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A study of oxidative stress induced by two polybrominated diphenyl ethers in the rotifer *Brachionus plicatilis*

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ABSTRACT

Polybrominated diphenyl ethers (PBDEs) are widely dispersed persistent organic pollutants in the marine ecosystem. However, their toxic mechanisms in marine organisms, especially invertebrates, remain poorly understood. Two common congeners of PBDEs, tetrabrominated diphenyl ether-47 (BDE-47) and decabrominated diphenyl ether-209 (BDE-209), were investigated. Their toxic mechanisms, with a focus on oxidative stress, were examined in rotifer *Brachionus plicatilis*. Overproduction of reactive oxygen species (ROS) was induced by two PBDEs. The expression of superoxide dismutase (SOD) mRNA was increased, suggesting SOD play a main role in ROS-scavenging. The intercellular concentrations of calcium ($[Ca^{2+}]_{in}$) and the expression of calmodulin (CaM) mRNA were increased. This indicates the calcium ion (Ca^{2+}) signaling channel is involved in PBDEs stress. Further analysis showed that the reproductive system might be the target site for toxicity of PBDEs. Moreover, high value of detection indexes in BDE-47 experimental groups suggested BDE-47 might cause higher oxidative damage than BDE-209 in rotifers.

1. Introduction

Brominated flame retardants, polybrominated diphenyl ethers (PBDEs), have been widely used for decades in various commercial products (de Wit, 2002). Two common congeners of PBDEs, tetrabrominated diphenyl ether-47 (BDE-47) and decabrominated diphenyl ether-209 (BDE-209), are ubiquitous environmental contaminants found in the marine ecosystem (Ross et al., 2009; Zeng et al., 2013; Lavandier et al., 2015). Exposure to some PBDE congeners is associated with several health effects, including reproductive and developmental effects, neurobehavioral toxicity, endocrine disruption, immunotoxicity and possibly cancer (Dingemans et al., 2011; Hu et al., 2011; Lee et al., 2012; Sha et al., 2015a). A primary focus of current PBDE research is how toxicity pathways and which mechanisms are applicable in marine biota. Currently, the most plausible mechanism of PBDE toxicity is thought to be *via* oxidative stress (Yan et al., 2011; Wang et al., 2012; Lv et al., 2015). A report by Chen et al. (2010) suggested that PBDE-209 could affect secondary messengers, cause oxidative stress in rat. The recently study also showed that BDE-47 induced oxidative stress and ensuing apoptotic cell death in *in vitro* (Costa et al., 2015). The effective processes involved in oxidative stress are reactive oxygen species (ROS) generation and detoxification (antioxidant defense; Kim et al., 2011; Lushchak, 2011; Zhao et al., 2011). To resist and remove ROS, organisms typically evolve antioxidant systems (Kanerva et al., 2012; Vardi et al., 2013). Antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) work as the first line of ROS-scavenging (Schvezov et al., 2013). SOD and CAT are the major antioxidant enzymes responsible for the elimination of ROS to protect cells against hazardous oxidative stress. The primary ROS produced by mitochondria is superoxide (O_2^-), which is converted to the less reactive hydrogen peroxide (H_2O_2) either by the enzyme SOD or by spontaneous dismutation. CAT then detoxifies H_2O_2 to H_2O and O_2^-. Otherwise, H_2O_2 will be further transformed into a hydroxyl free radical (OH•) in the presence of metal ions (Poletta et al., 2015).

In addition, oxidative stress can disrupt normal physiological pathways and even induce cell death by affecting the calcium ion (Ca^{2+}) signaling pathway (Chen et al., 2012). The biological functions of Ca^{2+} are extremely versatile, controlling multiple processes such as cellular metabolism, signal transduction, and gene expression (Zhang et al., 2011). The Ca^{2+} control mechanism in the cell is both sensitive to oxidative stress and able to modulate it (Gordeeva et al., 2003; Kelly et al., 2010).

To our knowledge, the toxic mechanisms of PBDEs in aquatic invertebrates have rarely been studied, particularly in zooplankton such as rotifers. Rotifers are expected to play an important ecological role because of their high growth and turnover rates (Dahms et al., 2011). Previous rotifer research has demonstrated that PBDEs may cause a dose-dependent rise in ROS levels and intercellular concentrations of calcium ($[Ca^{2+}]_{in}$; Wang et al., 2015; Zhang et al., 2013). Therefore, the objective of this study was to investigate the toxic mechanisms of PBDEs, with a

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focus on oxidative stress, using the rotifer *Brachionus plicatilis* as an experimental model.

2. Material and methods

2.1. Chemicals

BDE-47 and BDE-209 (gas chromatography mass spectrometry, > 99.99% purity, white solid powder) were purchased from AccuStandard Inc. (New Haven, CT, USA), and were stored in dimethyl sulfoxide (DMSO, GC grade, >99.0%, liquid; Sigma-Aldrich, St. Louis, MO, USA) as stock solutions with a concentration of 2000 mg L^{-1} at room temperature in darkness.

2.2. Rotifer cultures

Female rotifers (*B. plicatilis*) were provided by the Fisheries College, Ocean University of China (Qingdao, Shandong, China). Rotifers were fed daily with *Chlorella* sp. at a density of 100 cells mL^{-1} and kept on a 12-h light/dark cycle (illumination intensity 1000–1200 Lx) at a temperature of 20 ± 1 °C.

2.3. Experimental design

During the toxicity experiments, *B. plicatilis* were exposed to a blank control; solvent control; 0.05 mg L^{-1}, 0.1 mg L^{-1}, and 0.2 mg L^{-1} BDE-47; and 0.05 mg L^{-1}, 0.1 mg L^{-1}, and 0.2 mg L^{-1} BDE-209 without feeding. All of the exposures occurred in beakers at a density of 100 ind mL^{-1}. Each replicate consisted of tens of thousands of rotifers, with three replicates per treatment. The concentrations of BDE-47 and BDE-209 were designed based on the 24 h–EC50 value of BDE-47 (7.923 mg L^{-1}; Zhang et al., 2016). The actual concentrations of BDE-47 and BDE-209 were measured in a previous study using gas chromatography mass spectrometry (Zhang et al., 2016). The samples were analyzed on a TRACE 1310 gas chromatography system coupled with an ISQ MS (Thermo Scientific Inc., Waltham, MA, USA) in the same procedure as reported by Sha et al. (2015b). The group without PBDEs and DMSO additions was used as the blank control, and the group with 0.01% DMSO (v/v) added was regarded as the solvent control. The maximum volume ratio of DMSO used in our study (0.01%, v/v) was well below the no observed effect concentration of 7.038% (v/v; Zhang et al., 2016). Finally, rotifers were filtered using a 400-mesh sieve and washed with a 0.22-μm membrane filter and autoclaved seawater after 24-h exposure.

2.3.1. Analysis of ROS and Ca^{2+} levels

Total ROS production was labeled with DCFH-DA (Beyotime, Jiangsu, China) as described by Yan et al. (2011), with some modifications as follows: The samples were treated with 100 nmol L^{-1} DCFH-DA and incubated for 40 min at 37 °C in darkness. The change in $[Ca^{2+}]_{in}$ was determined using Fluo-3/AM (Beyotime) following Garcia-Prieto et al. (2013), with the following modifications: The samples were incubated with 2 μmol L^{-1} Fluo-3/AM for 40 min at 37 °C in darkness. *B. plicatilis* were normalized with protein content using a Coomassie protein assay (Beyotime). The fluorescence was detected using a Zeiss Imager A1 fluorescence microscope (Zeiss Co., Oberkochen, Germany). The mean intensity value in a region of interest was calculated using Image-Pro Plus 6.0 system (Media Cybernetics, Silver Spring, MD, USA).

2.3.2. Analysis of gene expressions using real-time quantitative PCR

The gene-specific primers of *SOD*-F (5′-AGCCTTGAAGTTTAATGGTGG-3′)/*SOD*-R (5′-GTGGCAACTAACAAATCTACC-3′), *CAT*-F (5′-TCCTGGACTCTTTACATTCAA-3′)/*CAT*-R (5′-TCAAATGTAAGGTGCGAAGG-3′), and *CaM*-F (5′-GGAGTGATAACATCGTGTGA-3′)/*CaM*-R (5′-CTTTTCTCCTCAGTCATCAG-3′) were used for real-time quantitative PCR for the SOD, CAT and CaM genes, respectively. Translation elongation factor-1 alpha (*TEF-1α*) was used as an internal standard (Oo et al., 2010), which was amplified with the primers *TEF-1α*-F (5′-GACGCCATTGTTCCACCATCA-3′)/*TEF-1α*-R (5′-GGCTGGAGCAAAAGTGACAAC-3′). The GenBank accession numbers of SOD, CAT, CaM, and TEF-1α are AB111351.1, FJ555373.1, AB491768.1 and AB513493.1, respectively.

Total RNA extraction was performed using an ultra pure RNA extraction kit (CWbio Co. Ltd., Beijing, China). cDNA was prepared from 20 μL of total RNA using a Verso cDNA Kit (CWbio Co. Ltd.) with a mix of random primers and anchored oligo-dT primer. Diluted cDNA (1.0 μL cDNA from each RT reaction) were run in triplicates on 96-well reaction plates with the Light Cycler 480II real-time PCR System (Roche Applied Sciences, Basel, Switzerland). Real-time quantitative PCR analysis was performed using an Ultra SYBR Mixture (CWbio Co. Ltd.). PCR was carried out with 10 min activation and a denaturizing step at 95 °C, followed by 40 cycles of a 15-s denaturing step at 95 °C, and a 60-s annealing step at 60 °C. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative abundance of the target genes transcripts using the reference gene. The values of the target genes in the control were set as the normalized values by the reference gene (Table 1).

2.4. Statistical analyses

Data were expressed as means ± standard error (S.E.). Normality was assessed using Shapiro-Wilk *W* test. As the analytical data showed a normal distribution. Data were analyzed by using univariate analyses of variance (ANOVA) to test the effect of PBDE congeners and concentrations. Then, Tukey HSD post-hoc tests were used to describe significant differences. A value of $P < 0.05$, $P < 0.01$ or $P < 0.001$ was considered significant. SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) was used for analyses.

3. Results

3.1. ROS levels

As shown in Fig. 1, the fluorescence imaging exhibited lower intensities in the control and higher intensities in the experimental groups, especially BDE-47 groups. Furthermore, fluorescence intensity in the ovary was higher than in other organs such as the mastax and stomach.

BDE-47 and BDE-209 increased the formation of ROS in the rotifer ovary, which was positively correlated with the exposure concentration (Fig. 2). Significant increases occurred in the 0.1 mg L^{-1} and 0.2 mg L^{-1} BDE-47 treatments ($P < 0.001$) compared with the control. Additionally, there was a significant elevation in intracellular ROS levels when *B. plicatilis* was exposed to the 0.2 mg L^{-1} BDE-209 treatment ($P < 0.01$) compared with the control. No significant

Table 1
PCR primer sequences and qRT-PCR efficiencies.

Accession no.	Gene	Primer sequences (5′ to 3′)	Amplicon size	PCR efficiency
AB111351.1	*sod* F	AGCCTTGAAGTTTAATGGTGG	156 bp	1.97
	sod R	GTGGCAACTAACAAATCTACC		
FJ555373.1	*cat* F	TCCTGGACTCTTTACATTCAA	128 bp	1.99
	cat R	TCAAATGTAAGGTGCGAAGG		
AB491768.1	*cam* F	GGAGTGATAACATCGTGTGA	128 bp	1.97
	cam R	CTTTTCTCCTCAGTCATCAG		
AB513493.1	*tef-1a* F	GACGCCATTGTTCCACCATCA	153 bp	1.97
	tef-1a R	GGCTGGAGCAAAAGTGACAAC		

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