Full Length Article

Fenobucarb-induced developmental neurotoxicity and mechanisms in zebrafish

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ABSTRACT

Fenobucarb (2-sec-butylphenyl methylcarbamate, BPMC) is an extensively used carbamate insecticide. Its developmental neurotoxicity and the underlying mechanisms have not been well investigated. In this study, zebrafish embryos were exposed to various concentrations of BPMC from 6 hpf (hours post fertilization, hpf) to 120 hpf. BPMC induced developmental toxicity with reduced motility in larval zebrafish. The spinal cord neutrophil infiltration, increased ROS production, caspase 3 and 9 activation, central nerve and peripheral motor neuron damage, axon and myelin degeneration were observed in zebrafish treated with BPMC generally in a dose-dependent manner. The expression of eight marker genes for nervous system function or development, namely, *a1-tubulin, shha, elavl3, gap43, syn2a, gfap, mbp* and *manf*, was significantly downregulated following BPMC exposure. AChE activity reduction and *ache* gene expression suppression was also found significantly in BPMC-treated zebrafish. These results indicate that BPMC is highly toxic to zebrafish and that BPMC induces zebrafish developmental neurotoxicity through pathways involved in inflammation, oxidative stress, degeneration and apoptosis.

1. Introduction

Fenobucarb (2-sec-butylphenyl methylcarbamate, BPMC) is a WHO (World Health Organization) Class II toxicity ('moderately hazardous') agricultural insecticide derived from carbamic acid (NH2COOH) and available in liquid formulations as 50 % emulsifiable concentrates (EC) (Lamb et al., 2016). BPMC competitively inhibits cholinesterase (AChE) (Zheng et al., 2017; Wood and Goulson, 2017; Yang et al., 2017) and causes muscle spasm and paralysis of insects, fish and mammals (Fahmy et al., 1970; Sastry and Siddiqui, 1982), posing potential risks to wild animals and human health (Kim et al., 2014). BPMC is applied for pest control to rice paddy soils where the irrigation water is used, increasing hazard to the aquatic environment (Kim et al., 2014). Several studies indicate that a fraction of the BPMC is transported from rice paddy effluent to receiving water bodies, including surface and ground water (Phong et al., 2010). Heong et al. (1994) found that about 32.2 % of the insecticides used in Vietnam was carbamates (CMs), such as fenobucarb. In a field study, Toan et al. (2013) reported that concentrations of CMs, such as fenobucarb, in surface water near rice fields varied between 0.11–5.0 μg/L.

Less attention has been paid to the toxicity of fenobucarb used alone. Kobayashi and colleagues reported that BPMC caused an increase in acetylcholine content and a decrease in AChE activity in the forebrain of 8–10 weeks old female ICR mice at 10 min after a single injection at 10 mg/kg (Kobayashi et al., 1985). Tam et al. reported that the sequential applications of fenobucarb caused significant inhibition on the brain AChE activity of climbing perch fingerlings (weight 4–5 g, length 5.4–6.7 cm) (Tam et al., 2016). Although the BPMC has been shown to induce AChE inhibition in animals, but the adverse effects of BPMC on the developing nervous system as well as the underlying mechanisms are not well investigated yet.

Zebrafish are emerging as a promising animal model for evaluating substance toxicity on development, and are particularly useful in studying the impacts of environmental pollutants to the nervous system and behaviors of vertebrates (Guo et al., 2018; Li et al., 2018; Shi et al., 2018; Qian et al., 2018). This transparent vertebrate offers numerous compelling experimental advantages for toxicity assessment not found in other model systems (Zhu et al., 2014; Chen et al., 2014; Duan et al., 2015; Zhu et al., 2015). Furthermore, there are high similarities in the nervous system development between zebrafish and mammals (Canestro et al., 2007; Tropepe and Sive, 2003). A number of laboratories have employed the zebrafish to investigate the toxic effects of

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model chemicals released into the environment on the development of the nervous system, including nanoparticles, pesticides and various organic pollutants (Guo et al., 2018; Li et al., 2018; Shi et al., 2018; Qian et al., 2018).

The present study was aimed to evaluate systematically and comprehensively developmental neurotoxicity and the potential mechanisms produced by BPMC exposure. We found that BPMC was highly toxic to zebrafish embryos, with concentration-dependent toxic effects in embryonic development, as well as decreased locomotor speed in zebrafish larvae. Our results also demonstrated that BPMC could induce inflammation and degeneration of the peripheral motor neuron, myelin, axon and the largest vagus sensory ganglion in a dose-dependent manner. In addition, the expression of eight marker genes for the nervous system functions or development, namely, *a1-tubulin*, *shha*, *elavl3*, *gap43*, *syn2a*, *gfap*, *mbp* and *manf*, was significantly downregulated following BPMC exposure. Reduced AChE activity and *ache* gene expression suppression, which is considered as another biomarker of neurotoxicant exposure, was also shown in zebrafish.

2. Materials and methods

2.1. Zebrafish care and maintenance

Four lines of zebrafish were used in this study: wild-type AB line zebrafish, Tg(Isl1:CMICP-GFP) transgenic zebrafish that express GFP in the cranial motor neurons, Tg(NBT:MAPT-GFP) (neural specific beta tubulin promoter)zc1 transgenic zebrafish that express green fluorescent protein (GFP) in the peripheral motor neurons, and Tg(mpx:GFP) transgenic zebrafish that express GFP exclusively in neutrophils. Zebrafish were housed in a light and temperature controlled aquaculture facility with a standard 14: 10 h light/dark photoperiod and fed with live brine shrimp twice daily and dry flake once a day. Four to five pairs of zebrafish were set up for natural mating every time. On average, 200–300 embryos were generated per pair of zebrafish. The embryos were maintained at 28 °C in fish water (0.2 % Instant Ocean Salt in deionized water, pH 6.9–7.2, conductivity 480–510 $\mu S.cm^{-1}$ and hardness 53.7–71.6 $mg.L^{-1}$ $CaCO_3$). The embryos were washed and staged at 6 and 24 hpf (hours post fertilization). The animal care and use for this study was approved by the Institutional Animal Care and Use Committee (IACUC) of Hunter Biotechnology, Inc. The zebrafish facility and laboratory at Hunter Biotechnology, Inc. is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International (Zhu et al., 2016a, 2016b; Zhou et al., 2015) and by the China National Accreditation Service for Conformity Assessment (CNAS).

2.2. Chemicals

Fenobucarb (BPMC) (purity = 99 %) dissolved in emulsifiable oil (Ning-emulsifier 33#) at a 50 % concentration was bought from Jiangxi lvchuan biotechnology industrial Co., Ltd (Fuzhou, China). Stock solutions were prepared in ultrapure water and stored in the dark at 4℃. Ning-emulsifier 33# was ordered from Nantong Deyi Chemical Co., LTD (Nantong, China). Instant Ocean Salt was purchased from Aquarium Systems (Sarrebourg, France). BSA (bovine serum albumin) and paraformaldehyde at 4 % concentration in phosphate-buffered saline was bought from Aladdin holdings (group) Co., Ltd (Shanghai, China). Trizol reagent, reverse transcriptase kit and the SYBR Green system were purchased from Takara (Dalian, China). Triton-X 100 was bought from Dingguo company (Beijing, China). Goat serum was provided by Jackson Immunoresearch Laboratories, Inc (West Grove, the United States).

2.3. Developmental toxicity assessment

Thirty wild-type zebrafish were distributed into 6-well plates (Nest

Biotech., Shanghai, China) in 3 mL fresh fish water. Zebrafish were treated with BPMC at concentrations of 6.25, 12.5, 25, 50, 100, 200 and 400 μg/mL and Ning-emulsifier 33# at 31.25, 62.5, 125, 250, 500, 1000 and 2000 nL/mL from 6 hpf to 120 hpf. The embryos were observed under the dissecting microscope at 1, 2, 3, 4 and 5 days post treatment (dpt) and photographed with the digital camera. Dead zebrafish were recorded and promptly removed from the solution during observations. Mortality curves were generated using Origin 8.0 (OriginLab, USA) and LC_{50} was calculated with logistic regression. The following parameters were evaluated: pericardial and body edema, abnormal pigmentation, size of eye, body length, heart-, head-, tail-, otoliths- and muscle deformation, absence of liver and intestine, and bleeding. All 30 zebrafish in each group, including small, malformed and normal zebrafish, were used for imaging. At the end of the experiments, all the zebrafish were anesthetized with 0.25 g/L tricaine methanesulfonate, which conforms to the American Veterinary Medical Association (AVMA) requirements for euthanasia by anesthetic (Shen et al., 2015). The developmental toxicity and other zebrafish endpoints were assessed by two well-trained scientists, at least one scientist was blinded to the treatment group.

2.4. Developmental neurotoxicity

2.4.1. Brain apoptosis

After treatment for 24 h, zebrafish were stained with 2.5 μg/mL acridine orange for 30 min and observed for apoptotic cells that would display yellow-green fluorescent spots in the brain under a stereo fluorescence microscope (Nikon AZ100 fluorescence microscope). Nikon NIS-Elements D 3.10 Advanced image processing software was used to capture and analyze the images. The fluorescence signal (S) from apoptotic cells in the brain was measured and the apoptotic rate was calculated as reported previously by our group (Li et al., 2006). The induction % of brain apoptosis in zebrafish treated with BPMC was calculated based on the following formula: The induction % of apoptosis = $([S_{BPMC}/S_{Control}]-1) \times 100$ %.

2.4.2. Central nerve loss

Thirty Tg(Isl1:CMICP-GFP) transgenic zebrafish were treated with BPMC at concentrations of 6.25, 12.5, 25, 50, 100 and 200 μg/mL from 6 hpf to 120 hpf. After treatment, 10 zebrafish from each group were randomly selected and images of central nerve were acquired using a stereo fluorescence microscope. Nikon NIS-Elements D 3.10 software was used to quantify the central nerve loss displayed as central nerve fluorescence intensity (S) in zebrafish. The percentage loss of central nerve in zebrafish treated with BPMC was calculated based on the following formula: The loss % of central nerves = $(1-[S_{BPMC}/S_{Control}]) \times 100$ %.

2.4.3. Peripheral motor neuron damage

Thirty Tg(NBT:MAPT-GFP)zc1 transgenic zebrafish were treated with BPMC at concentrations of 6.25, 12.5, 25, 50, 100 and 200 μg/mL from 6 hpf to 120 hpf. After treatment, ten zebrafish from each group were randomly selected and images were acquired using a stereo fluorescence microscope. To analyze average motor neuron length (L), NIS-Elements D3.10 image analysis software (Nikon) was used. The reduction % of peripheral motor neuron length in zebrafish treated with BPMC was calculated based on the following formula: The reduction % of peripheral motor neuron length = $(1-[L_{BPMC}/L_{Control}]) \times 100$ %.

2.4.4. Axon degeneration

In order to assess BPMC-induced axon degeneration, whole mount anti-acetylated tubulin immunostaining was performed. Early studies have indicated that anti-acetylated tubulin antibody specifically stains zebrafish neuron axon (Murphey and Zon, 2006; Sylvain et al., 2011). After treatment for 120 h, zebrafish were fixed in 0.4 % paraformaldehyde for 1 h and washed with 0.1 M phosphate buffered saline

(PBS) for $2-3$ h. The preparations were then permeabilized for 30 min in 4% Triton-X 100 containing 2% bovine serum albumin (BSA) and 10 % goat serum. Zebrafish were incubated for 48 h at 4 ℃ in a mouse monoclonal antibody against sea urchin acetylated-tubulin (α-AT) (Sigma-Aldrich, St. Louis, MO) at 1:100. Zebrafish were then washed for $2-3$ h in PBS and incubated in Rhodamine RedTM-X-conjugated affiniPure goat anti-mouse IgG + IgM(H + L) antibody (Jackson Immunoresearch Laboratories, West Grove, PA) at 1:800 for 4 h at room temperature. Preparations were then washed for 7 h in PBS-T (0.1 % Tween-20 in PBS), followed by mounting in glycerol for viewing. Ten zebrafish from each group were randomly selected and images were acquired using a stereo fluorescence microscope. Nikon NIS-Elements D 3.10 software was used to quantify all the axon fluorescence intensity (S) in zebrafish. The degeneration % of axon in zebrafish treated with BPMC was calculated based on the following formula: The degeneration % of axon = $(1-[S_{BPMC}/S_{Control}]) \times 100$ %.

2.4.5. Myelin degeneration

After 120 h treatment, zebrafish were subject to stain with FluoroMyelin (Molecular Probes, Eugene, OR) and then 10 zebrafish from each group were randomly selected and images were acquired using a stereo fluorescence microscope. NIS-Elements D3.10 image analysis software (Nikon) was used to quantify the myelin fluorescence intensity (S) in zebrafish. Quantitative image analysis was performed using image-based morphometric analysis. The myelin degeneration of zebrafish was obtained based on the quantitative FluoroMyelin staining results. The demyelination % in zebrafish treated with BPMC was calculated based on the following formula: The demyelination % = (1-$[S_{BPMC}/S_{Control}]) \times 100$ %.

2.5. Neurobehavioral toxicity assessment

After BPMC treatment at 120 hpf, 10 zebrafish from each group were loaded into 96-well plates, 1 zebrafish per well. Before monitoring, the larvae were acclimated in the 96-wells at 28 ℃ for 10 min. All experiments consisted of 60 min containing 3 cycles of a light/dark phase (10 min light and 10 min dark each), and the light level was 800 lx. Zebrafish traveled distance and movement speed to light-dark and dark-light cycles were recorded by viewpoint behavior analyzer (Zebralab V3, ViewPoint Life Sciences Co., Ltd.) and analyzed as we reported before (Huang et al., 2015). The reduction % of total distance traveled in zebrafish treated with BPMC was calculated based on the following formula: The reduction % of total distance traveled = (1-$[S_{BPMC}/S_{Control}]) \times 100$ %.

2.6. Mechanisms of developmental neurotoxicity

2.6.1. Nerve inflammation

Thirty Tg(MPO::GFP) transgenic zebrafish were treated with BPMC at concentrations of 6.25, 12.5, 25, 50, 100 and 200 μg/mL from 6 hpf to 120 hpf. After treatment, 10 zebrafish from each group were randomly selected and images were acquired using a stereo fluorescence microscope. NIS-Elements D3.10 image analysis software (Nikon) was used to quantify the fluorescence intensity (S) of neutrophil cells in zebrafish along the trunk and tail. The nerve inflammation resolution % in zebrafish treated with BPMC was calculated based on the following formula: The nerve inflammation resolution % = (1-$[S_{BPMC}/S_{Control}]) \times 100$ %.

2.6.2. Reactive oxygen species (ROS) assay

The ROS levels in zebrafish treated with BPMC at concentrations of 6.25, 12.5, 25, 50, 100 and 200 μg/mL from 6 hpf to 120 hpf were analyzed using an oxidation sensitive probe, 5-(and 6-)-chloromethyl-20, 70-dichloro-dihydrofluoresceindiacetate (CM-H2DCFDA, Life Technologies, Carlsbad, CA). The treated zebrafish were incubated with 0.5 mg/mL CM-H2DCFDA in the presence of BPMC for 1 h in dark at 28

℃. After rinsing for 3 times using fish water, zebrafish were transferred into a 96-well microplate (1 zebrafish per well) and ROS was measured at 488 nm under a multimode microplate reader (MikroWin 2000; Berthold, Stuttgart Germany) (Duan et al., 2015). Treatment solution (fish water) respectively containing ultrapure water, zebrafish or BPMC alone was used as background controls for the ROS microplate assay.

2.6.3. Caspase activity measurement

Thirty wild-type AB line zebrafish were treated with BPMC at concentrations of 6.25, 12.5, 25, 50, 100 and 200 μg/mL from 6 hpf to 120 hpf. At the end of treatment, zebrafish from each group were loaded into 96-well plates, 1 fish per well. Caspase activities were measured using caspase-Glo reagents (Promega, USA) through cleavage of colorless substrates specific for caspase 3/7 and caspase 9 using a multifunction microplate reader MikroWin 2000. In each assay, at least six wells per sample were measured for each dose and the results were averaged. The relative caspase activity in zebrafish treated with BPMC was calculated based on the following formula: The relative caspase activity = $S_{BPMC}/S_{Control} \times 100$ %.

2.6.4. AChE activity measurements

Thirty wild-type AB line zebrafish were treated with BPMC at concentrations of 6.25, 12.5, 25, 50, 100 and 200 μg/mL from 6 hpf to 120 hpf. At the end of treatment, zebrafish from each group without homogenization were rinsed in fish water for 3 times and then loaded into 96-well plates, 1 zebrafish per well in 50 μl fish water. AChE activitiy was measured using Amplite™ Fluorimetric Acetylcholinesterase Assay Kit (AAT Bioquest, USA) according to the manufacturer's instructions using a multifunction microplate reader MikroWin 2000. Briefly, after adding 50 μl of acetylthiocholine reaction mixture into each well of the *AChE* standard blank control, and test samples to make the total AChE assay volume of 100 μl per well, the microplate was incubated at room temperature for 30 min with protection from light and monitored the fluorescence increase with the microplate reader at Ex/Em = 490/520 nm. In each assay, at least six wells per sample were measured for each dose and the results were averaged. The relative AChE activity in zebrafish treated with BPMC was calculated based on the following formula: The relative AChE activity = $S_{BPMC}/S_{Control} \times 100$ %.

2.7. Gene expression analysis

To further investigate the possible developmental neurotoxicity mechanism induced by BPMCin zebrafish, key marker genes of *ache*, myelin basic protein (*mbp*), *manf*, synapsin IIa (*syn2a*), *a1-tubulin*, sonic hedgehog a (*shha*), *gap43*, *elavl3*, and glial fibrillary acidic protein (*gfap*) were determined. These genes are of importance to the developmental neurotoxicity and are believed to be responsive to chemical exposure as previously reported (Wang et al., 2015a,b; Benowitz and Routtenberg, 1997). The mRNA levels of the genes related to apoptosis *bcl-2* and *bax* were also measured after BPMC exposure. We chose the primer sequences following the previous studies as presented in Table 1 (Guo et al., 2018). After BPMC treatment, total RNA was extracted from 50 homogenized zebrafish per group using Trizol reagent (Invitrogen Life Technologies), 3 pools of 50 zebrafish each were assayed. The quality of RNA samples was evaluated using the methods from Nano-Drop 2000 (Thermo Scientific). About 2 μg total RNA of each sample was used for cDNA synthesis using FastQuant RT Kit (with gDNase) (Tiangen) and Q-PCR amplifications were carried out with a CFX Connect detection system (Biorad) using the iTaq Universal SYBR Green Supermix (Biorad) in which there are three technical or biological replicates. The PCR protocol used was: 2 min at 95 ℃, 40 cycles of 5 s 95 ℃, and 30 s at 60 ℃. Melting curve analysis was performed to check the specificity of the primers. Expression data was normalized against the expression of β-actin, having a stable expression in all treatments. The relative quantification of each gene mRNA level among groups was

Table 1
Sequences of primer pairs used in the real-time quantitative PCR reactions.

Gene	Forward (5′-3′)	Reverse (5′-3′)
β-actin	tcgagcaggagatgggaacc	ctctgtggataccgcaagattc
bax	gacttgggagctgcacttct	tccgatctgctgcaaacact
bcl-2	cactggatgactgactacctgaa	cctgcgagtcctcattctgtat
ache	ccctccagtgggtacaagaa	gggcctcatcaaaggtaaca
mbp	aatcagcaggttcttcggaggaga	aagaaatgcacgacagggttgacg
a1-tubulin	aatcaccaatgcttgcttcgagcc	ttcacgtctttgggtaccacgtca
shha	gcaagataacgcgcaattcggaga	tgcatctctgtgtcatgagcctgt
elavl3	gtcagaaagacatggagcagttg	gaaccgaatgaaacctaccccc
gap43	tgctgcatcagaagaactaa	cctccggtttgattccatc
syn2a	gtgaccatgccagcatttc	tggttctcactttcacctt
manf	agatggagagtgtgaagtctgtgtg	caattgagtcgctgtcaaaacttg
gfap	ggatgcagccaatcgtaat	ttccaggtcacaggtcag

calculated using the 2-△△Ct method (Sharif et al., 2016).

2.8. Statistical analysis

Sigmoidal regression for concentration–response curves was used for LC_{50} estimation (Origin 8.0). One-way ANOVA followed by the Dunnett's test was used to compare differences among groups. All statistical analyses were performed using the SPSS 16.0 software (SPSS, USA), and $p < 0.05$ was considered statistically significant. For quantitative analysis, all data were presented as mean ± SE, and results were statistically compared between drug-treated and control zebrafish groups. All experiments were repeated for at least 3 times.

3. Results

3.1. Developmental toxicity

The mortality and toxic phenotypes of zebrafish at different stages (1–5 dpf) were recorded as demonstrated in Fig. 1. In this study, the tested BPMC concentrations were from 6.25 μg/mL to 400 μg/mL; and correspondingly, the concentrations of Ning-emulsifier 33# as its dissolvent in BPMC formulation were from 31.25 nL/mL to 2000 nL/mL. No morphological abnormality was observed at1 − 5 dpf zebrafish treated with Ning-emulsifier 33# at any concentrations up to 2000 nL/mL (Fig. 1H), implying that the emulsifiable oil Ning-emulsifier 33# did not have an adverse effect on zebrafish. No morphological abnormality was observed in 1–3 dpf embryos treated with BMPC at 6.25 and 12.5 μg/mL. However, trunk curvature and tail malformation occurred in the zebrafish treated with BMPC at 50, 100 and 200 μg/mL (Fig. 1). After BMPC exposure for 5 continuous days, severe pericardial edema, trunk curvature, tail malformation and muscle degeneration were found in 100 and 200 μg/mL treatment group. The embryo deaths occurred in 200 and 400 μg/mL treatment groups, with 23.3 % and 100 % mortality; whereas the survivors had severe trunk curvature, pericardial edema, obvious circulation abnormalities, and muscle degeneration in 200 μg/mL treatment groups (Fig. 1). The estimated LC_{50} of BMPC in the zebrafish was 205 μg/mL. As compared with untreated control zebrafish, the body lengths in zebrafish treated with BPMC at concentrations ranging from 6.25 μg/mL to 200 μg/mL were decreased by (4 ± 2.9)%, (9 ± 3.8)%, (16 ± 2.5)%, (21 ± 1.5)%, (23 ± 0.4)% and (26 ± 1.2)%. The heart rates were (162 ± 2.2)/min in control zebrafish, and (141 ± 1.1)/min, (116 ± 1.1)/min, (99 ± 2.6)/min and (64 ± 2.5)/min in the zebrafish treated with BPMC at concentrations of 25, 50, 100 and 200 μg/mL, respectively. Liver absence and delayed yolk sac absorption were found in all BPMC-treated zebrafish. The representative phenotype figures showing zebrafish treated with BPMC at
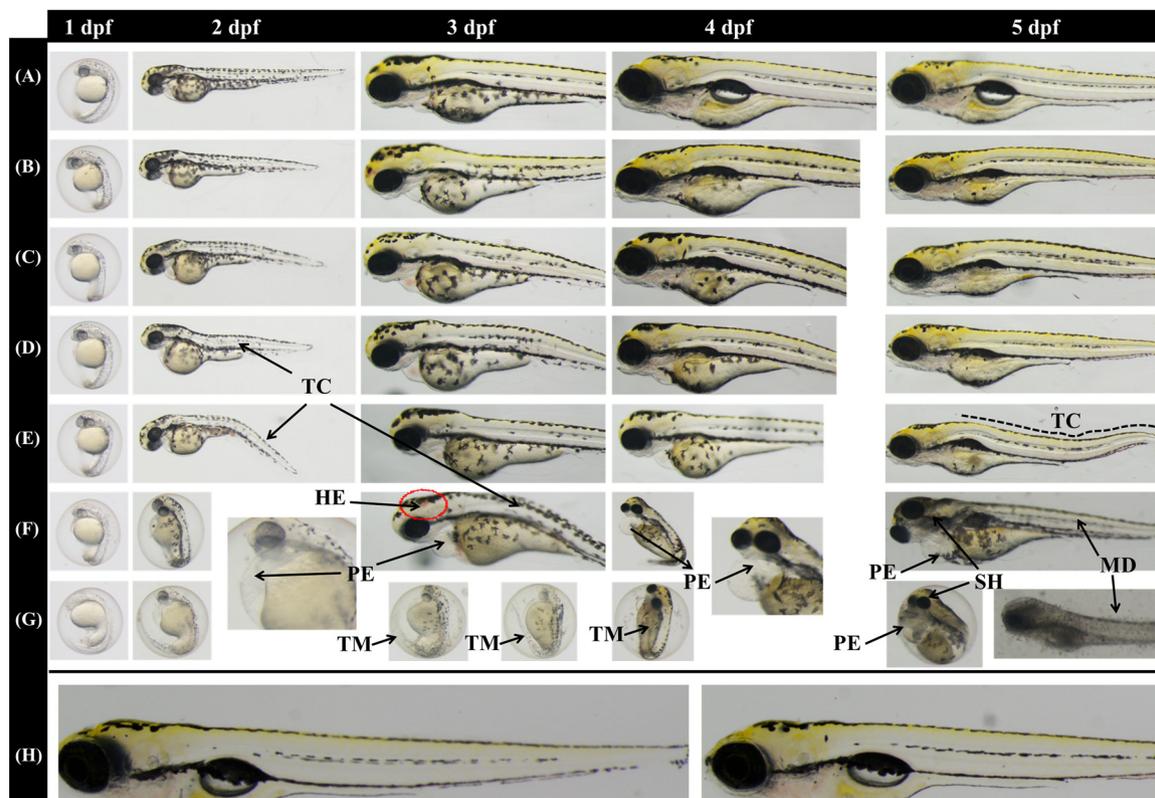
Fig. 1. Toxic effects of BPMC on zebrafish during the exposure at 1 dpf-5 dpf: (A) control zebrafish, (B) 6.25 μg/mL treatment, (C) 12.5 μg/mL treatment, (D) 25 μg/mLtreatment, (E) 50 μg/mLtreatment, (F) 100 μg/mL treatment, and (G) 200 μg/mL treatment. (H) 2000 nL/mL Ning-emulsifier 33# treatment. HE: hemorrhage, MD: muscle degeneration, PE: pericardial edema, SBL: short body length, TC: trunk curvature, TM: tail malformation, SH: small head.
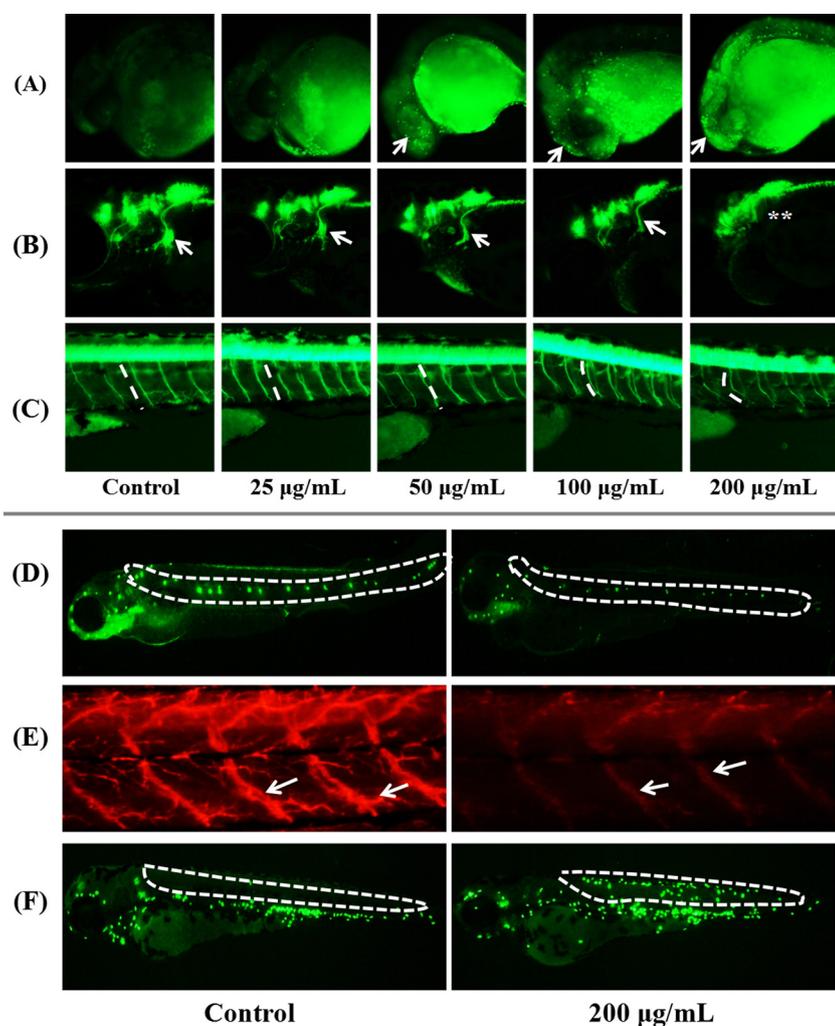
Fig. 2. BPMC exposure induced developmental neurotoxicity in zebrafish: (A) there were no obvious apoptotic cells observed in the control zebrafish, but considerable numbers of apoptotic cells were observed in BPMC-treated zebrafish; (B) central neurons were severely damaged at 50 μg/mL and 100 μg/mL, and disappeared at 200 μg/mL in Tg(Isl1:CMICP-GFP) transgenic zebrafish treated with BPMC; (C) peripheral motor neurons were bent and shorter in Tg(NBT:MAPT- GFP)zc1 transgenic zebrafish; (D) demyelination in wild type zebrafish stained with Fluoromyelin; (E) axon degeneration in wild type zebrafish stained with whole mount anti-acetylated tubulin immunostaining; (F) the control zebrafish showed the normal distribution of labeled neutrophil cells, whereas the zebrafish exposed to BPMC showed a general dispersal of fluorescent neutrophils.

various concentrations in different stages were presented in Fig. 1.

3.2. Developmental neurotoxicity

3.2.1. Brain apoptosis

The brain of zebrafish was chosen for the observation of apoptosis induction. There were no obvious apoptotic cells indicated in the control zebrafish, but considerable numbers of apoptotic cells were observed in BPMC-treated zebrafish brains at higher concentrations (50, 100 and 200 μg/mL) in a dose-dependent manner (Fig. 2A). The induction % of apoptosis was $(1 \pm 4.5)\%$, $(1 \pm 4.4)\%$, $(2 \pm 6.7)\%$, $(34 \pm 4.2)\%$, $(46 \pm 5.4)\%$ and $(53 \pm 5.7)\%$ in the zebrafish treated with BPMC at 6.25, 12.5, 25, 50, 100 and 200 μg/mL, respectively. Statistically significant apoptosis induction was demonstrated in the zebrafish treated with BPMC at 50 μg/mL and above ($p < 0.001$).

3.2.2. Central nerve and peripheral motor neuron damage

No morphological abnormality of central nerve and peripheral motor neuron were observed in the control zebrafish. As shown in Fig. 2B, we observed that the largest vagus sensory ganglion, which is likely to include neurons innervating visceral organs and the vagus nerve extending into the visceral organs was severely degenerated at 50 μg/mL and 100 μg/mL, and even disappeared at 200 μg/mL in Tg (Isl1:CMICP-GFP) transgenic zebrafish treated with BPMC. We also observed that the peripheral motor nerves were bent and shorter in Tg (NBT:MAPT- GFP)zc1 transgenic zebrafish exposed to BPMC in a dose-dependent manner (Fig. 2C). The loss of central nerves was from $(2 \pm 3.2)\%$ to $(94 \pm 0.2)\%$; and the peripheral motor neuron length

was reduced by $(9 \pm 1.9)\%$ - $(27 \pm 1.2)\%$ in BPMC-treated zebrafish at concentrations ranging from 6.25 μg/mL to 200 μg/mL. Statistically significant differences were observed between untreated control zebrafish and the zebrafish treated with 50, 100 and 200 μg/mL in all these assessments ($p < 0.05$ or $p < 0.001$) (Table 2).

3.2.3. Axon and myelin degeneration

No morphological abnormality of myelin and axon were observed in the control zebrafish. The demyelination (Fig. 2D) and axon degeneration (Fig. 2E) were demonstrated in wild-type zebrafish treated with BPMC in a dose-dependent manner. The demyelination was $(0 \pm 2.9)\%$ - $(59 \pm 1.7)\%$ and the axon degeneration was $(4 \pm 4.7)\%$ - $(57 \pm 1.7)\%$ in BPMC-treated zebrafish at concentrations ranging from 6.25 μg/mL to 200 μg/mL, respectively. As compared with the control group, demyelination and axon degeneration were statistically significant in the zebrafish treated with 50, 100 and 200 μg/mL BPMC ($p < 0.05$ or $p < 0.01$ or $p < 0.001$) (Table 2).

3.3. Neurobehavioral toxicity

We evaluated the locomotion activities of zebrafish under alternating light-dark stimulation. Distance traveled decreased significantly in a dose-dependent manner and the reduction % of total distance travelled was $(14 \pm 8.9)\%$, $(21 \pm 10.5)\%$, $(26 \pm 8.2)\%$, $(43 \pm 3.0)\%$, $(55 \pm 2.3)\%$ and $(59 \pm 3.1)\%$ after zebrafish exposed to BPMC at concentrations of 6.25, 12.5, 25, 50, 100 and 200 μg/mL, respectively (Fig. 3A and B). Average movement speeds of larval zebrafish during each 10-min photoperiod were presented in C. The activity of zebrafish

Table 2
Developmental neurotoxicity of BPMC on zebrafish.

Neurotoxicity (%)	Control	BMPC Concentration (μg/mL)					
		6.25	12.5	25	50	100	200
Brain apoptosis	0	1 ± 4.5	1 ± 4.4	2 ± 6.7	34 ± 4.2***	46 ± 5.4***	53 ± 5.7***
PMNL Reduction	0	9 ± 1.9*	12 ± 2.1*	17 ± 2.0***	20 ± 2.0***	23 ± 1.5***	27 ± 1.2***
Central neuron loss	0	2 ± 3.2	8 ± 3.3	11 ± 4.3	23 ± 2.8***	44 ± 3.8***	94 ± 0.2***
Axon degeneration	0	4 ± 4.7	8 ± 4.0	16 ± 4.7**	42 ± 1.3***	52 ± 1.9***	57 ± 1.7***
Demyelination	0	0 ± 2.9	6 ± 3.5	13 ± 4.1*	43 ± 1.4***	53 ± 2.1***	59 ± 1.7***
Nerve inflammation	0	2 ± 2.5	19 ± 7.2	48 ± 6.7***	60 ± 5.3***	71 ± 6.5***	82 ± 10.0***
ROS production	100 ± 4.5	98 ± 5.1	104 ± 0.7	125 ± 1.3	210 ± 9.4**	264 ± 9.0***	523 ± 21.2***
Caspase-3/7 activity	100 ± 3.8	100 ± 4.3	111 ± 1.1	112 ± 4.5	117 ± 3.7*	119 ± 1.7**	123 ± 4.4***
Caspase-9 activity	100 ± 6.4	118 ± 8.8	132 ± 6.5**	136 ± 5.6**	147 ± 3.8***	161 ± 3.8***	183 ± 6.5***
AChE activity	100 ± 4.2	99 ± 5.7	86 ± 5.0	83 ± 7.6	60 ± 2.9***	53 ± 3.4***	35 ± 2.0***

Compared with control: *p < 0.05, **p < 0.01, and ***p < 0.001.
PMNL: peripheral motor neuron length.
Note: Each data were calculated from 3 groups of 10 zebrafish.

in the light period was much slower than that in the dark period. Zebrafish with BPMC treatment showed a delayed decrease in activity than the controls when the photoperiod transitioned from dark to light (p < 0.05, p < 0.01 or p < 0.001). The activity increased much more slowly in BPMC treatment groups during the transition from light to dark. During the 10-min dark period, zebrafish from BPMC exposure groups showed significantly slower movement speed than controls (p < 0.05, p < 0.01 or p < 0.001).

3.4. Mechanisms of developmental neurotoxicity

3.4.1. Inflammation

As shown in Fig. 2F, the control zebrafish showed the normal distribution of labeled neutrophil cells, mostly localized in the ventral trunk and tail; whereas the zebrafish exposed to BPMC showed a general dispersal of fluorescent neutrophils, suggestive of active migration from their initial location to a few clusters along the horizontal midline
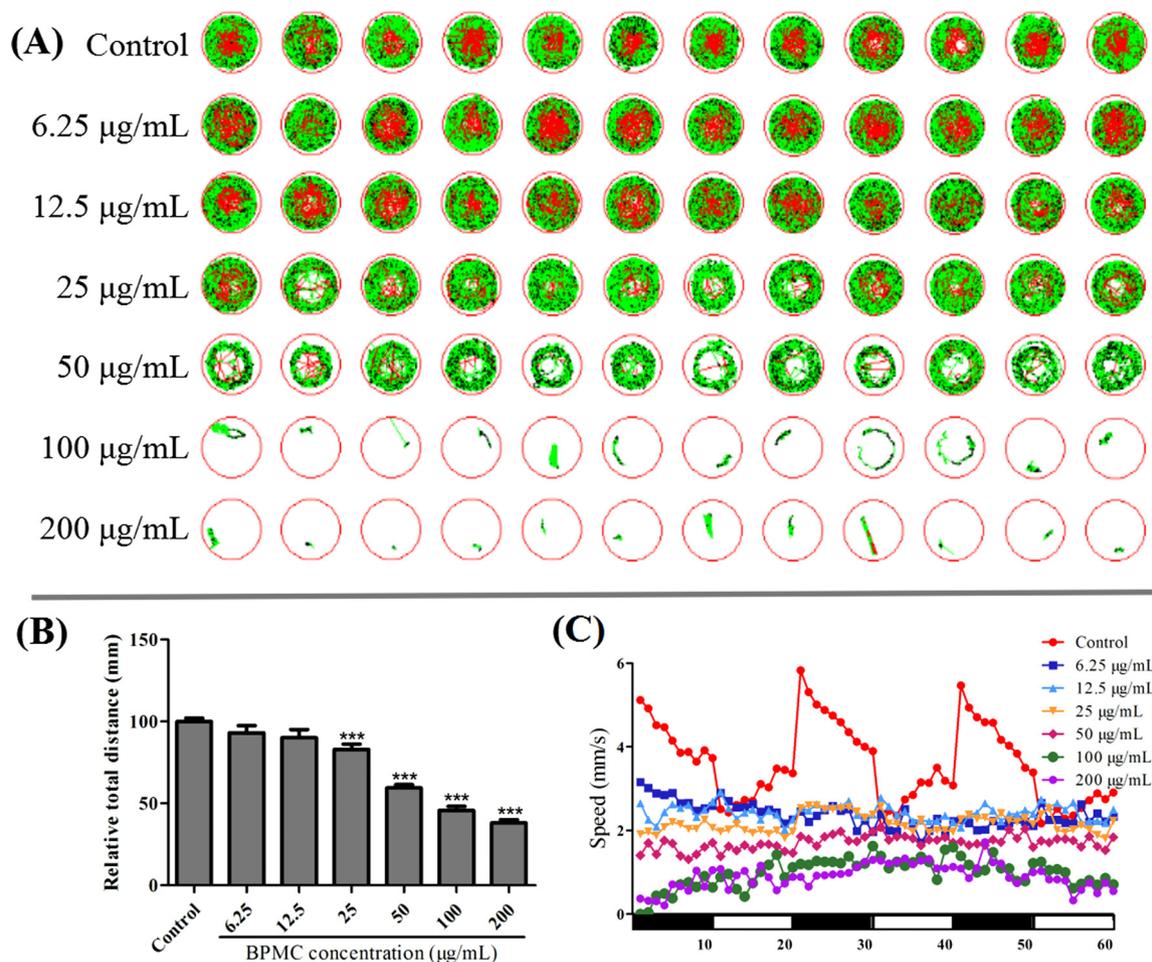
Fig. 3. Locomotor activity of zebrafish exposed to BPMC for 120 h was assessed. (A) Distance traveled was significantly decreased in a dose-dependent manner, and almost no movement at concentrations of 100 μg/mL and 200 μg/mL; (B) quantitative analysis of total distance traveled after zebrafish exposed to BPMC at concentrations of 6.25, 12.5, 50, 100 and 200 μg/mL; (C) locomotor patterns of zebrafish from the control and BPMC-treated zebrafish were displayed. Data were expressed as the mean ± SEM (n = 3) in 60-s intervals and analyzed by one-way ANOVA, followed by a LSD test. ***p < 0.001.

of the trunk and tail. The relative average fluorescence intensity of neutrophil cells was $(102 \pm 2.5)\%$, $(119 \pm 7.2)\%$, $(148 \pm 6.7)\%$, $(160 \pm 5.3)\%$, $(171 \pm 6.5)\%$ and $(182 \pm 10.0)\%$ in the zebrafish treated with BPMC at 6.25–200 μg/mL, respectively. Quantitative data showed that there was a significant difference in the fluorescence intensity of neutriphils localized to the lateral line between control and BPMC-treated zebrafish. BPMC exhibited significant dose-dependent promotion of leukocyte infiltration and the nerve inflammation induction % was $(2 \pm 2.5)\%$, $(19 \pm 7.2)\%$, $(48 \pm 6.7)\%$, $(60 \pm 5.3)\%$, $(71 \pm 6.5)\%$ and $(82 \pm 10.0)\%$ in the zebrafish treated with various concentrations of BPMC () ($p < 0.001$).

3.4.2. ROS production

BPMC treatment resulted in increased ROS production. ROS levels relative to control zebrafish were $(98 \pm 5.1)\%$, $(104 \pm 0.7)\%$, $(125 \pm 1.3)\%$, $(210 \pm 9.4)\%$, $(264 \pm 9.0)\%$ and $(523 \pm 21.2)\%$, respectively, in zebrafish treated with BPMC at concentrations from 6.25 μg/mL to 200 μg/mL. Statistically significant differences ($p < 0.01$ or $p < 0.001$) were found among the control zebrafish and the zebrafish treated with BPMC at 50, 100 and 200 μg/mL.

3.4.3. Caspase activity

After treatment with BPMC at 6.25, 12.5, 25, 50, 100 and 200 μg/mL, caspase-3/7 and -9 activities were all notably increased to $(100 \pm 4.3)\%$, $(111 \pm 1.1)\%$, $(112 \pm 4.5)\%$, $(117 \pm 3.7)\%$, $(119 \pm 1.7)\%$ and $(123 \pm 4.4)\%$; $(118 \pm 8.8)\%$, $(132 \pm 6.5)\%$, $(136 \pm 5.6)\%$, $(147 \pm 3.8)\%$, $(161 \pm 3.8)\%$ and $(183 \pm 6.5)\%$, respectively. Statistically significant elevations were found in the zebrafish treated with BPMC at 50, 100 and 200 μg/mL in caspase-3/7 and -9 activities ($p < 0.05$ or $p < 0.01$ or $p < 0.001$).

3.4.4. AChE activity

BPMC treatment led to AChE activity suppression. Total AChE activity relative to control group was reduced to $(99 \pm 5.7)\%$, $(86 \pm 5.0)\%$, $(83 \pm 7.6)\%$, $(60 \pm 2.9)\%$, $(53 \pm 3.4)\%$ and $(35 \pm 2.0)\%$, respectively, in zebrafish treated with BPMC at concentrations of 6.25, 12.5, 25, 50, 100 and 200 μg/mL. Statistically significant differences ($p < 0.001$) were found in zebrafish treated with BPMC at 50, 100 and 200 μg/mL.

3.4.5. Gene expression

3.4.5.1. Apoptosis related genes.
As shown in Fig. 4, a concentration-dependent upregulation of the *bax* gene expression was observed upon exposure to 12.5, 25, 50, 100 and 200 μg/mL BPMC, 1.0-, 1.2-, 1.6-, 2.0- and 2.1-fold increases relative to the control group. As for the expression of *bcl-2*, no significant alteration occurred in any treatment groups. Thus, the *bcl-2/bax* ratio decreased after BPMC exposure, especially at higher BPMC concentrations (50, 100 and 200 μg/mL).

3.4.5.2. Neurodevelopment related genes.
As shown in, the neurodevelopment and nerve functions-related genes were generally decreased in a concentration-dependent manner after zebrafish exposed to BPMC. The relative expression levels of *ache, mbp, a1-tubulin, elavl3, gap43, gfap, manf, shha* and *syn2a* were 0.16–1.00, 0.28−0.90, 0.40−0.94, 0.52−0.94, 0.43−0.99, 0.52−0.82, 0.58−0.88, 0.52−0.96 and 0.37–1.13, respectively. A statistically significant downregulation of 9 examined genes were observed in the zebrafish treated with BPMC ($p < 0.05$ or 0.01 or 0.001). In addition, the expression of *gfap* was also significantly reduced in zebrafish exposed to BPMC at concentration of 12.5 μg/mL with a relative expression level of 0.82 ($p < 0.01$).

4. Discussion

In the present study, we found that BPMC exposure to the zebrafish induced developmental toxicity, especially developmental neurotoxicity, including brain apoptosis, central nerve and peripheral motor neuron damage, axon and myelin degeneration with reduced motility. AChE activity as well as *ache* gene expression was also diminished. Pro-apoptotic protein Bax gene was elevated, whereas an array of neurodevelopment and functions related genes were downregulated. BPMC- induced inflammation, ROS production and caspase activation may contribute to the pathogenesis of its developmental neurotoxicity. To the best of our knowledge, this was the first report to provide solid evidence for developmental neurotoxicity and the possible mechanisms of BPMC in a whole animal model. These results provide valuable information for the neural health risk of BPMC and could facilitate environmental and ecological risk assessment of BPMC pollution.

General developmental toxicity induced by BPMC in zebrafish was indicated by an increase in malformation and reductions in hatchability, body length and the heart rate. Other developmental toxicity includes pericardial edema, circulation abnormalities, tail deformation, smaller head, trunk curvature and muscle degeneration. As indicated by many earlier studies, the abnormal development of the central nervous system (CNS) or peripheral nervous system (PNS) was important contributing factor to the toxic substance-induced neurotoxicity in the early life stage of zebrafish (Wang et al., 2015a, b). Here our results demonstrate that the primary and secondary neurons in BPMC-exposed zebrafish were adversely affected. In general, they exhibited a disordered pattern and partly loss of peripheral and central neurons. BPMC exposure also induced demyelination, axon loss, and nerve inflammation. In a previous investigation, all isolated bacteria were cultivated on a mineral medium containing BPMC at a concentration of 100 μg/mL (Kim et al., 2014). When used as an insecticide, the target concentrations were 20–1000 mg kg^{-1} (roughly corresponding to 20–1000 μg/mL) of BPMC in the treated soil (Kubota et al., 2007). We postulate that the neurotoxicity found in the zebrafish exposed to BPMC could be secondary to the developmental toxicity because the neurotoxicity was at concentrations higher than those affecting general development.

The behavioral response is related to the neuronal and physiological integrity of an organism and is highly sensitive to pollutants (Rihel et al., 2010; Nusser et al., 2016). Locomotor behavior of zebrafish has been widely utilized to study the neurological and developmental toxicity of various pollutants and toxins, especially since the introduction of automated video tracking systems (Ahmad et al., 2012; Noyes et al., 2015; Liu et al., 2016; Sun et al., 2016a, 2016b). In the current study, the zebrafish locomotor behaviors were assessed, and the results showed that BPMC exposure resulted in a notable decrease in total distance traveled and movement speed and finally led to hypoactivity, which was considered to represent developmental neurotoxicity caused by toxicants as well as an adaptive response for defense against toxic stress (Chen et al., 2012c; Jin et al., 2016). The reduced motility was caused, at least in part, by the potential neurological deficits that these BPMC-treated zebrafish had due to its neurotoxicological effects. The results were entirely consistent with previous reports on zebrafish exposed to other toxicants (Guo et al., 2018; Li et al., 2018; Shi et al., 2018; Qian et al., 2018).

Inflammation and oxidative stress have become important subjects in environmental and aquatic toxicology (Livingstone, 2003). Exposure to chemical pollutants may interfere the balance between endogenous and exogenous reactive oxygen species (ROS). In this study, neutrophil infiltration along the spinal cord with increased ROS generation and caspases 3/7 and 9 activities were detected, indicating that inflammation and oxidative stress may play important roles in the pathogenesis of BPMC-induced developmental toxicity and neurotoxicity, probably at least in part through apoptosis.

AChE activity has been extensively accepted as a biomarker of neurotoxicant exposure in aquatic organisms. Tam et al. reported that the sequential applications of BPMC caused significant inhibition on the brain AChE activity in the exposed fish (Tam et al., 2016). Chen et al. noticed that the inhibition of AChE activity and the change of *ache* gene
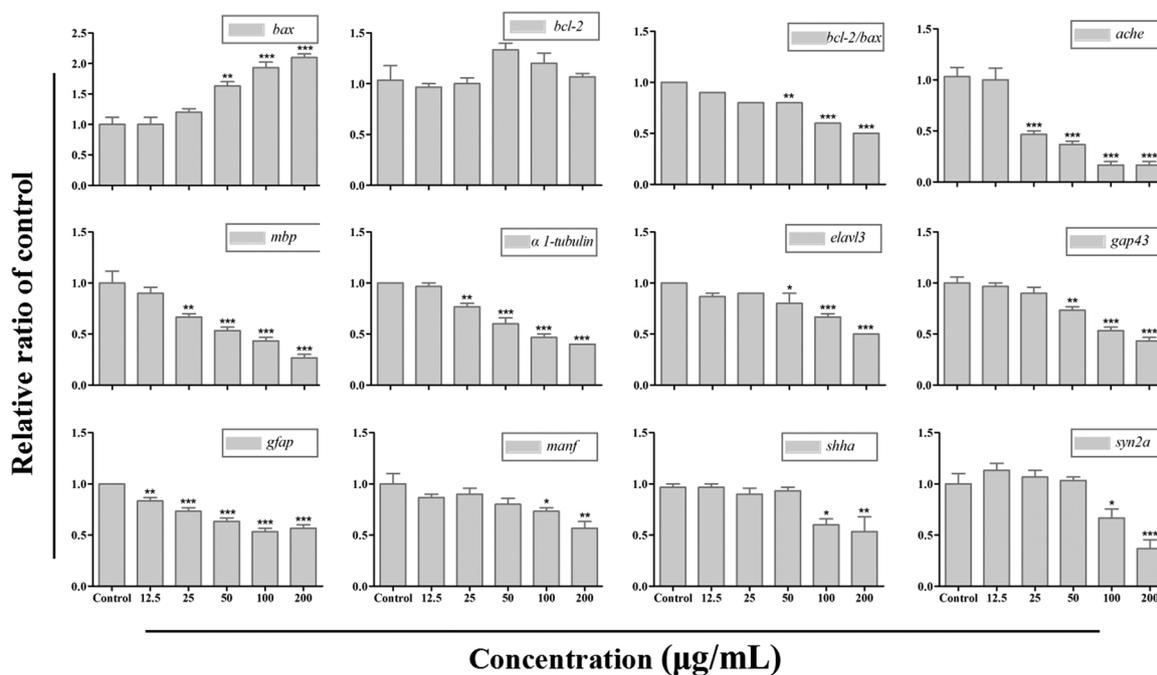
Fig. 4. Relative expression levels of *Bcl-2*, *Bax*, *Bcl-2/Bax* ratio, *ache*, and neurodevelopment-related genes including *mbp*, *a1-tubulin*, *elavl3*, *gap43*, *gfap*, *manf*, *shha* and *syn2a*, in the zebrafish after exposure to various concentrations of BPMC for 120 h. Total RNA was extracted from 50 homogenized zebrafish per group and 3 pools of 50 zebrafish each were assayed. Data were expressed as mean ± SE (n = 3). Compared with control group: *p < 0.05, **p < 0.01, ***p < 0.00.

could attribute to the alternation of locomotor behavior, ultimately leading to developmental toxicity in larval zebrafish. The decrease of AChE activity and the downregulation of *ache* gene were observed in this study after BPMC exposure, which was consistent with these previous reports (Tam et al., 2018; Chen et al., 2012a), suggesting that BPMC could exert developmental toxicity and alter locomotor behavior by disrupting the cholinergic nerve system.

The altered expression of CNS genes could contribute to developmental neurotoxicity (Chen et al., 2012b). The *α1-tubulin* encoded intermediate filament protein, which constitutes an essential part of structural and functional elements of microtubule cytoskeleton, axons and dendrites (Alm et al., 2008). *Elavl3* plays an important role in the neuronal development and individual behavior (Okano and Darnell, 1997; Pascale et al., 2004). The gene *manf* can maintain and regulate dopaminergic neurons and to direct the dopaminergic precursor cells differentiating into mature neurons (Chen et al., 2012c). *Gfap* is a sensitive and reliable astrocyte marker and a regulator of the astrocyte cytoskeleton differentiation (Nielsen and Jorgensen, 2003). *Syn2a* is associated with synapse formation during the period of zebrafish embryogenesis. *Mbp* play an important role as the primary protein constituent of the myelin sheath in the developing central nervous system of zebrafish (Lee and Fields, 2009). *Shha* is a signaling molecule which forms the nervous system (Muller et al., 1999) and also has an effect on axonal guidance cues in the spinal cord commissural axons and retinal ganglion cell axons (Kolpak et al., 2005; Charron et al., 2003). Moreover, *gap43* is integral when organisms offset the direct damage of toxicants (Alm et al., 2008). We found that the expression of all these eight marker genes for nervous system was significantly downregulated following BPMC exposure. These findings may imply that zebrafish are highly sensitive to BPMC that induces developmental neurotoxicity through pathways involved in inflammation, oxidative, apoptosis and degeneration.

5. Conclusions

BPMC was highly toxic to zebrafish with concentration-dependent neurotoxic effects on embryonic development and reduced motility.

BPMC induced oxidative stress, inflammation, central nerve and peripheral motor neuron damage, demyelination and axon degeneration. The expression of 8 marker genes for nervous system function or development, namely, *a1-tubulin*, *shha*, *elavl3*, *gap43*, *syn2a*, *gfap*, *mbp* and *manf*, was significantly downregulated following BPMC exposure. Marked inhibitions of AChE activity and *ache* gene expression were also observed in BPMC-treated zebrafish.

Credit author statement

Ping Li, Hua Yang and Chun-Qi Li designed the research; Xiao-Yu Zhu, Bo Xia, Yu-Ying Wu, Ming-Zhu Dai and Yan-Feng Huang performed the research; Xiao-Yu Zhu, Bo Xia and Yu-Ying Wu analyzed the data; Xiao-Yu Zhu and Chun-Qi Li wrote the paper.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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