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INCREASED APOPTOSIS AND ABNORMAL VISUAL BEHAVIOR BY HISTONE MODIFICATIONS WITH EXPOSURE TO PARA-XYLENE IN DEVELOPING *XENOPUS*

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Abstract—Xylene and its derivatives are raw materials widely used in industry and known to be toxic to animals. However, the mechanism underlying the neurotoxicity of para-xylene (PX) to the central nervous system (CNS) *in vivo* is less clear. Here, we exposed *Xenopus laevis* tadpoles to sub-lethal concentrations of PX during the critical period of brain development to determine the effects of PX on *Xenopus* development and visual behavior. We found that the abnormality rate was significantly increased with exposure to increasing concentrations of PX. In particular, the number of apoptotic cells in the optic tectum was dramatically increased with exposure to PX at 2 mM. Long-term PX exposure also resulted in significant deficits in visually guided avoidance behavior. Strikingly, co-incubation with PX and D-glucuronolactone (GA) decreased the number of apoptotic cells and rescued the avoidance behavior. Furthermore, we found that the acetylation of H4K12 (H4K12ac) and the dimethylation of H3K9 (H3K9me2) in the optic tectum were significantly increased in PX-treated animals, and these effects were suppressed by GA treatment. In particular, the increase in apoptotic cells in PX-treated brains was also inhibited by GA treatment. These effects indicate that epigenetic regulation plays a key role in PX-induced apoptosis and animal behavior. In an effort to characterize the neurotoxic effects of PX on brain development and behavior, these results suggest that the neurotoxicity of PX requires further evaluation regarding the safety of commercial and industrial uses.

Key words: *Xenopus*, para-xylene, apoptosis, histone modification, avoidance behavior, optic tectum.

INTRODUCTION

Para-xylene (PX) and its methylated derivatives m- and o-xylene are aromatic volatile organic compounds that are key chemical raw materials and daily necessities with increasing uses in a variety of commercial and industrial applications such as synthetic pesticides, plastics, fibers and petroleum chemistry. As a result of the expanding market, PX has been recognized as a harmful chemical that affects animal survival, growth and development in zebrafish and amphibians (Rajan and Malathi, 2014). Previous studies have shown that benzene, toluene or xylene exposure results in a considerable delay in embryonic development (Hudak and Ungvary, 1978; Brown-Woodman et al., 1991; Hass et al., 1995) and significant maternal toxicity (Saillenfait et al., 2003). Chronic exposure to xylene has been associated with neurophysiological dysfunction, anemia and leukopenia abnormalities (Langman, 1994). Experimental evidence for abnormal animal behavior (Ghosh et al., 1987; Bushnell, 1988) with subchronic exposure to m-xylene was thought to result from persistent changes in the function of the central nervous system (CNS) (Dudek et al., 1990; Gralewicz et al., 1995). However, the toxic effects of PX on brain development and animal behavior as well as the underlying cellular mechanisms remain unclear.

Several studies have shown that long-term xylene exposure increased the number of apoptotic cells in lung tissue (Sandikci et al., 2009), lymphocytes (Martinez-Velazquez et al., 2006) and renal proximal tubule cells (Al-Ghamdi et al., 2004). Volatile solvents are known to be lipophilic and to exert their effects via interaction with the lipids of cell membranes (Gustafson and Tagesson, 1985). Chronic inhalation exposure to p-xylene decreases axonal transport in rat retinal ganglion cells, and such defects can result in neuronal malfunction (Padilla and Lyerly, 1989; Padilla et al., 1992). Epigenetic modulation plays a key role in the regulation of transcriptional expression in neurotoxicity and neurological diseases (Nguyen et al., 2002; Cohen et al., 2004; MacDonald and Roskams, 2009; Peixoto and Abel, 2013). Deregulation of histone deacetylase activity results in neurotoxicity in cultured neurons (Kim et al., 2008) or neuronal death in rat cortical neurons (Bardai and D'Mello, 2011). Chromatin and gene regulation is attributable to the methylation and acetylation of lysine residues in histones by specific histone methyltransferases (HMTs)

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Abbreviations: AI, avoidance index; CNS, central nervous system; FETAX, frog embryo teratogenesis assay-*Xenopus*; GA, D-glucuronolactone; HDACs, histone deacetylases; HEPES, 4-(2-hydroxyethyl)-1-piperazinee thanesulfonic acid; PX, para-xylene.

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(Nguyen et al., 2002) and histone deacetylases (HDACs) (Nan et al., 1998). The dimethylation of H3K9 and the acetylation of H4K12 have been associated with gene silencing. However, there are no reports on the cellular effects of PX and the mechanisms underlying its histone modification in the developing brain.

A wide variety of organisms such as zebrafish, *Drosophila* and *Xenopus* have been used to assess the toxicity of various chemicals and samples (Cardellini and Ometto, 2001; Sharma et al., 2012; Spawn and Aizenman, 2012; Zhang et al., 2014). The embryos of *Xenopus laevis* (*X. laevis*) are widely used in laboratories for the evaluation of brain function and organ development (Buryskova et al., 2006; Zhang et al., 2014). The albino *X. laevis* is a transparent vertebrate model organism that is widely accepted for developmental and toxicological studies (Cardellini and Ometto, 2001; Buryskova et al., 2006). Albino *X. laevis* embryos are also easily manipulated for plasmid injection and *in vivo* time-lapse imaging. Furthermore, the frog embryo teratogenesis assay-*Xenopus* (FETAX) is a powerful bioassay for determining the lethal and teratogenic potential of chemical and drugs (Mantecca et al., 2007). To the best of our knowledge, impacts of PX exposure on cell survival and behavior have not been investigated in *X. laevis*, and such research has important implications in understanding the effects of deficits in neural circuit function and animal behavior caused by long-term PX exposure. In the current study, we aimed to determine the embryonic toxicity, cell survival and visual behavior responses following PX exposure during development in *X. laevis*. We further show the potential protective effect of GA against PX-induced toxicity *in vivo*.

EXPERIMENTAL PROCEDURES

Chemicals

Para-xylene (PX, 0.861 g/mL stock, Sangon Biotech) was stored in a brown bottle and diluted in Steinberg's solution (in mM: 10 HEPES, 58 NaCl, 0.67 KCl, 0.34 $Ca(NO_3)_2$, 0.83 $MgSO_4$, pH 7.4). D-(+)-Glucuronolactone (GA, Aladdin) was dissolved in Steinberg's solution as stock (10 mg/mL, pH 7.4).

Animals and FETAX test

Adult animals were purchased from Nasco (Fort Atkinson, WI, USA) and housed in the animal facility in standard *Xenopus* equipment (LY-ZC-9, China). All animal procedures were performed according to the requir ements of the 'Regulation for the Use of Experimental Animals in Zhejiang Province'. This study has been approved by the local ethics committee of the Hangzhou Normal University. Tadpoles were obtained by the mating of two adult albino *Xenopus* injected with human chorionic gonadotropin (HCG, Sigma–Aldrich, female 500 IU, male 200 IU) in the abdominal cavity and raised on a 12-h dark/light cycle in Steinberg's solution within a 20 °C incubator.

For FETAX test, the abnormally developed embryos were removed and healthy tadpoles were selected for further experiments. Tadpoles were placed in the Petri dishes at 15 cm diameter. Each well contained 160 mL control solution or PX at 1, 2, and 2.5 mM. The tadpoles were incubated in a constant temperature incubator at 20 °C. At the end of the test all tadpoles were evaluated for the survival rate and mortality. The survived tadpoles were anesthetized in 0.02% MS-222 (3-aminobenzoic acid ethyl ester methanesulfonate, Sigma–Aldrich) for further experiments. The morphology of the tadpoles was captured using a stereomicroscopes (Nikon SMZ1500) and images were processed with a NIS-Elements software. The number of malformed tadpoles as well as the percentage of single malformations was recorded. Each bioassay was repeated at least three times under the same experimental conditions. Embryos stages were identified according to significant developmental changes in the anatomy (Nieuwkoop and Faber, 1994).

Apoptosis assay

Cell death was visualized by a TUNEL (*in-situ* terminal deoxynucleotidyl transferase deoxyuridyl triphosphate nick-end labeling) technique according to the manufacturer's instructions (Beyotime, C1089, China). The tadpoles were fixed in 4% PFA overnight at 4 °C. Cryosections of full optic tectum were cut from the OCT-embedded brains and washed three times with PB (0.1 M) every 10 min. Slices were treated with 0.3% TritonX-100 in PBS for 30 min. After rinsing in PBS the sections were incubated with TUNEL reaction mixture (terminal deoxynucleotidyl transferase plus nucleotide mixture in reaction buffer) in dark for 60 min at 37 °C. After incubation, sections were rinsed with PBS for three times and counterstained with DAPI (1:10,000). The fluorescent images were taken using a confocal microscopy (LSM710, Zeiss, Germany).

Visual avoidance assay

All experiments were performed on tadpoles at developmental stages at 46–49. The visual avoidance behavior was measured with a custom-made equipment according to previous studies (Dong et al., 2009; Shen et al., 2011; McKeown et al., 2013). Single tadpoles were placed in a 9X8X3-cm chamber filled with ~1.5 cm Steinberg's rearing solution. Visual stimuli for moving spots were presented on the bottom of the chamber with a back-projection screen using a microprojector (3 M, MPro110). Tadpole was illuminated by infrared LEDs and the videos were recorded with a digital camera. Visual stimuli were generated and presented by MATLAB 2012a (The MathWorks, Psychophysics Toolbox toolbox). Randomly positioned moving spots of 0.4 cm diameter were presented for 60 s. Visual avoidance behavior was scored as a change in swim trajectory with the first ten encounters of the tadpoles and moving spots (plotted as avoidance index).

Western blot

Animals were anesthetized in 0.02% MS-222. The skin covered on the brain was peeled off to expose the