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Effects of Dechlorane Plus exposure on axonal growth, musculature and motor behavior in embryo-larval zebrafish☆

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ABSTRACT

Developmental neurobehavioral toxicity of Dechlorane Plus (DP) was investigated using the embryo-larval stages of zebrafish (*Danio rerio*). Normal fertilized embryos were waterborne exposed to DP at 15, 30, 60 μg/L beginning from 6 h post-fertilization (hpf). Larval teratology, motor activity, motoneuron axonal growth and muscle morphology were assessed at different developmental stages. Results showed that DP exposure significantly altered embryonic spontaneous movement, reduced touch-induced movement and free-swimming speed and decreased swimming speed of larvae in response to dark stimulation. These changes occurred at DP doses that resulted no significant teratogenesis in zebrafish. Interestingly, in accord with these behavioral anomalies, DP exposure significantly inhibited axonal growth of primary motoneuron and induced apoptotic cell death and lesions in the muscle fibers of zebrafish. Furthermore, DP exposure at 30 μg/L and 60 μg/L significantly increased reactive oxygen species (ROS) and malondialdehyde (MDA) formation, as well as the mRNA transcript levels of apoptosis-related genes *bax* and *caspase-3*. Together, our data indicate that DP induced neurobehavioral deficits may result from combined effects of altered neuronal connectivity and muscle injuries.

1. Introduction

Dechlorane Plus (DP), a chlorinated flame retardant, has been widely used in electronic equipment and furniture for nearly 50 years, with an estimated global annual production volume of 5000 tons (Wang et al., 2016). This chemical was not initially detected in environmental samples until it was identified in the Great Lakes of North America in 2006 (Hoh et al., 2006). Currently, DP receives increasing attention due to its similar characteristics as those of persistent organic pollutants (POPs), such as slow degradation, the potential for long-range transport and significant bioaccumulation (Feo et al., 2012; Möller et al., 2010). Technical DP is composed of two major isomers, i.e., *syn*- and *anti*-DP, and the two isomers have been widely detected in the air (Chen et al., 2011), soil (Zhang et al., 2012), dust (Li et al., 2015), sediment (Sverko et al., 2009) in hot spot areas. Although DP concentrations in surface water are usually very low (<100 ng/L) (Salamova and Hites, 2011; Wu et al., 2010), high levels of DP have been reported in tissues of aquatic species. For example, the DP concentrations ranged from 19 to 9630 ng/g lipid weight (lw) in aquatic species including water snake and mud carp from an electronic waste recycling workshop of South China (Wu et al., 2010). In addition, DP has also been detected in blood of occupational workers at up to 2958 ng/g lw (median concentration of 857 ng/g lw) from a DP manufacturing plant in China (Zhang et al., 2013).

Despite DP's prevalence in various environmental matrices, few studies explore its developmental neurobehavioral toxicity. Early study in adult zebrafish showed that DP disrupted thyroid hormones balance (Kang et al., 2016). Study in Chinese sturgeon revealed that DP affected intracellular Ca^{2+} signaling pathway (Liang et al., 2014). It has been reported that thyroid hormones and

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Ca^{2+} signaling pathway play important roles in maintaining normal function and development of nervous system (Gomez and Spitzer, 1999; Kodavanti and Curras-Collazo, 2010). Therefore, DP exposure may pose an adverse effect on nervous system development of aquatic species. Moreover, DP is used as a replacement of polybrominated diphenyl ethers (PBDEs) and has a similar chemical structure and high hydrophobicity as BDE-209 (Xian et al., 2011). A previous study with decabromodiphenyl ether (BDE-209) (He et al., 2011) showed that this flame retardant caused neurobehavioral deficits in developing zebrafish. Thus we speculate that DP may have a similar effect as BDE209 in developing zebrafish. Together, these results indicate that DP might be a potential developmental neurotoxicant.

Zebrafish (*Danio rerio*) is proving to be a reliable, sensitive and economic model for assessments of the developmental neurobehavioral toxicity of various chemicals (Bailey et al., 2013). It is easy to handle, has a small size and visible embryological phases, and importantly, it also exhibits similarities at the physiological and molecular levels with humans (Krishnaraj et al., 2016). Moreover, the development of motor activity in larval zebrafish and its locomotion network has been well known (Drapeau et al., 2002).

In the present study, zebrafish was used as an *in vivo* model to assess the developmental neurobehavioral toxicity of DP. The concentrations of DP that accumulated in larvae were measured and motor behavior, axonal growth of primary motoneuron and musculature morphology were examined at different developmental stages. Additionally, levels of reactive oxygen species (ROS) and lipid peroxidation product malonaldehyde (MDA), as well as mRNA transcript expression levels of axonal growth-related and apoptosis-related genes were further analyzed to elucidate the potential mechanisms of DP-induced developmental neurobehavioral toxicity.

2. Materials and methods

2.1. Fish husbandry

Healthy six-month-old adult zebrafish (wild-type; AB strain) were maintained at ambient temperature (28 ± 1 °C) with a 14-h/10-h light cycle in recirculating filtered water according to a standard protocol for raising zebrafish. Conductivity and pH were kept in range of 450–1000 μS/cm and 7.0–7.5, respectively. Adult fish were fed with freshly hatched brine shrimps twice per day. Embryos were collected from spawning, adults in an aquarium overnight with an optimal sex ratio of 1:1. Fertilized normal embryos were staged using a stereomicroscope (SMZ1500, Nikon, Japan) in accordance with standard procedures (Kimmel et al., 1995).

2.2. Chemical exposure and teratology screening

Dechlorane Plus (DP, CAS # 13560-89-9, purity > 97%) was purchased from Anpon Electrochemical Co., Ltd. (Jiangsu Province, China) and stock solutions of 15, 30, 60 mg/L were prepared by dissolving DP in dimethyl sulfoxide (DMSO). Working solutions of 15, 30, 60 μg/L were immediately prepared by a 1000-fold dilution of corresponding stock solution with embryo medium. The final DMSO concentration was 0.1% (v/v in embryo medium) across all exposure groups. The control also received 0.1% DMSO (v/v). Early study has demonstrated that 0.1% DMSO exposure, just like blank control, couldn't cause any development defect to zebrafish embryos (Hallare et al., 2006). The DP concentrations, exposure periods and endpoints used for each experiment were listed in Table 1. The DP concentrations used in this study were selected on the basis of previously published studies (Kang et al., 2016; Noyes et al., 2015). Furthermore, the content of DP in zebrafish (Fig. 1A) showed a similar DP body burden as previously published study (Wu et al., 2010), which fall into the same range as environmental samples from a reservoir near the e-waste recycling plant in south China. Thus DP concentrations used in this study generated a similar body burden relevant to environmental samples. The treatment solution was renewed every other day. Unless otherwise specified, experimental exposure in the present study began at 6 hpf, while they terminated at various time points. For teratology screens, hatching, malformation and survival were also recorded from 6 to 120 hpf.

2.3. Quantification of DP in exposure water and larval zebrafish

For quantification of DP concentrations in exposure water, the procedures performed according to previously published study (Wu et al., 2010). Briefly, water samples were spiked with recovery stand PCB-209 and extracted with solid-phase extraction columns (LC-C18; Sigma-Aldrich). Collected extracts were dehydrated, concentrated and re-dissolved in isooctane containing of internal standard ($^{13}C_{12}$-PCB-208) for subsequent analyses. Recoveries of PCB-209 were 90–106%.

For larval samples, approximately 100 larval zebrafish (n = 3 replicates) at 120 hpf were collected and rinsed three times with embryos medium, then freeze-dried to detect the bioaccumulation of DP as previously published methods (Kang et al., 2010). For isomer-specific DP measurements, individual standards of *syn*-DP and *anti*-DP (purity ≥ 98%, Accustandards Inc.; New Haven, CT, USA) were obtained. PCB-209 was added in larval samples as a surrogate standard and $^{13}C_{12}$-PCB-208 was spiked as an injection internal standard before instrument analysis. Quantifications of DP isomers were performed by an Agilent 7890A gas chromatograph coupled to a 5975C mass selective detector (GC-MS) equipped with a HP-5 MS capillary column (15 m × 0.25 mm × 0.1 μm; J&W Scientific). The recoveries of PCB-209 ranged from 85% to 103% for the spiked samples. A procedural blank was processed for each batch of fish samples and none of target substance was detected in the blank.

2.4. Behavioral assays

To determine whether DP exposure affected motor behavior of zebrafish larvae, four types of motor behavior assays, including embryonic spontaneous movement, touch-induced movement, free-swimming activity and locomotion behavior in response to dark-to-light transitions were tested. Unless otherwise specified, all behavior tests in the present study were performed with ambient temperature at 28 °C. For spontaneous movement test, embryos were exposed to DP in 6-well plate beginning at 6 hpf. Spontaneous movement (alternating tail bending or coiling) was videotaped for 1 min via a CCD camera (Nikon, Japan) mounted on a dissection microscope hourly from 22 to 25 hpf. All spontaneous movement recordings started after 5 min adaptation on the recording station. The time from the first to the last well was less than 8 min. A total of 60 embryos in each treatment group from three replicate experiments were used for data analysis.

For touch-induced movement at 36, 48 and 72 hpf, embryos were dechorionated using protease E (25 mL at 0.1 mg/mL; Roche, Germany) at the end of DP exposure. Larvae were allowed to adapt for 10 min in a 24-well plate before touching starts. Fish response was evoked by gentle touching the dorsal tail region only once with an eyelash probe. Touch-induced movement was recorded using camcorder software at a rate of 25 frames per second (fps). The distance of swimming after the initial touch was analyzed using Image-Pro Plus 6 based on previously described method (Tallafuss and Eisen, 2008). A total of 72 embryos in each treatment group