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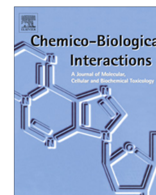

# Evaluation of tributyltin toxicity in Chinese rare minnow larvae by abnormal behavior, energy metabolism and endoplasmic reticulum stress

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A B S T R A C T

Tributyltin (TBT) is a ubiquitous contaminant in aquatic environment, but the detailed mechanisms underlying the toxicity of TBT have not been fully understood. In this study, the effects of TBT on behavior, energy metabolism and endoplasmic reticulum (ER) stress were investigated by using Chinese rare minnow larvae. Fish larvae were exposed at sublethal concentrations of TBT (100, 400 and 800 ng/L) for 7 days. Compared with the control, energy metabolic parameters (RNA/DNA ratio, $Na^+$-$K^+$-ATPase) were significantly inhibited in fish exposed at highest concentration (800 ng/L), as well as abnormal behaviors observed. Moreover, we found that the PERK (PKR-like ER kinase)–eIF2α (eukaryotic translation initiation factor 2α) pathway, as the main branch was activated by TBT exposure in fish larvae. In short, TBT-induced physiological, biochemical and molecular responses in fish larvae were reflected in parameters measured in this study, which suggest that these biomarkers could be used as potential indicators for monitoring organotin compounds present in aquatic environment.

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## 1. Introduction

Tributyltin (TBT), one of the organotin compounds, has been extensively used as an antifouling agent in paints applied to ships, boats and aquaculture nets [1]. Although the use of organotins in paints for vessels is virtually banned by the international treaty at present, its residue in the aquatic environment is still an important concern, due to its bioaccumulation potential, persistence in sediment and highly toxic effects on non-target aquatic life [2]. And fragmentary data about the occurrence of organotin compounds in China showed that some aquatic environment have been seriously polluted by TBT with concentrations from 0.5 ng/L (detection limited) to hundreds of ng/L as Sn [3].

Fish exposed to elevated TBT levels or other environmental alterations exhibit a variety of behavioral, physiological and biochemical responses, which provides a unique perspective linking the physiology and ecology between the organism and its environment [4]. Since behavior is not a random process, but rather a selective response that is constantly adapting through direct interaction with physical, chemical, social and physiological aspects of the environment, behavioral endpoints serve as valuable tools to discern and evaluate effects of exposure to environmental stressors and fish behavioral alterations can provide important indices for ecosystem assessment [4,5]. And environmental pollutants that interfere with energy-yielding reactions indirectly inhibit the synthesis of RNA, DNA and protein and some enzyme activity, e.g. ATPase [6]. Therefore, RNA to DNA ratio and ATPase activity have been extensively used to estimate energy metabolic conditions [7]. Additionally, the endoplasmic reticulum (ER) is the intracellular organelle responsible for various aspects of the quality control of biologically active proteins, such as synthesis, folding, posttranslational modification, and delivery [8]. It has been well documented that when cells are subjected to stress, some signal pathways are activated, such as the protein kinase RNA-activated-like ER kinase (PERK)–eukaryotic translation initiation factor 2 alpha (eIF2α) pathway, the inositolrequiring enzyme 1 (IRE1)–X-box binding protein 1 (XBP1) pathway and so on [2].

Although some available studies were focused on the adverse effects of TBT on fish, there is an information gap about the poten-

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tial contribution of behavioral responses, energy metabolism and ER stress in TBT toxicity. In our study, Chinese rare minnows (*Gobiocypris rarus*) are used as test fish, due to its small size, ease of culture, short life cycle and prolific egg production with high fertilization and hatching rates [9]. Our studies aimed at investigating the effects of TBT on behavioral responses, energy metabolism and ER stress in fish larvae, and try to clarify the possible molecular mechanisms.

## 2. Materials and methods

### 2.1. Chemicals

TBT, 90%, was purchased from Sigma–Aldrich Chemical Co. (USA). Suitable amount of this compound was directly weighed into a brown volume vessel and dissolved in 50 ml acetone–water (1:1) to form a concentration level of 1 mg TBT/ml as the stock solution, in which a little HCl (1.0 mol/L) was added to ensure its 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. Test fish and culture conditions

The rare minnow larvae (7 days after hatching, 9.65 ± 0.13 mm, 1.12 ± 0.17 mg) obtained from a local hatchery (Wuhan, China), was raised in a flow-through system with dechlorinated tap water (pH 7.2–7.6; hardness 44.0–61.0 mg $CaCO_3$/L) at a constant temperature (25 ± 1 °C) with a photoperiod of 16:8 h (light:dark) and has been fed with newly hatched brine shrimp (*Artemia nauplii*) two times daily. Waste and residue were removed daily while the test equipment and chambers were cleaned once a week.

### 2.3. Experimental design

To investigate the effects of sublethal TBT on physiological and biochemical parameters, larvae ($n = 20$) were transferred to 50 ml beakers equipped with mild aeration and exposed to several TBT concentrations (100, 400 and 800 ng/L) for a period of 7 days. TBT concentration with 100, 400 and 800 ng/L was used as environmental level, 10% 96 h-LC50 and 20% 96 h-LC50 (the 96 h-LC50 of TBT on Chinese rare minnow larvae was measured as 3.96 μg/L, unpublished), respectively. Another 20 test fish were raised in clean water for 1 week as the control group. Basic physical and chemical indices of the water used in the sublethal test were maintained as described for the acclimation period. Each group was duplicated. The beakers were checked twice a day. The behavioral changes of the healthy fish and the fish exposed to various doses of TBT were photographed with camera and evaluated for behavioral anomalies. The behavioral patterns changes of all tested fish were monitored according to the ASTM (E1711-95) Standard Guide for Measurement of Behavior during Fish Toxicity Tests. During the test, 80% of water was daily changed and replaced by new water containing the corresponding concentration of TBT. At the end of the exposure time, 6 larvae in each group were sampled, immediately frozen and stored at −80 °C until further study. As rare minnows larvae were too small, all the biochemical analysis and gene expressions were from a pool of whole larvae.

### 2.4. Biochemical assays

#### 2.4.1. RNA/DNA ratio assay

Each sample was weighted and homogenized (1:10 w/v) within Tris–HCl buffer (pH 7.4). Free nucleotides were removed using a series of washes with cold perchloric acid ($HClO_4$). RNA was then hydrolyzed with potassium hydroxide and the hydrolysate was acidified with cold $HClO_4$ to remove the RNA from the DNA and protein. Muscle RNA/DNA ratio was measured using the method according to Kuropat et al. [10] as modified by Mercaldo-Allen et al. [11].

#### 2.4.2. $Na^+$-$K^+$-ATPase assay

Frozen samples for analysis of $Na^+$-$K^+$-ATPase activity 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 enzyme activity assays of $Na^+$-$K^+$-ATPase, which was measured using the commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manual. $Na^+$-$K^+$-ATPase activity, expressed as μmol Pi liberated/mg protein/h in fish.

### 2.5. Gene expression

#### 2.5.1. RNA extraction and cDNA preparation

The larvae were washed twice with PBS (pH 7.4) and the total RNA of 10 homogenized larvae was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Each set of 10 larvae was pooled for RNA preparation. To remove the possibility of genomic DNA contamination, the RNA was digested by RNase-free DNase I (Promega Madison, WI, USA) and then purified. The total RNA recovered from the DNase I digestion process was measured at 260 and 280 nm using a spectrophotometer (M2, Molecular Devices, Sunnyvale, CA, USA). The 260 nm reading was used to estimate the concentration of total RNA. The RNA quality in each sample was verified by measuring the 260/280 nm ratios and by 1% agarose-formaldehyde gel electrophoresis with ethidium bromide staining. The cDNA was synthesized using 2 μg of RNA from each sample mixed with 500 ng of random primer (Takara, Kyoto, Japan) to which was added 10 mM of dNTPs diethylpyrocarbonate-(DEPC-) treated water to a final volume of 20 μl. The reaction was initiated using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) following the manufacturer's protocols.

#### 2.5.2. Quantitative real-time PCR (QPCR)

The QPCR was performed as previously described [12]. Gene names, accession numbers, forward and reverse primer sequences are listed in Table 1. PCR amplification was conducted on a Chrom 4TM detector (BioRad, USA) in sterile, 96-well PCR plates (Applied Biosystems, Foster City, CA). Every sample was analyzed individually and processed in triplicate. On the basis of the previous study, beta-actin (*β-actin*) expression levels were not significantly changed in different treated groups, and it was chosen as an internal control to normalize the data [13]. After verifying that the amplification efficiencies of the selected genes and *β-actin* were approximately equal, differences in expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [14].

**Table 1**
Sequences of primers for the tested genes.

| Gene name | Sequences of primers (5′-3′) | Accession number |
|---|---|---|
| β-actin | Forward: CTGTTCCAGCCATCCTTCTT<br>Reverse: TGTTGGCATACAGGTCCTTAC | DQ539421 |
| eif2ak3 | Forward: AGACAGCGACGAACCG<br>Reverse: ACCATAGGAGTTCGGAGT | BC122104.1 |
| atf4b1 | Forward: TTGGAGGGCTCGTGGTC<br>Reverse: TGGACTTACTGAAGCTGGAC | NM213233.1 |
| ern1 | Forward: GAAGGAGGATACGACTT<br>Reverse: CTCGGAGGGAAGAATAA | NM0010205301.1 |
| xbp1 | Forward: CTGTTGCCGAGACAAGACG<br>Reverse: TTCCTCGTCCAAGTCCATG | NM131874.1 |

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