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Fenobucarb induces heart failure and cerebral hemorrhage in zebrafish

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ABSTRACT

The potential risk and toxic mechanisms of fenobucarb (2-sec-butylphenyl methylcarbamate, BPMC) to animals and humans have not been fully elucidated. In this study, zebrafish embryos were exposed to various concentrations of BPMC from 48 hpf (hour post fertilization, hpf) to 72 hpf. We found that BPMC induced severe heart failure with bradycardia, reduced heart contractions, cardiac output and blood flow dynamics ; and myocardial apoptosis. BPMC also induced cerebral hemorrhages and blood erythrocyte reduction in a dose-dependent manner. Also observed were increased ROS production and capase 9 and 3/7 activation. The mRNA levels of the ATPase-related gene (*atp2a1l*), calcium channel-related gene (*cacna1ab*), sodium channel-related gene (*scn5Lab*), potassium channel-related gene (*kcnq1*), the regulatory gene (*tnnc1a)* for cardiac troponin C, and several apoptosis-related genes were significantly downregulated in zebrafish following BPMC exposure. These results suggest that exposure to BPMC is a possible risk factor to cardiovascular and cerebrovascular systems in animals.

1. Introduction

Fenobucarb (2-sec-butylphenyl methylcarbamate, BPMC) is widely applied on grasslands and farmlands to protect crops from plagues and it is available in liquid formulations as 50% emulsifiable concentrates (EC) (Lamb et al., 2016). Its careless use may give rise to the contamination of plants, and by grazing on these contaminated crops or through direct oral administration, animals may accumulate BPMC residues in their muscle tissues, milk, and eggs (Zheng et al., 2017). This increases the risk to human health via the food chain on a daily basis. BPMC is often applied for pest control to rice paddy soils where irrigation water is used, increasing risks to the aquatic environment (Kim et al., 2014). Several studies indicate that considerable fractions of BPMC is transported from rice paddy effluent to receiving water bodies, including surface and ground water, causing serious environmental problems (Phong et al., 2011).

So far most studies regarding BPMC toxicity refer to its joint toxicity with other pesticides (Miyaoka et al., 1984; Takahashi et al., 1987; Tam et al., 2018). Little investigation has been done on the toxicity and toxic mechanisms induced by BPMC itself. Kobayashi and colleagues reported that BPMC caused an increase in acetylcholine content and a decrease in AChE activity in the forebrains of mice (Kobayashi et al., 1985). Tam et al. noticed that the sequential applications of BPMC caused significant inhibition on the brain's AChE activity in exposed fish (Tam et al., 2016). BPMC exposure may pose a health risk to humans, particularly during sensitive windows of early development that can coincide with higher internal doses, due to the transfer ability of BPMC through the placenta and human dietary system (Tam et al., 2018). Besides this, Futagawa found that the primary cause of death in rabbits with BPMC at low doses is cardiovascular collapse mediated through direct inhibitory effects on cardiac and vascular smooth muscle contractions (Futagawa et al., 2000).

Zebrafish have been proposed as an alternative and predictive vertebrate animal model for in vivo assessments of toxicity and efficacy of compounds (McGrath and Li, 2008; Zhu et al., 2014), particularly in the cardiovascular system (Duan et al., 2013; McGrath and Li, 2008). Development of the two-chambered zebrafish heart is rapid: contractions begin by 26 h post fertilization (hpf); looping occurs by 48 hpf; and full vascular tree development by 72 hpf (Parker et al., 2014). Vascular development begins before 24 hpf with the migration of angioblasts, initiating formation of the two major axial vessels, the dorsal artery and posterior cardinal vein, which are fully formed and able to carry blood by 30 hpf (Parker et al., 2014). A number of studies have employed the zebrafish to investigate the toxic effects of chemicals released into the environment on the development of cardiovascular systems, including nanoparticles, pesticides and various organic pollutants (Duan et al., 2013; Liu et al., 2017; Zhu et al., 2014; Hu et al., 2017; Orfanidou et al., 2013; Huang et al., 2018; Kim et al., 2014).

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The present study was aimed to evaluate potential cardiovascular toxicity in zebrafish exposed to BPMC. We found that BPMC induced severe heart failure, with concentration-dependent negative effects on heart beats, heart contractions, cardiac output and blood flow dynamics, and myocardial cells. Our results also demonstrated that BPMC induced cerebral hemorrhages and blood erythrocyte reduction in a dose-dependent manner. Increased ROS production and capase 9 and 3/7 activation were also observed in the treated zebrafish. The mRNA levels of the ATPase-related gene (*atp2a1l*), calcium channel-related gene (*cacna1ab*), sodium channel-related gene (*scn5Lab*), potassium channel-related gene (*kcnq1*), regulatory gene (*tnnc1a)* for cardiac troponin C, and several apoptosis-related genes were significantly downregulated in the zebrafish following BPMC exposure.

2. Materials and methods

2.1. Zebrafish care and maintenance

Two lines of zebrafish were used in this study: wild-type AB line zebrafish and Tg(MPO::GFP) transgenic zebrafish. The zebrafish were housed in a temperature-controlled and light-controlled aquaculture facility with a standard 14: 10 h light/dark photoperiod. They were fed with live brine shrimp twice daily and dry flakes once a day. Four to five pairs of zebrafish were set up for natural mating every day. On average, 200–300 embryos were generated. 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 $mS.cm^{-1}$ 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 zebrafish facility 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).

2.2. Chemicals

Fenobucarb (BPMC) (99% purity) prepared in a 50% concentration dissolved in emulsifiable oil was bought from Jiangxi lvchuan biotechnology industrial Co., Ltd. Stock solutions were prepared in ultrapure water and stored in the dark at 4 °C. 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).

2.3. BPMC treatment

Cardiac phenotypes can appear as early as the 48 hpf embryonic stage. These changes include the distortion of the heart's shape, a decrease of the heart's size, and a gradual decrease of heart rate (Zhu et al., 2018). Because of this, we chose zebrafish in the 48 hpf stage as appropriate test subjects for BPMC treatment. Thirty 48 hpf 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 and 200 μg/mL (corresponding to 30, 60, 121, 241, 482 and 965 μM, respectively) from 48 hpf to 72 hpf. The highest tested concentration (200 μg/mL) used in this investigation was the maximum non-lethal concentration (MNLC) of BPMC in the zebrafish we identified in the pilot study.

2.4. Heart failure assessment

After BPMC treatment, ten zebrafish from each group were randomly selected for visual observation. Images were acquired of the heart beating of the zebrafish at the diastolic stage under a dissecting stereomicroscope without using anesthetics (Olympus, Japan). Quantitative image analyses of the area measurements of heart dilatation and venous congestion were performed using image-based morphometric analysis.

The resting zebrafish were subject to recorded videos under a Zebralab Blood Flow System (Viewpoint, France). To determine differential atrial beat rates (ABR) and ventricular beat rates (VBR), heart videos were analyzed using MicroZebraLab™ (v3.5, ViewPoint, Lyon, France). This software detects changes in pixel density associated with cardiac muscle contraction and chamber filling, and registers this as contractions of the cardiac muscle in beats per minute (bpm) (Parker et al., 2014). Compared with the heart beat rates in the control group, bradycardia or tachycardia or arrhythmia induced by BPMC could be assessed (Zhu et al., 2014).

Blood flow videos were analyzed using ZebraBlood™ (v1.3.2, ViewPoint, Lyon, France), which also works by detecting changes in pixel density and combining them with vessel diameter to generate a flow rate in nl/s for every frame (Parker et al., 2014). Quantitative assessments were performed using video-based analysis and cardiac output and blood flow dynamics were evaluated (Zhu et al., 2018).

Heart failure was induced by BPMC in zebrafish based on the qualitative and quantitative results of the ABR and VBR, the area measurements of heart dilatation and venous congestion, cardiac output, and blood flow dynamics.

2.5. Cardiac apoptosis assay

After BPMC treatment, zebrafish were stained with 2.5 μg/mL acridine orange for 30 min in the dark at 28 °C and then washed 3 times with fish water. Ten zebrafish from each group were observed and photographed for apoptotic cells that would display yellow-green fluorescent spots in the heart 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 from apoptotic cells in the heart was measured and the apoptotic rate was calculated as reported by us (Li et al., 2006).

2.6. Blood erythrocyte measurement

To measure the reduction of blood erythrocyte induced by BPMC, zebrafish were stained with o-dianisidine using a method reported by us previously (Zhu et al., 2016a) to quantify the erythrocyte in the heart area of zebrafish. Our previous patented technology suggested that zebrafish erythrocyte could be stained with o-dianisidine (China patent number: 201110126427.2, Zhu et al., 2016a). After BPMC treatment, zebrafsh from each group were incubated in 0.6 mg/mL o-dianisidine staining working solution (Sigma, USA) with 10 mM sodium acetate and 4% ethanol for 15 min in the dark at 28 °C and then washed 3 times with DMSO. After that, zebrafish with erythrocyte positive staining in cardiac section were observed under a stereomicroscope (Nikon, SMZ645, Tokyo, Japan). To quantify erythrocyte, the Image-pro Plus software (Media Cybernetics, Bethesda, MD) was utilized to calculate the average integrated optical density (IOD) for erythrocyte positive staining.

2.7. Cerebral hemorrhage assessment

After BPMC treatment, 30 zebrafish from each group were observed and photographed under a stereomicroscope (Nikon, SMZ645, Tokyo, Japan) to count the number of those with cerebral hemorrhaging (N) and to calculate the cerebral hemorrhaging incidence.

2.8. Reactive oxygen species (ROS) assay

The ROS levels in zebrafish treated with BPMC were analyzed using an oxidation sensitive probe, 5-(and 6-)-chloromethyl-20, 70-dichlorodihydrofluoresceindiacetate (CM-H2DCFDA, Life Technologies,

Carlsbad, CA). The treated zebrafish were incubated with 0.5 mg/mL CM-H2DCFDA for 1 h in dark at 28 °C. 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 multi-mode microplate reader (Duan et al., 2015).

2.9. Caspase activity measurement

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 to caspase 3/7 and caspase 9 using a multifunction microplate reader. 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.

2.10. Gene expression analysis

To further investigate the possible heart failure mechanism induced by BPMC, the mRNA levels of the genes related to apoptosis *bcl-2* and *bax,* the regulatory gene (*tnnc1a)* for cardiac troponin C, calcium channel-related gene (*cacna1ab*), ATPase-related gene (*atp2a1l*), sodium channel-related gene (*scn5Lab*), and potassium channel-related gene (*kcnq1*) were determined by qPCR and the primers used in this study were listed in Table 1.

After BPMC treatment, total RNA was extracted from fifty homogenized zebrafish per group using Trizol reagent (Invitrogen Life Technologies). The quality of RNA samples was evaluated using the methods from NanoDrop 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 °C, 40 cycles of 5 s each at 95 °C, and 30 s at 60 °C. 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 calculated using the 2-△△Ct method (Sharif et al., 2016).

2.11. Statistical analysis

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 analyses, all data was presented as mean ± SE, and results were statistically compared between drug-treated and control zebrafish groups. All experiments were repeated at least 3 times.

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

Gene	Forward (5'-3')	Reverse (5'-3')
β-actin	tcgagcaggagatgggaacc	ctcgtggataccgcaagattc
bax	gacttgggagctgcacttct	tccgatctgctgcaaacact
bcl-2	cactggatgactgactacctgaa	cctgcgagtcctcattctgtat
tnnc1a	ggcagagcaactcaccgat	gtagggttctggccaacat
cacna1ab	ggaatcggcagatgagggag	attgccgacagagccgtaat
atp2a1l	ccgacaaaactggcaccttg	ctgcagtcaactttggcacc
scn5Lab	ttcaacatcggtctgctgct	tcgtgcattagtgccagtgt
kcnq1	catcatcacaaccagcgacg	tctggacctgatccccatgt

3. Results

3.1. BPMC induced heart failure

3.1.1. Bradycardia

Zebrafish atrial and ventricular beat rates were recorded at 4 h after BPMC treatment and analyzed for statistical significance. BPMC exposure resulted in a concentration-dependent reduction in both ABR and VBR, with significantly lower rates detected as the mean across all time points. The heart rate was (162 ± 2.2)/min in control zebrafish, and in the zebrafish treated with BPMC at concentrations of 25, 50, 100 and 200 μg/mL it was (141 ± 1.1)/min, (116 ± 1.1)/min, (99 ± 2.6)/min, (64 ± 2.5)/min, respectively, indicating that BPMC treatment can result in bradycardia in a dose-dependent manner, but has no effect on heart rhythm, as ABR was the same as VBR in all BPMC treatment groups. The bradycardia incidences in zebrafish treated with BPMC at concentrations of 6.25, 12.5, 25, 50, 100 and 200 μg/mL were 0%, 0%, 13%, 29%, 39% and 60%, respectively. Statistically significant bradycardia was observed at 25 μg/mL and above concentrations ($p < 0.001$).

3.1.2. Heart dilatation and venous congestion

Heart dilatation and venous congestion were acquired at the diastolic stage of zebrafish heart beating without using anesthetics under a dissecting stereomicroscope (Fig. 1A). At 72 hpf, BPMC treated zebrafish showed a dose-dependent increase of heart size and venous congestion as compared with the control group. The relative sizes of hearts in zebrafish treated with BPMC at concentrations of 6.25, 12.5, 25, 50, 100, 200 μg/mL were 101%, 103%, 105%, 114%, 209%, 221%, respectively (Fig. 1B). The relative areas of venous congestion in zebrafish treated with BPMC at concentrations of 6.25, 12.5, 25, 50, 100 and 200 μg/mL were 104%, 111%, 121%, 143%, 268% and 435%, respectively (Fig. 1C). Statistically significant heart dilatation and venous congestion were observed at 100 μg/mL ($p < 0.001$) and 200 μg/mL ($p < 0.001$).

3.1.3. Cardiac output and blood flow dynamics reduction

The cardiac output and blood flow dynamics were detected based on analyses of the motion of erythrocytes within a tracking area by the ZebraBlood™ software (Fig. 2A–D). BPMC treatment resulted in a concentration-dependant decrease both in cardiac output and blood flow dynamics, and within a few days, circulation ceased and the animals died (not shown). The relative cardiac outputs were 97%, 92%, 87%, 84%, 54% and 31% in zebrafish treated with BPMC at concentrations of 6.25, 12.5, 25, 50, 100 and 200 μg/mL, respectively (Fig. 2E). The relative blood flow dynamics were 97%, 97%, 93%, 92%, 62% and 41% in zebrafish treated with BPMC at concentrations of 6.25, 12.5, 25, 50, 100 and 200 μg/mL, respectively (Fig. 2F). Statistically significant cardiac output and blood flow dynamics reduction were observed at 100 μg/mL ($p < 0.001$) and 200 μg/mL ($p < 0.001$).

3.1.4. BPMC- induced cardiac apoptosis

As shown in Fig. 3A, BPMC-treated cardiomyocytes showed apoptotic changes which appeared around the heart area. This might then reduce ventricular contraction. There were no obvious apoptotic cells indicated in the control zebrafish, but considerable numbers of apoptotic cells were observed in BPMC-treated groups at higher concentrations in a dose-dependent manner. The induction percentages of apoptosis were 1%, 1%, 7%, 36%, 48% and 51% for BPMC at 6.25, 12.5, 25, 50, 100 and 200 μg/mL, respectively. Statistically significant induced apoptosis was observed in zebrafish treated with BPMC at concentrations of 50 μg/mL ($p < 0.001$), 100 μg/mL ($p < 0.001$) and 200 μg/mL ($p < 0.001$) (Fig. 3C).

3.1.5. BPMC- induced blood erythrocyte reduction

As shown in Fig. 3B, at the lower concentration of BPMC (12.5 μg/

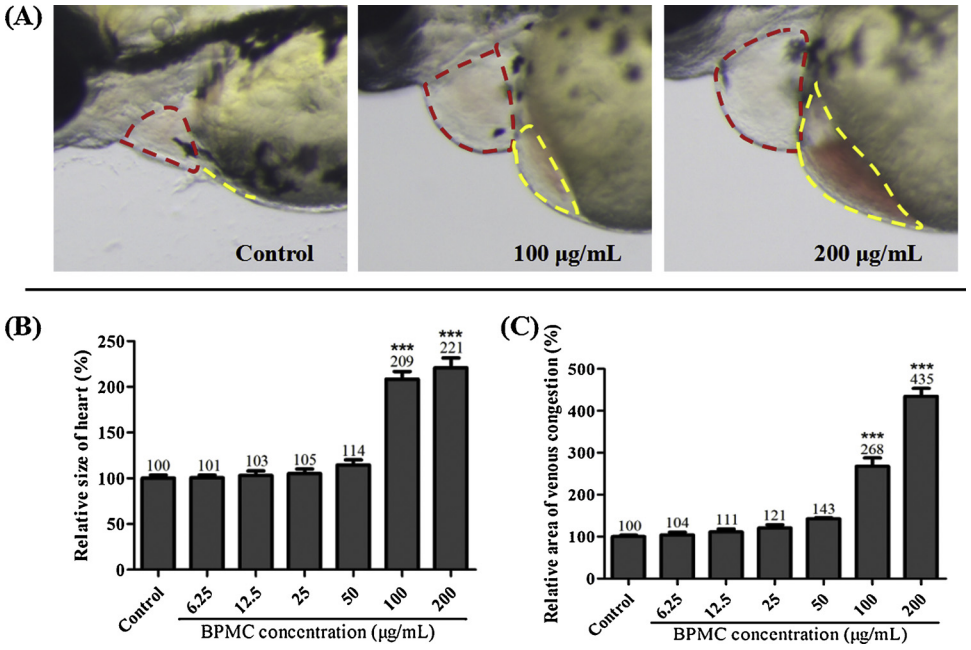

Fig. 1. Heart dilatation and venous congestion induced by BPMC in zebrafish.Heart dilatation and venous congestion images acquired at the diastolic stage of zebrafish heart beating without using an anesthetics under a dissecting stereomicroscope (A); relative size of heart in zebrafish treated with BPMC (B) ; and relative area of venous congestion in zebrafish treated with BPMC (C).

mL), the relative number of erythrocytes was decreased significantly, 85.23% lower than the control. The relative number of erythrocytes was 94%, 94%, 90%, 89%, 59% and 43% for BPMC at 6.25, 12.5, 25, 50, 100 and 200 μg/mL, respectively. Statistically significant reduction of erythrocytes was observed in zebrafish treated with BPMC at concentrations of 100 μg/mL ($p < 0.001$) and 200 μg/mL ($p < 0.001$) (Fig. 3D).

3.1.6. BPMC- induced cerebral hemorrhage

As shown in Fig. 4A, BPMC clearly induced cerebral hemorrhaging in zebrafish, with bleeding into the surrounding brain, blood accumulation through leakage in the cranial region, and erythrocyte accumulation in the cerebral hemorrhage region of the zebrafish head. The incidences of cerebral hemorrhaging were 0%, 0%, 0%, 0%, 13.3% and 20% for BPMC at 6.25, 12.5, 25, 50, 100 and 200 μg/mL, respectively (Fig. 4B). Statistically significant incidence of cerebral hemorrhaging was observed in zebrafish treated with BPMC at concentrations of 200 μg/mL ($p < 0.05$).

3.1.7. BPMC induced ROS production

BPMC treatment resulted in increased ROS production. ROS levels relative to control zebrafiash were 103%, 108%, 111%, 155%, 163% and 340%, respectively, in zebrafish treated with BPMC at concentrations of 6.25, 12.5, 25, 50, 100 and 200 μg/mL. Statistically significant differences were found in zebrafish treated with BPMC at 50 μg/mL ($p < 0.05$), 100 μg/mL ($p < 0.001$) and 200 μg/mL ($p < 0.001$) (Fig. 4C).

3.1.8. 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 102%, 105%, 108%, 123%, 125% and 131%; 104%, 108%, 110%, 145%, 157% and 175%, respectively. Statistically significant elevations were found in zebrafish treated with BPMC at 50 μg/mL ($p < 0.001$), 100 μg/mL ($p < 0.001$) and 200 μg/mL ($p < 0.001$) in caspase-3/7 and -9 activities (Fig. 4D, E).

3.1.9. Gene expression

As shown in Fig. 5, 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-, 1.9-, 2.1- and 2.3-fold increases relative to the control group. As for the expression of *bcl-2*, only 200 μg/mL BPMC showed significant alteration ($p < 0.05$). Thus, the *bcl-2/bax* ratio decreased after BPMC exposure at concentrations of 50, 100 and 200 μg/mL ($p < 0.001$).

The relative quantitative expression of *tnnc1a*, *cacna1ab*, *atp2a1l*, *scn5Lab* and *kcnq1* were 0.4–1.0, 0.5–1.0, 0.2-0.9, 0.4-0.9 and 0.4–1.0, respectively. A statistically significant downregulation of 5 examined genes were observed in the zebrafish treated with BPMC ($p < 0.05$ or 0.01 or 0.001).

4. Discussion

Fenobucarb (BPMC) is a WHO (World Health Organization) moderately hazardous agricultural insecticide derived from carbamic acid (NH2COOH) (Lamb et al., 2016). Carbamate pesticide sevin inhibits competitively cholinesterase (AChE) (Zheng et al., 2017; Wood and Goulson, 2017; Yang et al., 2017) and causes muscle spasms and paralysis for insects, mammals, and fish at a sublethal concentration of 1.05 mg/L (Sastry and Siddiqui, 1982; Fahmy et al., 1970). This poses potential risks to wild animals and human health (Kim et al., 2014). In the present study, we found that BPMC could induce severe heart failure, with reduced heart rate, heart contraction, cardiac output and blood flow dynamics, and increased venous congestion and myocardial cell apoptosis. BPMC could induce cerebral hemorrhaging, reduced blood erythrocytes, elevated ROS production and up- and down-stream caspase activation, generally in a dose-dependent manner. The mRNA levels of the ATPase-related gene (*atp2a1l*), calcium channel-related gene (*cacna1ab*), sodium channel-related gene (*scn5Lab*), potassium channel-related gene (*kcnq1*), and regulatory gene (*tnnc1a)* for cardiac troponin C were significantly downregulated following BPMC exposure. To the best of our knowledge, this was the first report to provide solid evidence for BPMC-induced heart failure and cerebral hemorrhaging as well as the possible cellular and molecular toxic mechanisms in a whole animal model. These results provide valuable information for the heart and brain health risk of BPMC and could facilitate environmental and ecological risk assessment of BPMC pollution.

Heart failure is characterized by the gradual deterioration of cardiac function, culminating in erratic heart rhythm, edema, and death. Approximately 50% of people diagnosed with heart failure will die within 5 years (Wang et al., 2015a). Weakening heart function often causes congestion, or fluid buildup in the lungs and other tissues, by

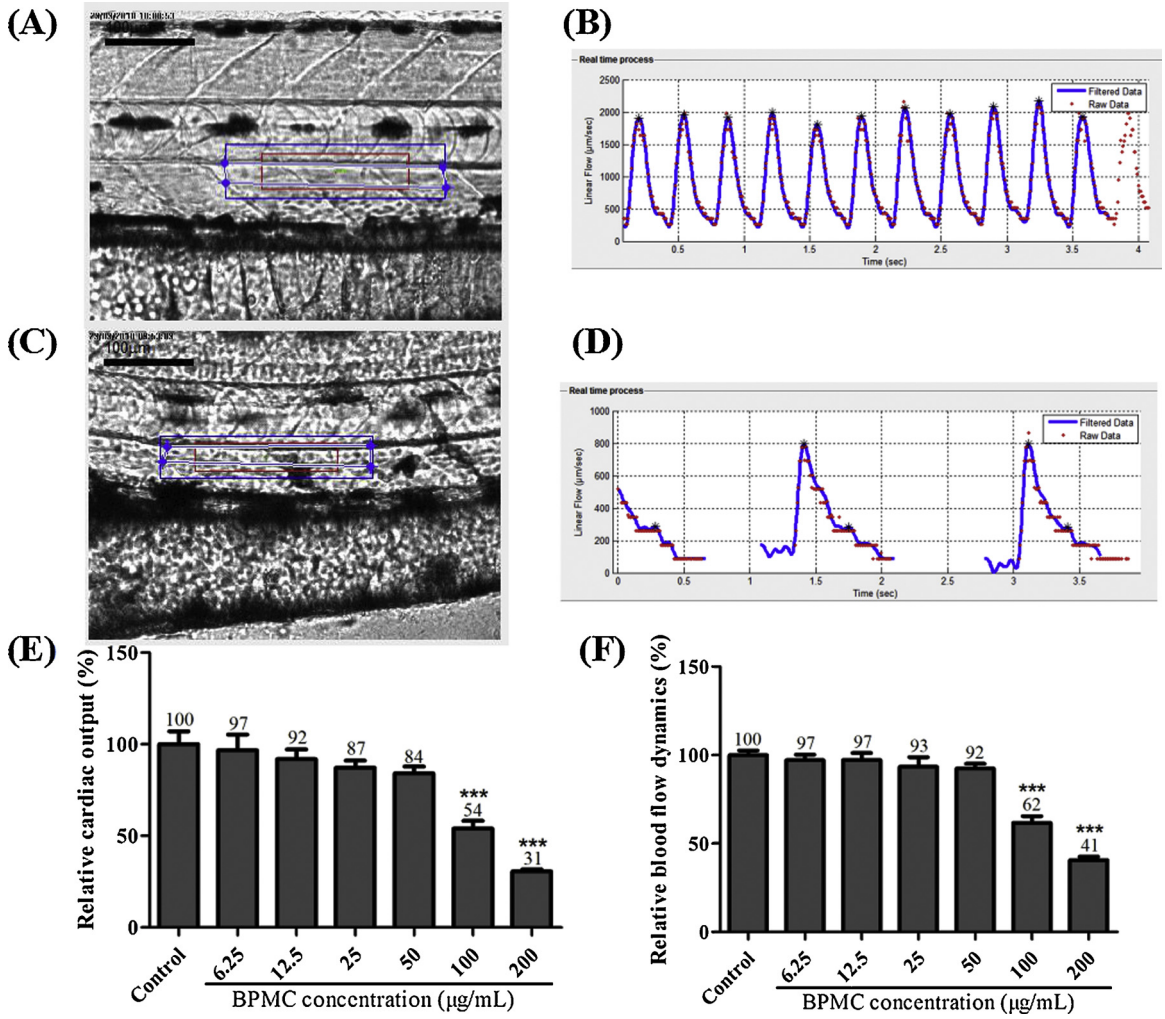

Fig. 2. Reduced cardiac output and blood flow dynamics in zebrafish treated with BPMC. Cardiac output and blood flow dynamics of control group detected based on analysis the motion of erythrocytes within tracking area by the ZebraBlood™ software (A); cardiac output and blood flow dynamics map in control zebrafish (B); Cardiac output and blood flow dynamics of zebrafish treated with 200 μg/mL BPMC detected based on analysis the motion of erythrocytes within tracking area by the ZebraBlood™ software (C); cardiac output and blood flow dynamics map in zebrafish treated with 200 μg/mL BPMC (D); relative cardiac output in zebrafish treated with BPMC (E); and relative blood flow dynamics in zebrafish treated with BPMC (F).

hindering the flow of blood through the chambers of the heart (Wang et al., 2015b). In the current study, we found that BPMC treatment at 100 and 200 μg/mL induced severely bradycardia, heart enlargement and venous congestion in zebrafish, while didn't show any uncoupled atrial-ventricular contraction, demonstrating no substantial blockage of atrioventricular electric conduction. In an earlier 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).

Blood circulation occurs early in the linear heart tube stage when diffused oxygen is still sufficient to support various physiological processes, suggesting that blood circulation is required for heart morphogenesis (Wu et al., 2011). By simple definition, heart failure is the progressive decrease in cardiac output (Wang et al., 2015a). Statistically significant reductions in cardiac output and blood flow dynamics were observed in zebrafish treated with BPMC. We observed circulation with only a few or no red blood cells in zebrafish treated with BPMC at concentration of 200 μg/mL. As erythrocytes influence blood viscosity, which is one of the main physical factors affecting blood fluidic shear stress on the blood vessel wall (Hierck et al., 2008; Papaioannou and Stefanadis, 2005), our relative wall shear stress approach also indicated a significant decrease in the wall shear stress in BPMC-treated embryos due to relatively few erythrocytes in the circulation. A decreased wall shear stress produced decreased hemodynamic forces, which further impacted heart development and function in BPMC-treated zebrafish.

Intracranial hemorrhage is a debilitating form of stroke, which accounts for 10–15% of all strokes and is strongly associated with mortality and morbidity worldwide (Yang et al., 2017). Hemorrhagic stroke occurs when a weakened vessel ruptures and bleeds into the surrounding brain, leading to high rates of death and disability worldwide. It is the second most common form of stroke, and no therapies have proven effective to preventing hemorrhagic stroke. Nonetheless, the entire suite of molecular and physiological events in the brain during cerebral hemorrhaging is still unexplored. Our results demonstrated that BPMC could induce cerebral hemorrhaging in a dose-dependent manner, which means that it would have potential to cause strokes in animals and humans.

Oxidative stress has become an important subject in and environmental and aquatic toxicology (Livingstone, 2003). Exposure to chemical pollutants may interfere with the balance between endogenous and exogenous ROS. BPMC treatment resulted in the formation of ROS such as superoxide (Wu et al., 2011). ROS can cause the release of cytochrome C from mitochondria to activate caspase, resulting in phosphatidylserine exposure, DNA fragmentation, and cell

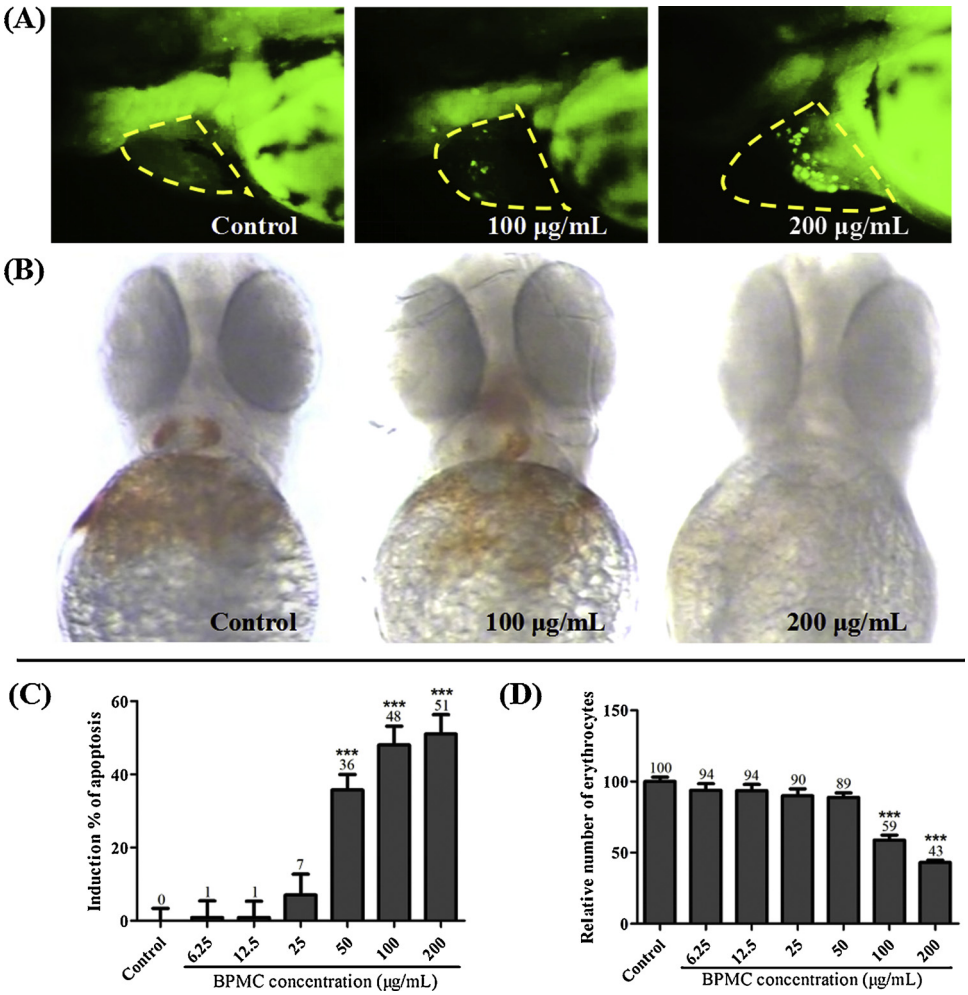

Fig. 3. BPMC-induced cardiac apoptosis and erythrocyte reduction in zebrafish. There were no obvious apoptotic cells indicated in the control zebrafish, while considerable numbers of apoptotic cells appeared around the heart area in BPMC-treated zebrafish at 100 and 200 μg/mL (A); o-dianisidine staining images of erythrocytes in cardiac region in the control and BPMC-treated zebrafish at 100 and 200 μg/mL (B); dose-dependent apoptosis induction in zebrafish treated with BPMC (C); relative number of erythrocytes in zebrafish treated with BPMC (D).

morphological changes (Hampton and Orrenius, 1998). In this study, cardiac apoptosis with increased ROS generation were detected, indicating that oxidative stress and apoptosis may play important roles in the pathogenesis of BPMC-induced heart failure and cerebral hemorrhage. The ratio of *bcl-2/bax* was decreased upon treatment with BPMC, suggesting that BPMC might change the ratio between the *bcl-2* and *bax*, likely leading to an induction of mitochondrial cytochrome c release and then activation of apoptosis (Hildeman et al., 2003; Zhu et al., 2015). Caspase-3 plays an essential role in apoptosis, mainly by catalyzing the specific cleavage of many key cellular proteins (Liu et al., 2007; Zeng et al., 2014). It is well established that pathways to caspase-3 activation either depend on or are independent of mitochondrial cytochrome c release and caspase-9 function (Porter and Janicke, 1999). Here both caspase-3 and caspase-9 were activated, implying that caspase-3 may play a pivotal role in BPMC induced apoptosis via caspase-9 pathway in zebrafish.

In this study, using qPCR we measured and quantified 7 genes in zebrafish exposed to BPMC, including the apoptosis-related genes *bcl-2* and *bax,* regulatory gene *tnnc1a* for cardiac troponin C, calcium channel-related gene (*cacna1ab*), ATPase-related gene (*atp2a1l*), sodium channel-related gene (*scn5Lab*), and potassium channel-related gene (*kcnq1*). The *tnnc1a* is a regulatory gene for cardiac troponin C; the *cacna1ab* is controlling gene for voltage-dependent calcium channel; the *atp2a1l* regulates ATPase and Ca^{2+} transportation (Duan et al., 2015); *scn5Lab* is required for zebrafish cardiogenesis at distinct phases of differentiation and proliferation. Zebrafish deficient in *scn5Lab* develop visibly smaller, un-looped hearts, and have slower heart rates and pericardial edema (Bennett et al., 2013); *kcnqx* genes encode slowly activating-inactivating K^+ channels, are linked to physiological signal transduction pathways, and *kcnq1* expression was highest in the heart and is associated with cardiac disorders such as long QT syndrome (Wu et al., 2014). We found that the expression of all these marker genes for heart function or development was significantly reduced following BPMC exposure. These new findings suggest that exposure to BPMC is a possible risk factor to cardiovascular and cerebrovascular system and further investigations in other animal models and in human beings are needed to confirm our discoveries.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

Ping Li, Hua Yang and Chun-Qi Li designed the research; Xiao-Yu Zhu, Bo Xia, and Yu-Ying Wu 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. This work was sponsored in part by the National Science & Technology Major Projects of China (No. 2017ZX09301-059) and the National Natural Science Foundation of China (No.

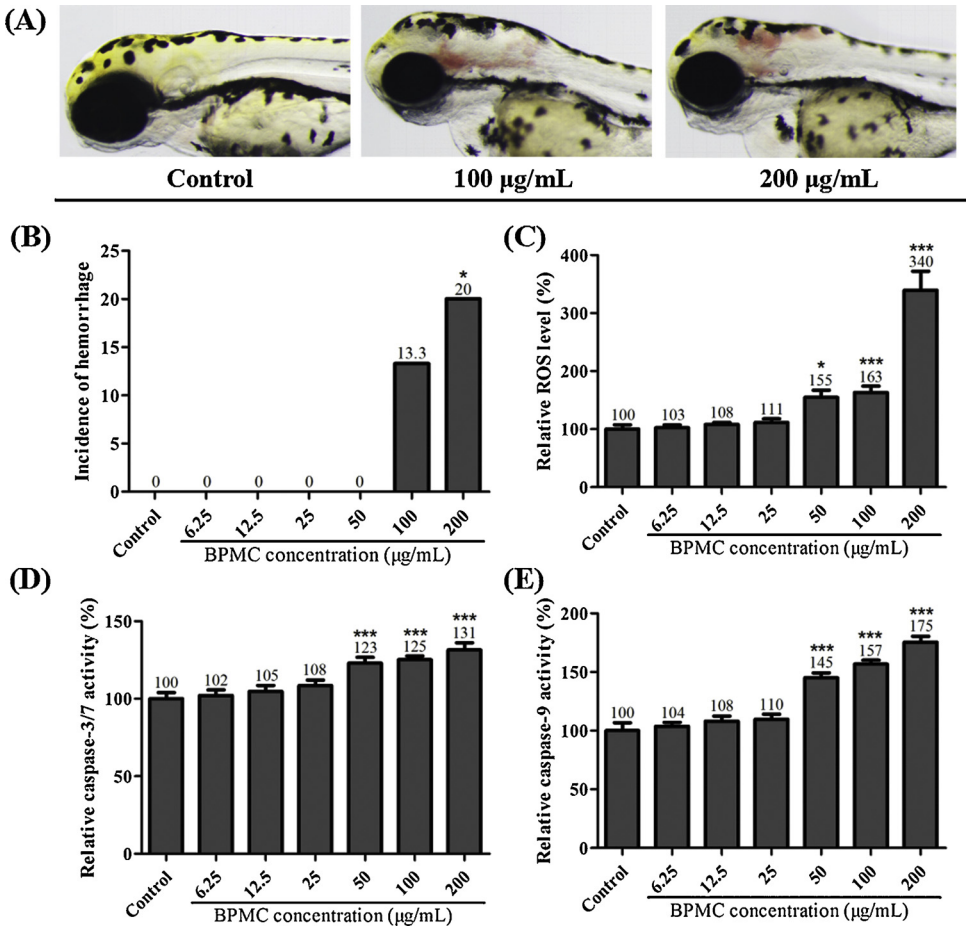

Fig. 4. Cerebral hemorrhage, elevated ROS production and caspase 3/7 and 9 acivaton in BPMC-treated zebrafish. BPMC at 100 and 200 μg/mL clearly induced cerebral hemorrhage in zebrafish, with bleeds into the surrounding brain and blood accumulated through leakage in the cranial region and erythrocyte accumulation in the cerebral hemorrhage region of the zebrafish head (A); cerebral hemorrhage percentage in zebrafish treated with BPMC (B) ; ROS levels relative to control group in zebrafish treated with BPMC (C); and relative caspase-3/7 and -9 activities in zebrafish treated with BPMC (D and E).

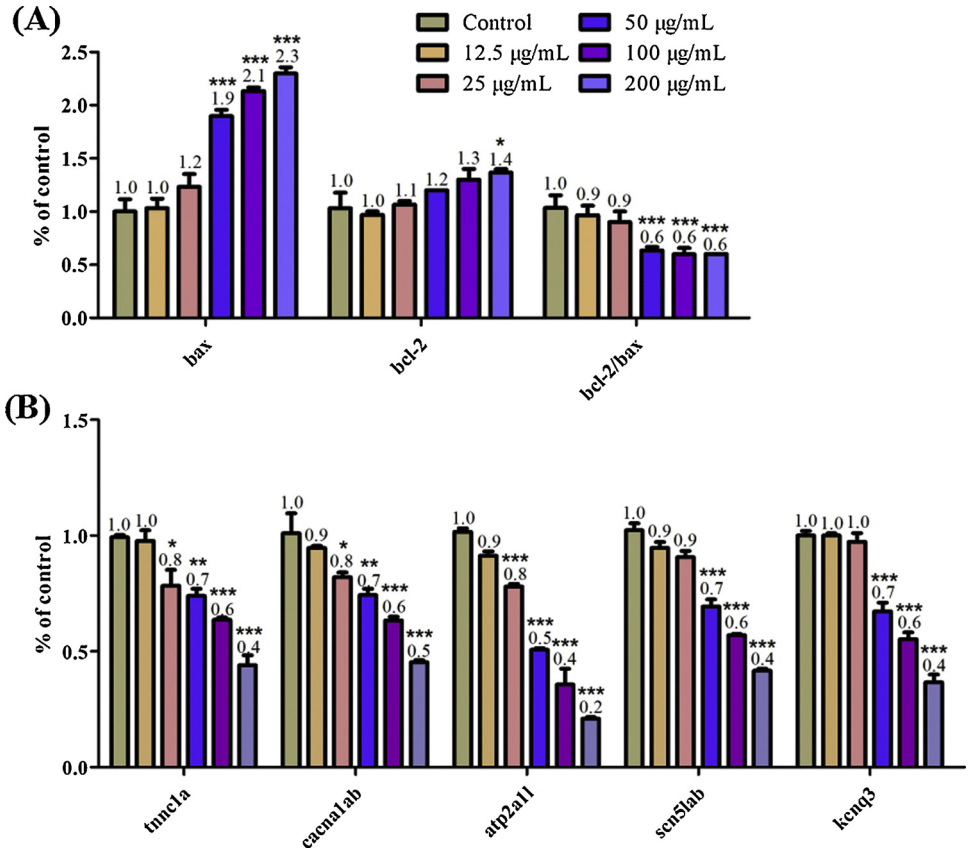

Fig. 5. Relative gene expression levels in zebrafish after exposure to various concentrations of BPMC for 24 h. Relative gene expression levels of *Bcl-2*, *Bax* and *Bcl-2/Bax* ratio in zebrafish after exposure to various concentrations of BPMC (A); Relative gene expression levels of *Bcl-2*, *Bax*, *Bcl-2/Bax* ratio, *tnnc1a*, *cacna1ab*, *atp2a1l*, *scn5Lab* and *kcnq1* in zebrafish after exposure to various concentrations of BPMC (B). Data are expressed as mean ± SE. Compared with control group: *p < 0.05, **p < 0.01, ***p < 0.001.

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References

Bennett, J.S., Stroud, D.M., Becker, J.R., Roden, D.M., 2013. Proliferation of embryonic cardiomyocytes in zebrafish requires the sodium channel scn5Lab. Genesis 51, 562–574.

Duan, J., Yu, Y., Li, Y., Sun, Z., 2013. Cardiovascular toxicity evaluation of silica nanoparticles in endothelial cells and zebrafish model. Biomaterials 34, 5853–5862.

Duan, J.C., Yu, Y., Li, Y., Li, Y.B., Liu, H.C., Jing, L., Yang, M., Wang, J., Li, C.Q., Sun, Z.W., 2015. Low-dose exposure of silica nanoparticles induces cardiac dysfunction via neutrophil-mediated inflammation and cardiac contraction in zebrafish embryos. Nanotoxicology 10, 575–585.

Fahmy, M.A., Fukuto, T.R., Myers, R.O., March, R.B., 1970. The selective toxicity of new N-phosphorothioyl-carbamate esters. J. Agric. Food Chem. 18, 793–796.

Futagawa, H., Kakinuma, Y., Takahashi, H., 2000. The role of cholinergic and non-cholinergic mechanisms in the cardiorespiratory failure produced by N-methylcarbamate cholinesterase inhibitors in rabbits. Toxicol. Appl. Pharmacol. 165, 27.

Hampton, M.B., Orrenius, S., 1998. Redox regulation of apoptotic cell death in the immune system. Toxicol. Lett. 102–103, 355–358.

Hierck, B.P., Van der Heiden, K., Poelma, C., Westerweel, J., Poelmann, R.E., 2008. Fluid shear stress and inner curvature remodeling of the embryonic heart. Choosing the right lane!. Sci. World J. 8, 212–222.

Hildeman, D.A., Mitchell, T., Kappler, J., Marrack, P., 2003. T cell apoptosis and reactive oxygen species. J. Clin. Invest. 111, 575–581.

Hu, Q., Guo, F., Zhao, F., Tang, G., Fu, Z., 2017. Cardiovascular toxicity assessment of poly (ethylene imine)- based cationic polymers on zebrafish model. J. Biomater. Sci. Polym. Ed. 28, 768–780.

Huang, M., Jiao, J., Wang, J., Xia, Z., Zhang, Y., 2018. Characterization of acrylamide-induced oxidative stress and cardiovascular toxicity in zebrafish embryos. J. Hazard. Mater. 347, 451–460.

Kim, I., Kim, D.U., Kim, N.H., Ka, J.O., 2014. Isolation and characterization of fenobucarb-degrading bacteria from rice paddy soils. Biodegradation 25, 383–394.

Kobayashi, H., Yuyama, A., Kajita, T., Shimura, K., Ohkawa, T., Satoh, K., 1985. Effects of insecticidal carbamates on brain acetylcholine content, acetylcholinesterase activity and behavior in mice. Toxicol. Lett. 29, 153–159.

Kubota, S., Shono, Y., Matsunaga, T., Tsunoda, K., 2007. Response of the subterranean termite Coptotermes formosanus (Isoptera: Rhinotermitidae) to soil treated with microencapsulated fenobucarb. Pest Manag. Sci. 63, 1224–1229.

Lamb, T., Selvarajah, L.R., Mohamed, F., Jayamanne, S., Gawarammana, I., Mostafa, A., Buckley, N.A., Roberts, M.S., Eddleston, M., 2016. High lethality and minimal variation after acute self-poisoning with carbamate insecticides in Sri Lanka-implications for global suicide prevention. Clin. Toxicol. 54, 624–631.

Li, C.Q., Pang, B., Kiziltepe, T., Trudel, L.J., Engelward, B.P., Dedon, P.C., Wogan, G.N., 2006. Threshold effects of nitric oxide-induced toxicity and cellular responses in wild-type and p53-null human lymphoblastoid cells. Chem. Res. Toxicol. 19, 399–406.

Liu, C., Yu, K., Shi, X., Wang, J., Lam, P.K., Wu, R.S., Zhou, B., 2007. Induction of oxidative stress and apoptosis by PFOS and PFOA in primary cultured hepatocytes of freshwater tilapia (Oreochromis niloticus). Aquat. Toxicol. 82, 135–143.

Liu, H.C., Chu, T.Y., Chen, L.L., Gui, W.J., Zhu, G.N., 2017. The cardiovascular toxicity of triadimefon in early life stage of zebrafish and potential implications to human health. Environ. Pollut. 231, 1093–1103.

Livingstone, D., 2003. Oxidative stress in aquatic organisms in relation to pollution and aquaculture. Rev. Elev. Med. Vet. Pays Trop. 154, 427–430.

McGrath, P., Li, C.Q., 2008. Zebrafish: a predictive model for assessing drug-induced toxicity. Drug Discov. Today 13, 394–401.

Miyaoka, T., Takahashi, H., Tsuda, S., Shirasu, Y., 1984. Potentiation of acute toxicity of 2- sec -butylphenyl N -methylcarbamate (BPMC) by fenthion in mice. Fundam. Appl. Toxicol. 4, 802–807.

Orfanidou, T., Papaefthimiou, C., Antonopoulou, E., Leonardos, I., Theophilidis, G., 2013. The force of the spontaneously contracting zebrafish heart, in the assessment of cardiovascular toxicity: application on adriamycin. Toxicol. Vitr. 27, 1440–1444.

Papaioannou, T.G., Stefanadis, C., 2005. Vascular wall shear stress: basic principles and methods. Hellenic J. Cardiol. 46, 9–15.

Parker, T., Libourel, P.A., Hetheridge, M.J., Cumming, R.I., Sutcliffe, T.P., Goonesinghe, A.C., Ball, J.S., Owen, S.F., Chomis, Y., Winter, M.J., 2014. A multi -endpoint in vivo larval zebrafish (Danio rerio) model for the assessment of integrated cardiovascular function. J. Pharmacol. Toxicol. Methods 69, 30–38.

Phong, T.K., Vu, S.H., Ishihara, S., Hiramatsu, K., Watanabe, H., 2011. Exposure risk assessment and evaluation of the best management practice for controlling pesticide runoff from paddy fields. Part 2: model simulation for the herbicide pretilachlor. Pest Manag. Sci. 67, 70–76.

Porter, A.G., Janicke, R.U., 1999. Emerging roles of caspase-3 in apoptosis. Cell Death Differ. 6, 99–104.

Sastry, K.V., Siddiqui, A.A., 1982. Chronic toxic effects of the carbamate pesticide sevin on carbohydrate metabolism in a freshwater snakehead fish, Channa punctatus. Toxicol. Lett. 14, 123–130.

Sharif, F., Steenbergen, P.J., Metz, J.R., Champagne, D.L., 2016. Long-lasting effects of dexamethasone on immune cells and wound healing in the zebrafish. Wound Repair Regen. 23, 855–865.

Takahashi, H., Tanaka, J., Tsuda, S., Shirasu, Y., 1987. Contribution of monoaminergic nervous system in potentiation of 2-sec-butylphenyl N-methylcarbamate (BPMC) toxicity by malathion in male mice. Fundam. Appl. Toxicol. 8, 415–422.

Tam, N.T., Berg, H., Cong, N.V., 2016. Evaluation of the joint toxicity of chlorpyrifos ethyl and BPMC on climbing perch (Anabas testudineus) from rice fields in the Mekong Delta, Vietnam. Environ. Sci. Pollut. Res. 25, 1–9.

Tam, N.T., Berg, H., Cong, N.V., 2018. Evaluation of the joint toxicity of chlorpyrifos ethyl and BPMC on climbing perch (Anabas testudineus) from rice fields in the Mekong Delta, Vietnam. Environ. Sci. Pollut. Res. Int. 25, 13226–13234.

Wang, Q., Lam, J.C.W., Man, Y.C., Lai, N.L.S., Kwok, K.Y., Guo, Y., Lam, P.K.S., Zhou, B., 2015a. Bioconcentration, metabolism and neurotoxicity of the organophorous flame retardant 1, 3-dichloro 2-propyl phosphate (TDCPP) to zebrafish. Aquat. Toxicol. 158, 108–115.

Wang, X.F., Yang, L.H., Wu, Y.Y., Huang, C.J., Wang, Q.W., Han, J., Guo, Y.Y., Shi, X.J., Zhou, B.S., 2015b. The developmental neurotoxicity of polybrominated diphenylethers: effect of De-71 on dopamine in zebrafish larvae. Environ. Toxicol. Chem. 34, 1119–1126.

Wood, T.J., Goulson, D., 2017. The environmental risks of neonicotinoid pesticides: a review of the evidence post 2013. Environ. Sci. Pollut. Res. 24 17285–173.

Wu, Y.T., Lin, C.Y., Tsai, M.Y., Chen, Y.H., Lu, Y.F., Huang, C.J., Cheng, C.M., Hwang, S.P., 2011. β-Lapachone induces heart morphogenetic and functional defects by promoting the death of erythrocytes and the endocardium in zebrafish embryos. J. Biomed. Sci. 18, 70.

Wu, C., Sharma, K., Laster, K., Hersi, M., Torres, C., Lukas, T.J., Moore, E.J., 2014. Kcnq1-5 (Kv7.1-5) potassium channel expression in the adult zebrafish. BMC Physiol. 14, 1–11.

Yang, R., Zhang, Y., Huang, D., Luo, X., Zhang, L., Zhu, X., Zhang, X., Liu, Z., Han, J.Y., Xiong, J.W., 2017. Miconazole protects blood vessels from MMP9-dependent rupture andhemorrhage. Dis. Model. Mech. 10, 337–348.

Zeng, C., Sun, H., Xie, P., Wang, J., Zhang, G., Chen, N., Yan, W., Li, G., 2014. The role of apoptosis in MCLR-induced developmental toxicity in zebrafish embryos. Aquat. Toxicol. 149, 25–32.

Zheng, W., Park, J.A., Zhang, D., Abd, E.A.M., Kim, S.K., Cho, S.H., Choi, J.M., Shim, J.H., Chang, B.J., Kim, J.S., Shin, H.C., 2017. Determination of fenobucarb residues in animal and aquatic food products using liquid chromatography-tandem mass spectrometry coupled with a QuEChERS extraction method. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 1058, 1–7.

Zhou, J., Xu, Y.Q., Guo, S.Y., Li, C.Q., 2015. Rapid analysis of hypolipidemic drugs in a live zebrafish assay. J. Pharmacol. Toxicol. Methods 72, 47–52.

Zhu, J.J., Xu, Y.Q., He, J.H., Yu, H.P., Huang, C.J., Gao, J.M., Dong, Q.X., Li, C.Q., 2014. Human cardiotoxic drugs delivered by soaking and microinjection induce cardiovascular toxicity in zebrafish. J. Appl. Toxicol. 34, 139–148.

Zhu, L., Mu, X., Wang, K., Chai, T., Yang, Y., Qiu, L., Wang, C., 2015. Cyhalofop-butyl has the potential to induce developmental toxicity, oxidative stress and apoptosis in early life stage of zebrafish (Danio rerio). Environ. Pollut. 203, 40–49.

Zhu, X.Y., Liu, H.C., Guo, S.Y., Xia, B., Song, R.S., Lao, Q.C., Xuan, Y.X., Li, C.Q., 2016a. A zebrafish thrombosis model for assessing antithrombotic drugs. Zebrafish 13, 335–344.

Zhu, X.Y., Xia, B., Liu, H.C., Xu, Y.Q., Huang, C.J., Gao, J.M., Dong, Q.X., Li, C.Q., 2016b. Closantel suppresses angiogenesis and cancer growth in zebrafish models. Assay Drug Dev. Technol. 14.

Zhu, X.Y., Wu, S.Q., Guo, S.Y., Yang, H., Xia, B., Li, P., Li, C.Q., 2018. A zebrafish heart failure model for assessing therapeutic agents. Zebrafish 15, 243–253.