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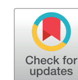
Chronic toxicity of hexabromocyclododecane(HBCD) induced by oxidative stress and cell apoptosis on nematode *Caenorhabditis elegans*



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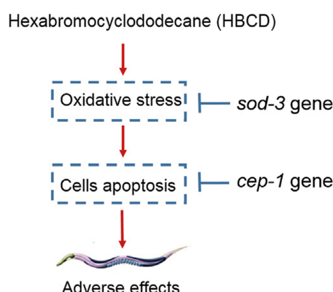
HIGHLIGHTS

- Low concentrations of HBCD caused adverse effects in *C. elegans.*
- The oxidative stress and cell apoptosis contributed to HBCD toxicity formation.
- The *sod-3* and *cep-1* gene protected *C. elegans* against HBCD-induced toxicity.

GRAPHICAL ABSTRACT



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ABSTRACT

In order to gain insights into the chronic effects and mechanisms of hexabromocyclododecane (HBCD), the animal model *Caenorhabditis elegans* (*C. elegans*) was chosen for toxicity study. Multiple endpoints, including the physiological (growth and locomotion behaviors), biochemical (reactive oxygen species (ROS) production, lipofuscin accumulation, and cell apoptosis), and molecular (stress-related gene expressions) levels, were tested by chronic exposure for 10 d to low concentrations of HBCD (0.2 nM-200 nM). The results revealed that chronic exposure to HBCD at concentrations more than 20 nM would significantly influence the growth, locomotion behaviors, ROS formation, lipofuscin accumulation, and cell apoptosis of nematodes. Treatment with antioxidants of ascorbate and N-acetyl-L-cysteine (NAC) suppressed the toxicity induced by HBCD. The integrated gene expression profiles showed that the chronic exposure to 200 nM of HBCD significantly increased the expression levels of stress-related genes (e.g., *hsp-16.2*, *hsp-16.48*, *sod-1*, *sod-3*, and *cep-1* genes). Among these genes, the *sod-1*, *sod-3*, and *cep-1* gene expressions were significantly correlated with HBCD-induced physiological effects by the Pearson correlation test. The mutations of *sod-3* and *cep-1* induced more severe toxicity compared to wild-type nematodes. Therefore, HBCD exposure induced oxidative stress by ROS accumulation and cell apoptosis,

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which resulted in HBCD-induced toxicity on nematodes, and *sod-3* and *cep-1* played important roles in protecting nematodes against HBCD-induced toxicity.

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1. Introduction

Hexabromocyclododecanes (HBCD), additive brominated flame retardants (BFRs), is widely used in polystyrene foams, packaging material, and electric products (Covaci et al., 2006). In the processes of production, consumption, and disposal of these products, emission of HBCD takes place abruptly to the environment through various industrial activities (Gao et al., 2011). Therefore, it has been reported that HBCD has been widely identified in environmental matrices, including water, sediments, soil, and even human milk (Gikas, 2007; Marvin et al., 2011; Law et al., 2014). Moreover, HBCD has multiple toxic effects, including developmental toxicity (Du et al., 2012), neurotoxicity (Al-Mousa and Michelangeli, 2014), and reproductive toxicity (Marteinson et al., 2012). Currently, several toxicological studies of HBCD have been performed on aquatic organisms and terrestrial species (Hong et al., 2014; Olivier et al., 2016). However, the chronic effects and toxic mechanisms of HBCD exposure on nematodes have rarely been reported. To obtain further insights into the different chronic effects and mechanisms of HBCD exposure on nematodes, it is necessary to choose *Caenorhabditis elegans* (*C. elegans*) for toxicity study.

C. elegans, a free-living nematode, is found abundantly in ecosystems. *C. elegans* has been successfully used in the environmental and toxicological studies of toxicants due to its translucent body, short life cycle, high fecundity, and sensitivity to toxicants (Leung et al., 2008). Endpoints, including development, locomotion behavior, reproduction, reactive oxygen species (ROS), lipofuscin, cell apoptosis, and stress response were used to assess the toxicity of environmental toxicants (Zhang et al., 2011; Zhou et al., 2016a, 2016c). In addition, toxicological study of toxicants employing invertebrates with chronic exposures have been recommended (Gikas et al., 2009). Some studies performed *in C. elegans* are focused on toxicology of toxicants to chronic exposure (Shen et al., 2009; Li et al., 2012b; Wu et al., 2012a). Therefore, *C. elegans* was an excellent model organism for the study of chronic toxicity along with the mechanisms of HBCD exposure.

Previous reports suggested that concentrations of HBCD range from 0.01 to 100 μg/L in various kinds of water samples (Morris et al., 2004; Remberger et al., 2004; Harrad et al., 2009; Ichihara et al., 2014; Oh et al., 2014). In addition, previous research has proven that exposure to HBCD at concentrations of 1–100 μg/L caused adverse effects on aquatic organisms (Wu et al., 2013; Hong et al., 2014; Shi et al., 2017). In this study, nematodes were exposed to HBCD at the environmentally relevant concentrations of 0, 0.2, 2, 20 nM and 200 nM (0, 0.128, 1.28, 12.83 and 128.3 μg/L) for 10 d. The expected adverse physiological effects of HBCD were assessed by growth and locomotion behavior of nematodes. The possible biochemical effects of HBCD were evaluated by lipofuscin, ROS, and cell apoptosis. The effects of antioxidant treatment were also investigated on nematodes. The expression levels of genes related to stress response were examined by quantitative real-time polymerase chain reaction (PCR, Supplementary data Table S1). Moreover, some mutations were also performed to determine the importance of certain genes. The main aim of this study was to investigate the mechanisms of oxidative stress and cell apoptosis in *C. elegans* exposed to HBCD. The results are helpful for understanding the toxic effects of HBCD along with the evaluation of the various potential risks of HBCD.

2. Materials and methods

2.1. Strain preparation and exposure conditions

Nematodes (wild-type N2, GA186 [sod-3*(tm760)*], GA187 [sod-1*(tm776)*], VC172 [cep-1 *(gk138)*]), obtained from the *Caenorhabditis* Genetics Center (CGC), were cultured on nematode growth plates (NGM, 3 g/L NaCl, 2.5 g/L peptone, 17 g/L agar, 25 mM potassium phosphate, 1 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, and 1 mL cholesterol) plates seeded with *Escherichia (E.) coli* strain OP50 as a food source (Brenner, 1974). Synchronized populations of L4 larval stages were acquired with bleaching mixture (0.45 M NaOH, 2% HOCl) (Donkin, 1993), and washed three times with K medium (32 mM KCl, 51 mM NaCl) (Williams, 1990).

HBCD (1,2,5,6,9,10-hexabromocyclododecane) was dissolved in dimethyl sulfoxide (DMSO) to prepare the desired HBCD stock solutions, and then was diluted with K medium at HBCD concentrations of 0, 0.2, 2, 20, and 200 nM. Chronic exposures to HBCD at environmentally relevant concentrations were performed with L4 larvae for 10 d in plate wells containing 10 mL of treatment solutions. All the exposures were performed in an incubator at 20 °C in the presence of food. To prevent the production of offspring, 5-fluoro-2′-deoxyuridine (5-FUDR) was added to the test plates at a final concentration of 25 mM (Shen et al., 2009). The exposed nematodes were transferred to a new plate well with fresh treated solutions every day. Replications were carried out four times in each exposure group.

2.2. Evaluation of physiological indicators

The nematodes' growth was assessed by measuring the body length of nematodes killed by heat under a microscope (Nikon Eclipse 80i) equipped with a graduated eyepiece. In addition, locomotion behaviors were assessed by the body bend and head thrash. To assay body bends, the nematodes were transferred into a second plate without food, and body bends were counted for 20 s. A body bend referred to a change in the direction of nematodes corresponding to the posterior bulb of the pharynx along the y axis, assuming that the nematode was traveling along the x axis as explained in the literature (Zhou et al., 2016b). To assay the head thrash, the nematodes were picked onto 60 μL of K medium on top of agar without food. After *C. elegans* recovered for 1 min on nematode NGM, head thrashes were counted for 1 min. A thrash was defined as a change in the direction of bending at the middle body. 40 worms were assayed for each treatment, and 4 independent experiments were performed.

2.3. Evaluation of biochemical indicators

The exposed nematodes were treated with formaldehyde (4%) for 10 min. Then, fluorescent images were collected by a fluorescence microscope (Nikon Eclipse 80i) with a suitable filterset (UV-2A) (Zhou et al., 2016c). The lipofuscin accumulation was expressed as relative fluorescent units (RFU) by Image J software. 40 worms were assayed for each treatment, and 4 independent experiments

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