent as reflected by the significant higher MDA content in the fish intestine, associated with an inhibition of T-AOC activities. After 60 days, the RNA/DNA ratio in fish intestine was significantly lower in groups exposed to TBT at higher concentrations (0.75 and 7.5 μg/L). In addition, the expression levels of *try, lipc* and *amy* in intestine of all treated fish were inhibited, even at the environmental concentration (75 ng/L). Our results suggest that long-term exposure to TBT could result in different responses of intestine-related biochemical biomarkers in fish, which could be used as new potential indicators for monitoring residual TBT present in aquatic environment.

1. Introduction

Nowadays, a series of toxicological and environmental problems that resulted from the widespread use of organotin compounds have received extensive concerns, in particular the appearance of potential toxic effects in man and animals (Hall and Pinkney, 1985). Particularly, a great deal of attention has been paid to accumulation and toxic effects of butyltin compounds in aquatic system (Kannan et al., 1996; Kannan and Falandysz, 1997; Kannan et al., 1997, 1998; Hong et al., 2002). Among them, tributyltin (TBT), as antifouling paints, should need more attention because of its high stability and toxicity to the aquatic organisms (Qun-Fang et al., 2002; Antizar-Ladislao, 2008). Literature on the effects of TBT in biota has focused primarily on reproductive toxicity. It has been demonstrated that TBT can induce imposex in female mollusks (Morcillo and Porte, 2000). In fish, it has been reported that TBT can affect sexual behaviour and reproduction (Nakayama et al., 2004), change the oestrogen/androgen levels and inhibit gonad development (Zhang et al., 2007). Moreover, TBT-induced neurotoxicity, developmental toxicity and endocrine dysfunction have been reported in previous studies (St-Jean et al., 2002; Horiguchi, 2006; Porte et al., 2006). Despite the efforts to reduce its use, considerable levels of TBT are still detected in aquatic ecosystems exceeding toxicity levels (Antizar-Ladislao, 2008). In China, the environmental concentrations of TBT were recorded as 0.5 ng/L (detection limited) to hundreds of ng/L as Sn in surface water (Gao et al., 2006).

It is well known that many environmental contaminants could produce severe damage in different organs of fish and alter the activities of enzymes (Li et al., 2011). Intestine is an important organ in charge of digestive and absorbable function, although it is not directly exposed to environmental toxicants and not mainly responsible for detoxification (Sastry and Gupta, 1979). If intestinal function was damaged, the biotransformation and other physiological process *in vivo* would be disordered and impaired, which finally might lead to organismal death (Leaner and Mason, 2002). Nonetheless, the related information about the effects of organotin compounds on intestinal function of fish is largely lacking, so it is necessary to fill the gap in this field.

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The enzymatic systems, including digestive enzymes and antioxidant enzymes, not only play a key role in maintaining the normal physiological metabolic regulation *in vivo*, but also are very sensitive to environmental stress (Lionetto et al., 1998; Li et al., 2010a). Therefore, enzymatic alterations in aquatic organisms are always used as bio-indicators for monitoring environmental pollutants. In addition, toxic pollutants that interfere with energy-yielding reactions indirectly inhibit the synthesis of RNA, DNA and protein (Kim and Kang, 2004). Hence, RNA to DNA ratio not only provides a measure of synthetic capacity of cell, but also could be a potential tool for reflecting environmental stress (Li et al., 2010d).

The objectives of this study were to investigate the effects of the TBT on the digestive-related biochemical parameters in juvenile common carp (*Cyprinus carpio*), by analysing the digestive enzymes trypsin, lipase and amylase, antioxidant response (MDA and T-AOC) and RNA/DNA ratio, as well as the expression of digestive-related genes (*try, lipc* and *amy*) in intestine of fish.

2. Materials and methods

2.1. Chemicals

TBT, 90%, was purchased from Sinopharm Chemical Reagent Co. Ltd (Beijing, China). Suitable amount of this compound was directly weighed into a brown volume vessel and dissolved in 50 ml acetone (ACT)-water (1:1) to form a concentration level stability. This stock solution was sealed and kept at 4 °C in a refrigerator until used. Working standard solution (100 μg/ml) was freshly prepared by diluting the stock solution with deionized water before use.

2.2. Fish

The juvenile common carp (9.65 ± 0.13 cm, 22 ± 1.8 g) obtained from a local hatchery (Jingzhou, China) was raised in a flow-through system with dechlorinated tap water (pH 7.4 ± 0.2; hardness 42.5 ± 1.3 $CaCO_3$/L) at a constant temperature (20 ± 1 °C) with a photoperiod of 12:12 h (light:dark). Fish were acclimatized for 14 days before the beginning of the experiment and were fed commercial fish food (Tongwei, China). Wastes and residues were removed daily while the test equipment and chambers were cleaned once a week. The fish were starved for 24 h prior to experimentation to avoid prandial effects during the assay. Experiments were carried out in accordance with our institute rules approved by a local ethics committee.

2.3. Exposure to TBT

A 100 L semi-static system was used in which 20 juvenile common carp were randomly distributed to each of ten aquaria. The nominal concentrations of TBT used were 75 ng/L (E1 group, according to environmental concentration), 0.75 μg/L (E2 group, 1% 96 h-LC50) and 7.5 μg/L (E3 group, 10% 96 h-LC50). TBT was dissolved in ACT with a final concentration less than 0.01%. Two other groups were used as contrast groups, a control group exposed to clean freshwater and a ACT group exposed to the volume of ACT (v/v, 0.01%) used for the highest TBT concentration. Each experimental condition was duplicated. The fish were fed daily with commercial fish pellets at 1% total body weight at a fixed time and the extra food was removed. Eighty percent of the exposed solution was renewed each day after 2 h of feeding to maintain the appropriate concentration of TBT and ACT and to maintain water quality. The test equipment was cleaned every 7 days. The test fish were exposed to TBT for 60 days. At the end of the exposure time, 6 fish in each group were sampled, immediately frozen and stored at −80 °C until further study.

To ensure agreement between nominal and actual compound concentrations in the aquaria, water samples were analysed during the experimental period by LC-MS/MS. Water samples were collected from the test aquaria after 1 h and 24 h of renewing the test solutions. The mean concentration of TBT in the water samples was always within 20% of the intended concentration.

2.4. Biochemical parameters measurement

2.4.1. Digestive enzyme activities

The activities of trypsin, lipase and amylase and protein concentration in the intestine of fish were determined by spectrophotometry using kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The units of the digestive enzymes were defined based on the manufacturer's recommendations. Briefly, 1 U of trypsin activity was expressed as the equivalent enzyme activity required to generate an optical density (OD) change of 0.003 at pH 8.0 and 37 °C. Amylase activity was calculated as the activity required to hydrolyse 10 mg of starch in 30 min at 37 °C. One unit of lipase activity was defined as the micromole of substrate hydrolysed per minute at 37 °C. Enzyme activities were expressed as specific activity (U/mg protein).

2.4.2. Antioxidant parameters

Frozen samples for analysis of enzyme activities were defrosted and homogenized on ice with 10 volumes of cold 0.86% physiological saline. The homogenate was centrifuged at 3000 rpm at 4 °C for 10 min to obtain the supernatant for the MDA content and T-AOC activities. All the biochemical parameters were measured by following the instruction of the kit (Nanjing Jiancheng Bioengineering Institute, China). MDA content, as a biomarker for lipid peroxidation, was examined, which is based on 2-thiobarbituric acid (2,6-dihydroxypyrimidine-2-thiol; TBA) reactivity, and the results were expressed as nmol/mg protein (Jain et al., 1989). The measurement of T-AOC activities was based on the detection of ROS by fluorometry (ex/em: 485/520 nm) employing 2′,7′-dichlorofluorescein diacetate (H(2)DCF-DA) as substrate, and the activity was expressed as U/mg protein (Amado et al., 2009).

2.4.3. RNA/DNA ratio

Each frozen sample was weighted and homogenized (1:10 w/v) with Tris–HCl buffer (pH 7.4). Free nucleotides were removed using a series of washes with cold perchloric acid ($HClO_4$). RNA was then hydrolysed with potassium hydroxide and the hydrolysate was acidified with cold $HClO_4$ to remove the RNA from the DNA and protein. Intestinal RNA/DNA ratio was measured using the method according to Kuropat et al. (2002) as modified by Mercaldo-Allen et al. (2006).

2.5. Total RNA extraction and real-time PCR

Total RNA was extracted from fish tissues using a Trizol kit (TaKaRa, Dalian, China). After the total RNA was incubated with deoxyribonuclease I (TaKaRa, Dalian, China), the reverse transcription was performed on 1 μg of total RNA following a PrimeSript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) using the manufacturer's recommended procedure. Levels of *try, lipc* and *amy* in the fish tissues were determined using quantitative RT-PCR with SYBR Green chemistry on a Rotor-Gene 3000 (Applied Biosystems, USA) using the housekeeping gene *β-actin* as internal control according to the method of our laboratory (Li et al., 2014). The differences in expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The quantitative RT-PCR primers are shown in Table 1.

2.6. Integrated biomarker response (IBR)

Biomarkers were combined into one general "stress index" termed IBR (Beliaeff and Burgeot, 2002). The result is directly dependent on the number of biomarkers (n) in the set and thus, IBR values were presented divided by n as suggested by Broeg and Lehtonen (2006). Results of data standardization procedure needed for IBR calculation were presented in site star plots.

2.7. Data statistical assays

All values were expressed as mean ± SD and analysed by SPSS for Win 13.0 software. After the normality of the data was verified, one-way ANOVA with Tukey's test was used to determine whether the results of treatments were significantly different from the control group ($p < 0.05$ or $p < 0.01$).

3. Results

3.1. Digestive enzymes

Changes of activities of three different digestive enzymes in fish intestine were shown in Table 2. Although a slight hint for a decrease of activities of all digestive enzymes were observed in E1 group, there was no significant change ($p > 0.05$). However, all digestive enzymes activities were significantly inhibited ($p < 0.05$ or $p < 0.01$) at the higher TBT concentrations (E2 and E3 groups).

3.2. Antioxidant responses

The oxidative stress and antioxidant responses in fish intestine were evaluated in this study (Fig. 1). Although there was no significant induction ($p > 0.05$) in MDA formation in lower concentration of TBT (E1 and E2 groups), a significant higher ($p < 0.05$) MDA level was observed in E3 group after 60 days of exposure compared with the control. In the study, T-AOC activities were slightly induced ($p > 0.05$)