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Biological effects of tris (1-chloro-2-propyl) phosphate (TCPP) on immunity in mussel *Mytilus galloprovincialis*

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ABSTRACT

Organophosphate flame retardants (OPFRs) are increasingly produced and used as alternatives of brominated flame-retardants (BFRs) and have become emerging marine environmental contaminants. So far, however, little is known regarding the biological effects of OPFRs in marine organisms. In this study, the biological effects of one of the most abundant OPFRs, tris (1-chloro-2-propyl) phosphate (TCPP), on the immunity in mussel *Mytilus galloprovincialis* were characterized by testing the reactive oxygen species, apoptosis, antioxidant system and immunity related gene expressions. Results indicated that both TCPP exposures (10 and 100 nmol L^{-1}) significantly ($p < 0.01$) enhanced reactive oxygen species production and the high dose of TCPP induced more apoptosis and oxidative stress in mussel hemocytes. TCPP also induced an obvious hormesis phenomenon (low dose inhibition and high dose stimulation) in mussel hemocytes, as indicated by the gene expression profiles of *caspase 8* and *mytimacin*. The down-regulated gene expression levels of lysozymes suggested that both TCPP exposures inhibited the innate immunity in mussel *M. galloprovincialis*. The significantly ($p < 0.01$) increased gene expression levels of *TLR*, *galectin*, *PGRP* and *LITAF* demonstrated that TCPP induced dose-dependent immune stress in mussels. Overall, this work suggested that TCPP could influence the immune system in marine mussel *M. galloprovincialis*.

1. Introduction

Organophosphate flame retardants (OPFRs) are increasingly produced and used as alternatives of brominated flame-retardants (BFRs), such as the polybrominated diphenyl ethers (PBDEs) (Wang et al., 2015). Nowadays, the OPFRs can be frequently detected in seawaters and sediments from the Bohai Sea (Zhong et al., 2018; Zhong et al., 2017), which has posed a risk on marine organisms and subsequent human health via food chains. Among the OPFRs, the tris (1-chloro-2-propyl) phosphate (TCPP) is one of the most frequently detected halogenated OPFRs in both waters and sediments due to its wide usage as flame retardants in polyurethane foam and epoxy resin, with a concentration up to 31.4 ng L^{-1} in the seawater from the Bohai Sea in China (Zhong et al., 2018; Zhong et al., 2017; Andresen et al., 2004; Bacaloni et al., 2008; Martinez-Carballo et al., 2007; Regnery and Puttmann, 2010). Although the adverse effects to human health and ecosystem of OPFRs have been reported in a few researches (Reemtsma et al., 2008; Ren et al., 2008; Pillai et al., 2014), there is a lack of studies on the biological effects of OPFRs in marine organisms. Compared with brominated flame retardants, such as PBDEs and tetrabromobisphenol A (TBBPA), however, OPFRs have received little attention with regard to the adverse biological effects and ecological risk. Therefore, it is necessary to characterize the biological effects of OPFRs in marine organisms.

The marine bivalve, mussel *Mytilus galloprovincialis*, distributes widely in the Bohai Sea and is consumed as popular seafood by local residents. Therefore mussel *M. galloprovincialis* has been an important species in marine aquaculture industry in China. In addition, as a filter-feeder, mussel *M. galloprovincialis* plays an important role in coastal ecosystem. Due to its high capacity to accumulate environmental contaminants, this bivalve is also a preferable bioindicator used in marine biology and ecotoxicology, as well as in 'Mussel Watch Program' (Goldberg et al., 1983; Jernelov, 1996). However, there was a lack of investigations on the toxicological effects of OPFRs in marine mussels.

As an emerging class of marine environmental contaminants in the Bohai Sea, the OPFRs have been of great concern to researchers. In this work, the mussel *M. galloprovincialis* was used as experimental animal to study the chronic biological effects of one of the most frequently

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detected OPFRs, TCCP. The immune system of *M. galloprovincialis* is innate and consists of an open and vascular system including hemocytes and humoral components (Tanguy et al., 2018). The hemocytes provide the first line of defense against immune stressors by phagocytosis and encapsulation. Then, the humoral components including lysosomal enzymes, aminopeptidases, lectins and antimicrobial molecules may be produced to destroy immune stressors (Tanguy et al., 2018). In this study, therefore, the reactive oxygen species, apoptosis, antioxidant system and immunity-related gene expressions in hemocytes were tested to elucidate the biological effects of TCCP on the immunity in mussel *M. galloprovincialis*.

2. Materials and methods

2.1. Animal culture and experiment design

Adult mussels *Mytilus galloprovincialis* (shell length: 4–6 cm) were purchased from a local culturing farm (Yantai, China). After transported to laboratory, the mussels were acclimatized in filtered and aerated seawater in glass containers for 7 days and then were randomly divided into four groups (seawater control, solvent control, low and high doses of TCPP treatments). For each group, there were two replicate containers each containing 20 individual mussels. The mussels cultured in the filtered seawater and filtered seawater containing 0.00025% DMSO (v/v) were used as seawater control and solvent control groups, respectively. TCPP (CAS No. 13,674-84-5) was purchased from J&K Chemical Co., Ltd. The TCPP-treated mussels were exposed to two concentrations (10 and 100 nmol L^{-1}) of TCPP, respectively. The concentrations of TCPP stock solutions were 1 and 10 mg L^{-1} in DMSO to ensure the same DMSO concentrations in the TCPP-exposed groups to that of solvent control group. During the periods of acclimation and exposure, the mortalities for all groups were recorded and found to be less than 5% without significant differences among the groups. All the animals were kept at 18–20 °C under a photoperiod of 12 h light and 12 h dark, and fed with 1.5 g *Chlorella vulgaris* per day. After a chronic exposure for 42 days, the mussels were taken out of the exposure tanks and opened by using scalpels to cut off the adductor muscle carefully. Then, the hemolymph was collected from the adductor by using sterile injection syringes and mixed immediately with equivoluminal anticoagulant (20.80 g of glucose, 8.00 g of sodium citrate, 3.36 g of ethylene diamine tetraacetic acid (EDTA), and 22.50 g of sodium chloride in 1 L of pure water). Then the hemolymph samples were filtrated by screen spun silks of 300 meshes to exclude impurities and used in subsequent experiments. For all the experiments, 4 biological replicates were used in each group and each biological replicate consisted of blood from four individual mussels.

2.2. Hemocytes RNA extraction, cDNA synthesis and gene expression analysis

Approximately, 10 mL of mussel blood was centrifuged (5000 rpm for 5 min) to enrich hemocytes and TRIzol Reagent (Invitrogen) was used in total RNA extraction. The extracted RNA was purified with RNeasy mini kit (Qiagen) and resuspended in purified water (RNase free). Total RNA quality and concentrations were evaluated by the A260/280 and A260/230 spectrophotometric ratios using the NanoDrop 1000 spectrophotometer (Thermo Scientific). By employing M-MLV reverse transcriptase (Promega) and Oligo dT primers, 500 ng of total RNA was used to synthesize cDNA. All the processes were followed the manufacturers' instructions.

For gene expression analysis, 12 immunity related genes were selected and the corresponding primers were referred to the publications (Wang et al., 2013, 2012; Martins et al., 2014; Gerdol et al., 2012; You, 2013) and listed in Table 1. The 7300 Real Time PCR System (Applied Biosystems) was employed to conduct qPCR analysis. Briefly, 6 μL of 50 times diluted cDNA, 10 μL of SYBR green PCR master mix (Applied Biosystems), 0.8 μL (10 mM) of forward and reverse primer and 3.2 μL of nuclease-free water (Qiagen) were added to 20 μL of reaction volume. The qPCR program was designed as 95 °C for 7 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s and 60 °C for 10 min. The *M. galloprovincialis* 18S rRNA gene was chosen as reference gene and the comparative CT method ($2^{-\Delta\Delta Ct}$ method) was used to analyze the expression level of the genes (Livak and Schmittgen, 2001).

2.3. Analysis of reactive oxygen species (ROS) and apoptosis

Approximately, 600 μL of mussel blood was centrifuged (5000 rpm for 5 min) to enrich hemocytes. Following the manufacturer's instructions, the ROS Assay Kit (the method of 2′, 7′-dichlorofluorescin (DCF) labeling, Beyotime) and the One Step TUNEL Apoptosis Assay Kit (the method of fluorescein isothiocyanate (FITC) labeling, Beyotime) were used in pretreatment of the ROS and apoptosis analysis, respectively. Then the treated hemocytes (10,000 individuals for one sample) were screened and sorted by the flow cytometer (FACSAria) at excitation wavelength of 488 nm and emission wavelength of 525 nm.

2.4. Analysis of superoxide dismutase (SOD) activity and malondialdehyde (MDA) content

About 600 μL mussel blood was centrifuged (5000 rpm for 5 min) to enrich hemocytes. Following the manufacturer's instructions, SOD Assay Kit (Nanjing Jiancheng Bioengineering Institute) and MDA Assay Kit (Nanjing Jiancheng Bioengineering Institute) were used in pretreatment of the SOD and MDA analysis, respectively. Then the treated samples were analyzed by using Infinite M200 microplate spectrophotometer (Tecan Infinite).

2.5. Statistical analysis

The statistical analysis of all data was performed by the one-way analysis of variance (one-way ANOVA) using the statistical software, SPSS 13.0. All the data analyzed followed normal distributions and variances. The p values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Effects of TCPP on ROS production, apoptosis and antioxidant system

The mussels *M. galloprovincialis* were exposed to two concentrations of TCCP (10 and 100 nmol L^{-1}) for 42 days. The reactive oxygen species (ROS) and apoptosis in mussel hemocytes were measured by flow cytometry. Basically, both ROS and apoptosis levels were not significantly different between seawater control and solvent control groups. Therefore, only the mussel samples from solvent control group were used for comparisons in subsequent analysis.

As shown in Fig. 1, ROS productions were highly significantly ($p < 0.01$) elevated in both TCPP-treated mussel samples. In addition, the ROS level in the high dose (100 nmol L^{-1}) of TCPP treatment was significantly higher ($p < 0.01$) than that in the low dose (10 nmol L^{-1}) of TCPP treatment. Obviously, both TCPP exposures enhanced ROS production in mussel hemocytes. As it is known, excessive ROS production may cause cytochrome-c release from mitochondria and induce oxidative and immune stresses, which may lead to cell apoptosis (Zorov et al., 2006). From Fig. 2, the significantly ($p < 0.01$) increased apoptosis ratio was observed in the high concentration (100 nmol L^{-1}) of TCPP-exposed group. However, the apoptosis ratio in the low concentration (10 nmol L^{-1}) of TCPP-exposed group was not significantly altered. After TCPP exposures for 42 day, interestingly, hemocytes superoxide dismutase (SOD) activity and malondialdehyde (MDA) levels represented similar alterations to that of apoptosis ratio in mussel samples (Fig. 3). Both SOD and MDA play important roles against to the