THE NEUROTOXICITY OF PHTHALATE ESTERS IN ZEBRAFISH EMBRYOS

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Introduction

Phthalate esters (PAEs), including di-n-butyl phthalate (DBP) and diethyl phthalate (DEP), are plasticizers that have been extensively used in a variety of consumer products such as sealants, paints, adhesives, cosmetics and food packaging [1]. To date, limited studies on PAEs in mammals and humans suggest that prenatal exposure may adversely impact congitive and neurobehavioral parameters in rats [2] and children [3]. However, the potential effects on nervous system in fish are virtually unknown. In the present study, we assessed the expression patterns of following genes exclusively in the nervous system in zebrafish embryos/larvae [4] after exposure to DBP, DEP and their mixture until 96 hpf: *growth associated protein 43* (*gap43*), *embryonic lethal abnormal vision –like 3* (*elavl3*), *α1-tubulin*, *glial filbrillary acidic protein* (*gfap*), *myelin basic protein* (*mbp*) and *neurogenin1* (*ngn1*).

Materials and methods

Normal zebrafish embryos were randomly distributed into a dish as a group and exposed to a dilute toxicant solution (500 mL) until 96 hpf in triplicate at each treatment concentration. The single chemical exposure was designed at 5, 50 and 500 μg/L for DBP, and 5, 50 and 500 μg/L for DEP. The DBP-DEP co-exposure was set at 5:5 μg/L and 500:500 μg/L for the DBP and DEP mixture. The solvent control group received 0.005% DMSO (v/v), while the aqueous control received dechlorinated tap water only. Fresh solutions were replaced 50% every 12 h.

Total RNA was extracted from 20 homogenized zebrafish larvae using the RNAprep pure Tissure kit (TIANGEN Biotech, Shanghai, China) according to the manufacturer's protocol. The quality and quantity of RNA were detemined by UV spectrophotometry and by 1% agarose gel electrophoresis. For each sample, cDNA was synthesized from 100 ng total RNA using iScript™ cDNA Synthesis kit (Bio-Rad, USA) following the manufacturer's introduction. Quantitative realtime PCR was performed using the SsoFast™ EvaGreen® Supermix kit (Bio-Rad, USA) and an iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA). A Ct-based relative quantification with efficiency correction normalizing to *ribosomal protein L13A* (*rpl13a*) was calculated by the $2^{-\Delta\Delta Ct}$ method. Sequences of primers are shown in Table 1.

Table 1. Primers sequences for quantitative reverse transcription polymerase chain reaction used in this study

Gene	Forward primer (from 5′ to 3′)	Reverse primer (from 5′ to 3′)	GenBank accession No.
ribosomal protein L13A	tctggaggactgtaagaggtatgc	agacgcacaatcttgagagcag	NM_212784
growth associated protein 43	tgctgcatcagaagaactaa	cctccggtttgattccatc	NM_131341
embryonic lethal abnormal vision -like 3	agacaagatcacaggccagagctt	tggtctgcagtttgagaccgttga	NM_131449
glial fibrillary acidic protein	ggatgcagccaatcgtaat	ttccaggtcacaggtcag	NM_131373
myelin basic protein	aatcagcaggttcttcggaggaga	aagaaatgcacgacagggttgacg	AY860977
α1-tubulin	aatcaccaatgcttgcttcgagcc	ttcacgtctttgggtaccacgtca	NM_194388
neurogenin1	tgcacaaccttaacgacgcattgg	tgcccagatgtagttgtgagcgaa	NM_131041

Experimental data were checked for normality and homogeneity of variance using Kolmogorov–Smirnov one-sample test and Levene's test. Intergroup differences were assessed using one-way analysis of variance (ANOVA) followed by Duncan's test, using SPSS Statistics 18 (SPSS Inc., Chicago, IL, USA). The level for statistical significance was set at $p<0.05$ or 0.01. All data are shown as mean ± standard error (S.E.M.).

Results and discussion

The expression profiles of selected neuron-related genes previously reported as potential markers for rapid developmental neurotoxicity screening were determined in zebrafish embryos/larvae after DBP and DEP exposure. Fig. 1 shows the relative expressions of significantly upregulated genes in zebrafish embryos exposed to series of concentrations of DBP, DEP and DBP-DEP mixture.

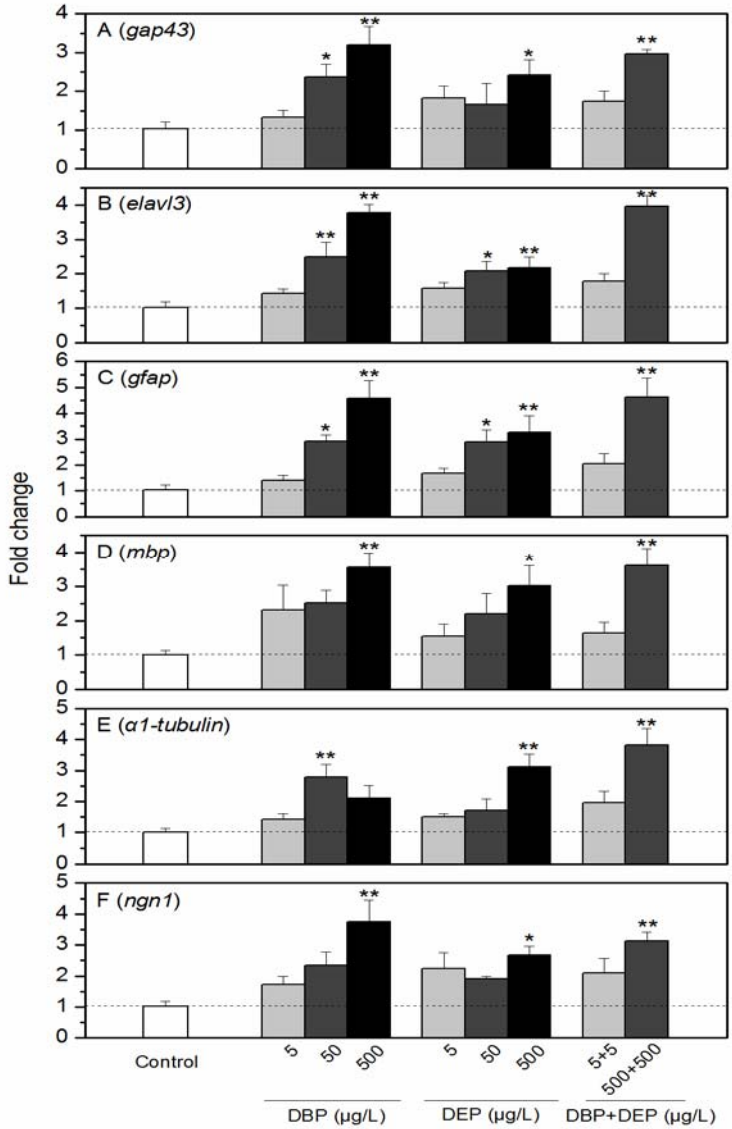

Fig. 1 Expression of neuron-related gene *growth associated protein 43* (*gap43*, A), *embryonic lethal abnormal vision -like 3* (*elavl3*, B), *glial fibrillary acidic protein* (*gfap*, C), *myelin basic protein* (*mbp*, D), *α1-tubulin* (E) and *neurogenin1* (*ngn1*, F) in zebrafish larvae after exposure to various concentrations of DBP, DEP and DBP-DEP. The results are means ± S.E.M. of triplicate samples. * indicated significant difference at $P<0.05$, and ** indicated significant difference at $P<0.01$.

The mRNA expression of *gap43* was significantly up-regulated 2.37-, 3.19-fold in 50, 500 μg/L DBP, 2.41-fold in 500 μg/L DEP and 2.97-fold DBP-DEP mixture-treated embryos relative to the control group (Fig. 1A). *Gap43* is a memberane-associated protein which is known as neuromodulin and highly expressed in neuronal growth cones during synaptogenesis in the central nervous system (CNS). The induction of *gap43* marks

metabolic changes that enhance the potential of a central neuron for neurite outgrowth and formation of new connctions.

The mRNA expression of *elavl3* was up-regulated by 2.50-, 3.77-fold in 50, 500 μg/L DBP, 2.09-, 2.17-fold in 50, 500 μg/L DEP and 3.96-fold DBP-DEP mixture treatments relative to the control group (Fig. 1B). *Elavl3* is one of the *elavl*-like gene family members that are proposed to function by binding to specific mRNAs and regulaing their expression to control a developmental program [5]. The overexpression of elav-like genes results in an alteration of the developmental fates of some cells [6].

The transcription of *gfap* was significantly up-regulated by 2.91-, 4.56-, 2.88-, 3.37, 4.63-fold in the treatment groups of DBP (50 μg/L and 500 μg/L), DEP (50 μg/L and 500 μg/L), and DBP-DEP (500 μg/L for each) as compared to the control (Fig. 1C). *Gfap* is a member of the family of intermediate filament structural proteins and highly expressed in astrocytes of the CNS. Due to its induced expression after cerebral injury in animals, *gfap* is considered to be a biological marker of neurotoxicity [7]. For example, increased protein content of *gfap* and up-regulation mRNA level of gfap gene has been found in experimental models after chemical insult [8]. Our finding of enhanced *gfap* transcription after DBP and DEP exporsue was consistent with these previous studies.

A significant rise in *mbp* mRNA levels was observed in the 500 μg/L of DBP, 500 μg/L of DEP and 500 μg/L (each) of DBP-DEP treatment groups, by 3.55-, 3.02-, 3.61-fold of that of the control, respectively (Fig. 1D). *Mbp* is a marker gene for myelinizetion of central nerves and robustly expressed in myelinating glia in the CNS. The change of *mbp* mRNA levels are related to brain injury. It has been reported that *mbp* protein levels was inhibited following PCB insult in the rats [9], whereas it enhanced after ethanol exposure in zebrafish embryos [4].

The transcription of *α1-tubulin* was significantly up-regulated by 2.79-, 3.11-, 3.83-fold in 50 μg/L of DBP, 500 μg/L of DEP and 500 μg/L (each) of DBP-DEP treated embryos compared to the control (Fig. 1E). The *α1-tubulin* is a neuron-specific microtubule protein and expressed during the development and regeneration of fish CNS [10,11]. Many previous reports showed that toxic chemicals could afftect *α1-tubulin* mRNA levels in mammals. For example, a proteomics study showed BDE-99 given to male mice at day 10 postnatally caused changes in brain protein expression after 24 h [12]. In the present study, exposure to DBP and DEP increased the *α1-tubulin* transcription levels at various concentration of DBP and DEP, suggesting the optic axons are vulnerable to DBP and DEP exposure.

Ngn1 gene expression increased significantly by 3.74-, 2.67-, 3.13--fold in the 500 μg/L of DBP, 500 μg/L of DEP and 500 μg/L (each) of DBP-DEP treated embryos relative to the control group (Fig. 1F). *Ngn1* is a basic helix-loop-helix transcription factor, which is essential for the specification of cranial neurogenic placodes. In mammal studies, mouse embryos lacking *ngn1* failed to generate the proximal subset of cranial sensory neurons [13]; consistent with which, zebrafish that lack *ngn1* activity were deficient in sensory neurons [14]. The up-regulation of *ngn1* expression observed in our study indicates that ngn1 in DBP/DEP-treated zebrafish embryos might play a protecting role in an attempt to repair the damaged cranial ganglia.

In addition, we analyzed the neuro-related gene expression in zebrafish embryos after exposure to DBP-DEP mixture. Interestingly, we found that the co-exposure resulted in induction of the nervous system responsive genes in embryonic zebafish. However, no significant difference of the selected genes, at least partly, were found in the DBP-DEP coexposure group comparing with the DBP/DEP individual exposure group. Thus, it can not be speculated whether the synergism, antagonism, or other joint action of DBP-DEP co-exposure affect the nervous system of zebrafish. Therefore, further study is needed to understand the mechanism underlying the neurotoxicology effects caused by multiple chemicals.

In summary, DBP and DEP exposure caused changes of transcriptional levels of selected neuron-related genes. Our findings revealed a susceptibility of the developmental nervous system in fish embryos to PAEs, thus, the chronic effects of these compounds on nervous system of aquatic organisms need more attention. In addition, the tested neuronal genes should be useful as biomarkers for the risk assessment of PAEs in the aquatic environment. However, further work is required to advance our understanding of the effects of PAEs on neurodevelopment.

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