

Research Article

Diazepam and Its Disinfection Byproduct Promote the Early Development of Nervous System in Zebrafish Embryos

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The widely used diazepam, as central nervous system inhibitor, has found to be ubiquitous in surface water and drinking water. Moreover, a series of byproducts such as 2-methylamino-5-chlorobenzophenone (MACB) were generated after the chlorine disinfection process. However, little information is available about the neurobiological effects of these emerging chemicals at low doses, especially on infants and children. Here, we exposed zebrafish (*Danio rerio*) embryos to diazepam and MACB at 0.05, 0.5, and 5 nM, which were equivalent to environmental levels. Both diazepam and MACB increased the somite number and promoted nervous development of transgenic zebrafish [Tg (elavl3: EGFP) larvae] at 72 hours postfertilization (hpf). Both diazepam and MACB also disrupted the homeostasis of adenosine monophosphate, valine, methionine, and fumaric acid in zebrafish embryos at 12 hpf. Additionally, the locomotor behavior activity of zebrafish was significantly enhanced after 120-hour sustained exposure to diazepam or MACB. Moreover, the mRNA expression levels of *oct4*, *sox2*, and *nanog*, modulating the pluripotency and self-renewal, were upregulated by diazepam and MACB in zebrafish embryo. Altogether, diazepam and MACB stimulate developmental neurogenesis and may induce neuronal excitotoxicity at quite low doses. These results indicated that the chronic exposure to psychoactive drugs may pose a potential risk to the development of the nervous system in infancy.

1. Introduction

Drinking water pollution by pharmaceuticals and personal care products (PPCPs) poses global concerns since it exhibit pharmacological action potencies even at low concentrations [1–3]. Psychoactive drugs are one kind of PPCPs and ubiquitously found in the environment [4, 5]. Diazepam (DZP), as one of the mostly prescribed psychoactive drug, was routinely used to treat anxiety disorders [6] which troubled about 14% people worldwide [7]. With the widespread application or abuse [8], a large amount of DZP was released into environment by excretion and discard. Thus, DZP has been detected in wastewater, surface water, and drinking water and persists at levels as high as hundreds nanograms per liter [9–11].

As an antianxiety and sedative drug, DZP can inhibit different parts of the central nervous system (CNS) by enhancing the postsynaptic inhibition of gamma-aminobutyric acid type A (GABAA) [12]. However, there have been concerns

that the drug causes adverse effects on fetal development because of its fast oral absorption, high bioavailability, long half-life, and easy penetration of the blood-brain barrier and blood-fetal barrier [13]. The offspring of Wistar rats presented neurobehavioral toxicity after prenatal exposure to different doses of DZP, manifesting as early physiological development and delayed neurobehavioral development [14], although the malformation evidences for newborns when maternal exposed to diazepam are of contradictory [13]. The floppy infant syndrome of newborn was well documented after prenatal exposure to diazepam during the last trimester of pregnancy, the newborns characterized as hypotonia, hypothermia, lethargy, respiratory distress, suckling difficulties, lower self-regulation, and more CNS stress signs across the first postnatal month [15, 16]. A survey study in 2016 showed that prenatal exposure to benzodiazepine would cause severe damage to the hearing and visual cognition of newborns [17]. All above-mentioned evidences are

based on prescribed dose exposure to DZP. Few information is available on adverse effects of DZP at low-dose exposure. As the same family drug of DZP, oxazepam was found to alter behavior and the feeding rate of wild European perch (*Perca fluviatilis*) at concentrations encountered in effluent-influenced surface waters [1], while wild-caught zebrafish exposed to concentrations of the anxiolytic drug oxazepam as low as 0.57 $\mu\text{g/L}$ showed a reduction in the response to conspecific alarm pheromone [18]. The brain neurochemistry for wild-type zebrafish was also altered after 28 days of exposure to oxazepam at 0.57 $\mu\text{g/L}$ level, and the serotonin turnover (ratio 5-HIAA/5-HT) was reduced [19]. As an important source of drinking water, PPCPs can transfer to tap water since the treatment techniques are not specifically aimed to trace drugs. It is particularly worrying that chlorination of these drugs can produce a series of byproducts that were more serious toxic effects than their precursors [20–22]. 2-Methylamino-5-chlorobenzophenone (MACB) was identified as predominate chlorinated chemical of DZP after chlorination disinfection process [5, 23] and ubiquitously found in tap water of Beijing. Even so, there is little information on the developmental and ethological effects of the DZP and MACB at low-dose exposure for newborn and infant, although MACB was predicted to be more likely to accumulate in nervous system and toxic/mutagenic than the precursor drug base on quantitative structure-activity relationship [24].

Here, we utilized zebrafish as a neonatal model to determine the potential effects of DZP and MACB at environmental concentration. We show that DZP and MACB increased the somite number of zebrafish embryos after 10-hour exposure. Peripheral and central neurons were quantified in embryo of HuC-GFP line. The enhancement of neuronal proliferation was verified in DZP- and MACB-treated zebrafish larvae. Moreover, zebrafish larvae exhibited hyperactivity after sustained exposure to DZP and MACB. The transcription factors in the pluripotency regulatory network were also upregulated in the treated zebrafish embryo. These findings supported the notion that psychoactive drugs at environmental concentration posed a serious risk to the neurodevelopment of infancy.

2. Materials and Methods

2.1. Chemicals and Reagents. DZP with purity > 98% was purchased from the National Institutes for Food and Drug Control (Beijing, China). MACB with purity > 98% was synthesized in laboratory. Dimethyl sulfoxide (DMSO) was supplied by Sigma-Aldrich (purity > 99.7%, St. Louis, USA). The stock solution of DZP and MACB (10 mg/mL) was prepared in DMSO and stored at -20°C .

2.2. Zebrafish and Treatment. Wild-type (WT) and transgenic (TG) line CZ160 [Tg (elavl3: EGFP)] zebrafish were purchased from China Zebrafish Research Centre (Wuhan, China) at 3 months old and maintained on aquatic habitat recirculation systems (Pentair Aquatic Habitats, Apopka, FL). The neuronal Elav-like (nElavl or neuronal Hu) proteins are RNA-binding proteins, which are early neuronal markers in the early development and are crucial for the maintenance

of axonal homeostasis in mature neurons [25, 26]. In transgenic HuC-GFP [Tg (elavl3: EGFP)] line, GFP was expressed in all neurons [27]. HuC-GFP expression could be occurred in all neuronal process and is important marker in early development of brain and spinal cord [28]. Water was maintained at 28.5°C , with a pH of 7.5, and a conductivity of 500–780 μS . Zebrafish were held at a light cycle of 14:10 light-dark, fed with *Artemia nauplii* twice daily.

To fertilize embryos, adult zebrafish were separated with 2 males to 1 female in spawning boxes overnight. The division plate was removed at the next beginning of light cycle. Then, incubation ended in 30 minutes, and the embryos were collected and washed three times with embryo medium. Subsequently, embryos were selected for exposure experiments at 1 hour postfertilization (hpf) in 6 cm high borosilicate glass dish with DZP and MACB. Our study had indicated that the concentration of DZP or MACB was about 0.05 nM in drinking water [5]. Zebrafish embryos ($n = 45 \pm 5$) were exposed to MACB at 0.05 nM, 0.5 nM, and 5 nM with three replicates. The vehicle group is 0.01% DMSO. During the experiment periods, fifty percent of EM was renewed to keep optimum and equal concentrations of tested material. The embryos incubated in a thermostatic climate incubator at 28°C (Jiangnan Instrument Co., Ltd., Ningbo, China). At 12 hpf, 60 embryos were taken to detect adenosine monophosphate, valine, methionine, and fumaric acid. Embryos were extracted with acetonitrile and analyzed by LCMS-8045 (Shimadzu Corporation, Japan).

2.3. Developmental Assessment. Embryos were viewed under the stereomicroscope (SZM76, UOP, China), and photographs were taken in 1280×1024 image resolution for counting somites at 10, 12, and 16 hpf, respectively. The key components of the HuC promoter drive expression of green fluorescent protein (GFP) expressed specifically in neurons. The impact of DZP and MACB on neuronal development was assessed at low concentration using the TG embryos [Tg (elavl3: EGFP)]. Adult male (or female) WT and female (or male) TG were separated in spawning boxes overnight. The TG larvae were collected at 72 hpf and imaged using laser confocal fluorescence (TCS SP8, Leica, Germany) with the excitation wavelength at 425 nm and emission at 600 nm. The length of HuC-GFP was measured accurately.

2.4. Locomotor Behavior Assays. Embryo was exposed to embryo medium with different DZP or MACB concentrations at 1 hpf. Zebrafish (120 hpf) behavior was assessed using either a light/dark cycle. Briefly, at 104 hpf, zebrafish larvae were loaded into 96-well plates in 300 μL of embryo media and allowed to acclimate overnight at 28.5°C . At 120 hpf, zebrafish were placed into a box tracking system and allowed to acclimate for 10 min, following which they were exposed to alternating 10 min cycles of dark and light for an hour. The speed per minute of larva moved in the well was calculated for each individual larva ($n = 24$ larvae).

2.5. Total RNA Extraction and Quantitative Real-Time PCR (qRT-PCR). In order to investigate whether *oct4* expression was associated with high hatch rate before 50 hpf, RT-qPCR

TABLE 1: Primers used in real-time PCR analyses of mRNA expression.

Gene name	Sequence of the primers (5'-3')	
	Forward	Reverse
<i>rpl13</i>	TCTGTCCCATGCCAACCAT	TCAGACGCACAATCTTGAGAGCAG
<i>oct4</i>	TGTAGTGC GTGTATGGTTCTGC	GGTGGTGGACTCTGCTCGTAA
<i>sox2</i>	TGAACGCCCTTCATGGTGTGGTC	TGAATGGTCGCTTCTCGCTCTC
<i>Nanog</i>	AAGACTGAGCCCGACCAAAA	GTCAGAGGAACCCCTTCTCG

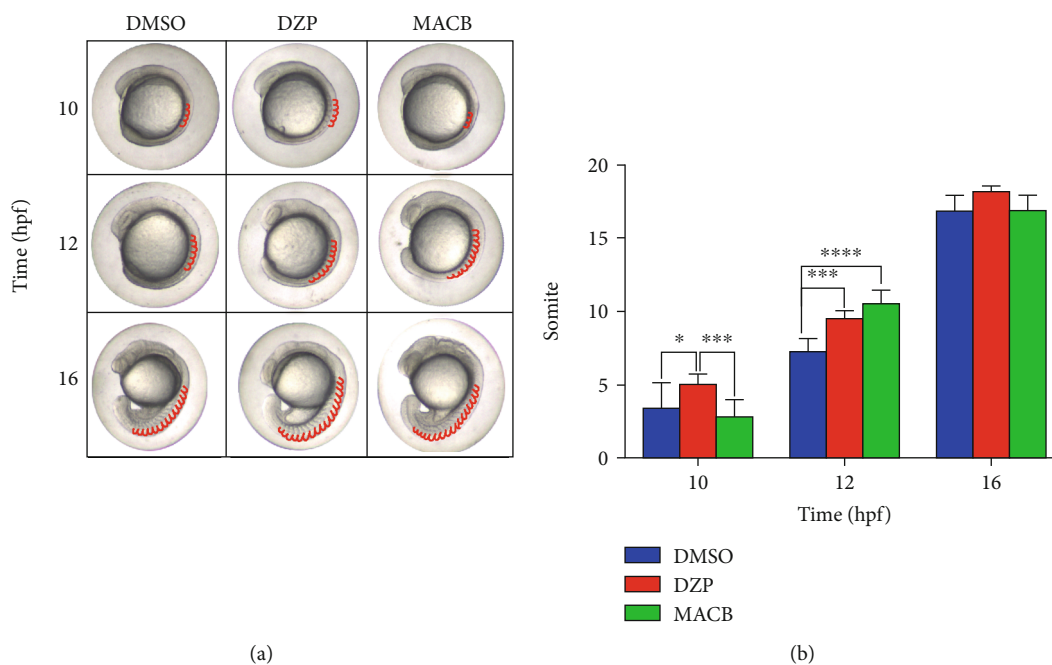


FIGURE 1: DZP and MACB accelerated the development of embryo somite. (a) Early somite development of zebrafish embryos. (b) Total somite of embryo treated with DZP and MACB at 5 nM for 10 hpf, 12 hpf, and 16 hpf. Data was analyzed by nonparametric, one-way analysis of variance (ANOVA) test, $n = 7 \sim 10$ larvae per group, followed with Tukey's multiple comparisons test, expressed as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$; NS: not significant.

was used to detect the gene expression of *oct4*, *sox2*, and *nanog* in zebrafish embryos. A total of 30 embryos (treated with 5 nM) or larvae were collected at 24, 48, and 72 hpf, respectively. Total RNA of zebrafish larvae was extracted using Trizol reagents (Thermo Fisher Science, USA) following the manufacturer's instruction. The concentrations of RNA samples were measured by Nano Drop, and genomic DNA was removed by RNase-free DNase I. 1 μ g RNA of each sample was used to synthesize first-strand cDNA using cDNA Synthesis kit (Takara, Japan). qRT-PCR was performed using SYBR Green qPCR Master Mix (BioRad, USA) in real-time PCR system (Applied Biosystem, Roche Life Science, USA). In 20 μ L of PCR reaction system, 1 μ L of cDNA template, 0.8 μ L of forward primer, 0.8 μ L of reverse primer, 7.4 μ L of nuclease-free water, and 10 μ L of SYBR Green qPCR Master Mix were mixed. The PCR reaction condition was set at 50°C for 2 min, 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 2 min. The corresponding pair primer sequences were designed by NCBI (Table 1). Quantitative polymerase chain reaction (qPCR) was performed to evaluate the expression

level of *oct4*, *sox2*, and *nanog* genes in real-time PCR system for three times.

2.6. Statistical Analysis. Prism 7.0 (Graphpad Prism Software, CA) was used for statistical analysis. The data analysis was carried out using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test to compare significance. All data were shown as mean \pm SEM, and $P < 0.05$ was defined as statistically significant.

3. Results

3.1. DZP and MACB Promote Somite Development in Zebrafish. To determine the developmental effects of DZP and MACB, we exposed zebrafish embryos to these two chemicals at 5 nM and then monitored the growth from 1 hpf (hour postfertilization) to end of the experiment. We found that developmental rates of somite were different among groups (Figure 1(a)). DZP significantly ($P < 0.05$) increased the number of embryonic segments from 4 to 5 compared with DMSO-treated zebrafish embryos at 10 hpf (Figure 1(b)).

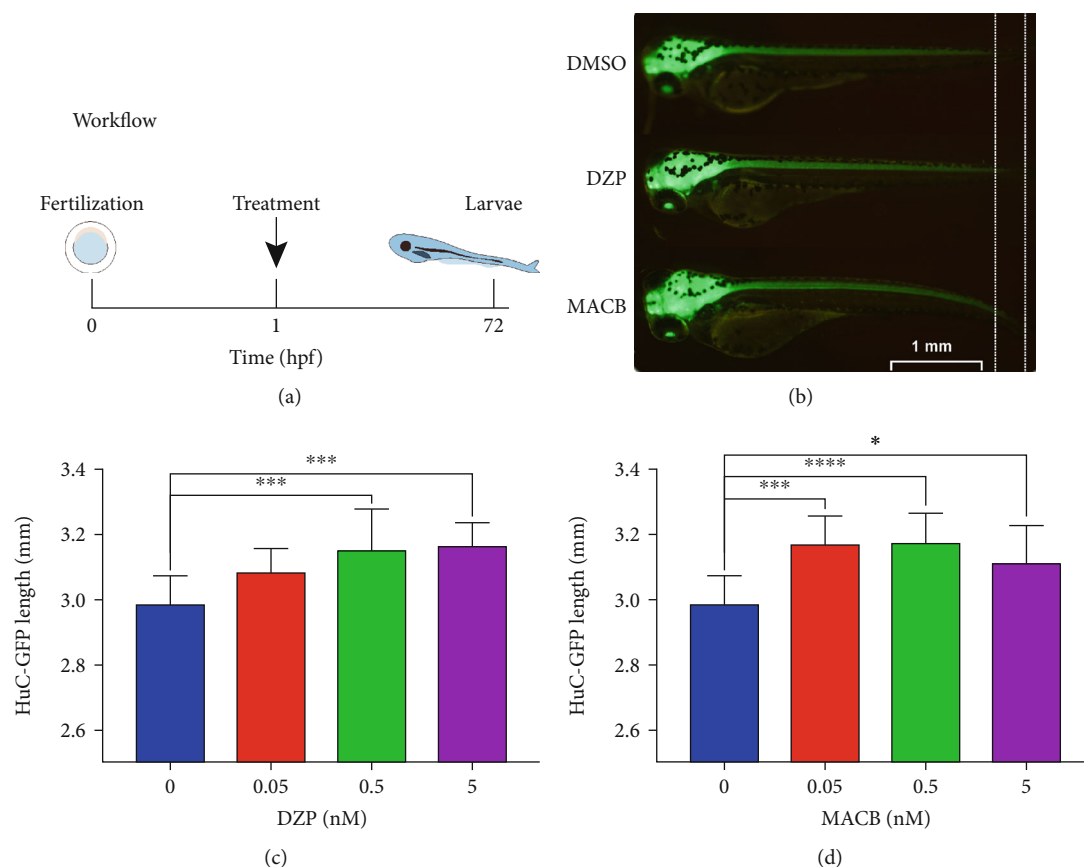


FIGURE 2: DZP and MACB stimulate neurogenesis. (a) Treatment flowchart for DZP and MACB exposure in embryonic zebrafish. (b) Representative images for the protein expression of HuC-GFP in the transgenic (TG) zebrafish treated with 0.05 nM DZP or MACB. The intensity of HuC was the indicator of neurogenesis and quantified by measuring the fluorescence length. The zebrafish larvae of HuC-GFP line treated with (c) DZP or (d) MACB for 72 hpf. Data was analyzed by nonparametric, one-way analysis of variance (ANOVA) test, $n = 12$ larvae per group, followed with Tukey's multiple comparisons test, expressed as mean \pm SEM. * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$; NS: not significant.

Somite increased to 7 in DMSO-treated zebrafish at 12 hpf. DZP and MACB further accelerated the embryonic segment development at this stage. However, no differences were observed at 16 hpf for the embryonic somites among groups (Figure 1(b)). Thus, zebrafish were sensitive to DZP and MACB in somite development at early stage.

3.2. Assessment of Impact on Nervous Development. For somitic organization underlies the segmental organization of nerves [29], we further determined neural effects of DZP and MACB in GFP zebrafish embryos labeled the neural RNA-binding protein HuC. Embryos were incubated in DZP or MACB solutions during the period of neural development from 1 hpf to 72 hpf (Figure 2(a)) [30, 31]. The expression levels of the HuC-GFP were measured by the length of body with green fluorescence at 72 hpf (Figure 2(b)). DZP significantly ($P < 0.001$) promoted neuron proliferation at 0.5 and 5 nM (Figure 2(c)). The nervous system was more sensitive to MACB, and the body length significantly increased at 0.05 nM exposure. These results indicated that DZP and MACB could speed up the neurodevelopmental process at low concentration.

3.3. The Locomotor Behavior of Larvae. Previous studies have shown that DZP and MACB at drinking water pollution level interfere with the neurodevelopment of juvenile fish; we further explored the behavioral effect of DZP and MACB on the larval fish using the dark-light test [32, 33]. The eyes of larvae could detect light signals and made a judgement [34]. For example, the larval locomotion was high in dark and low in light when exposed a dark-light cycle [35]. The movement distance of larvae treated with DZP and MACB was presented in the dark-light cycles (Figures 3(a) and 3(c)). Our results showed that DZP and MACB treatment significantly excited the activity level of larvae in the light (Figures 3(b) and 3(d)), and the excitatory effect of MACB on motor neurons was obvious over time (Figure 3(c)). There were no behavioral differences among all groups in dark periods (Figures 3(e) and 3(f)). Therefore, DZP and MACB stimulated hyperactivity for zebrafish larvae at environmental doses.

3.4. The Level of Nutrients in Embryos. To further study the effects of DZP and MACB on neurometabolic, we analyzed embryonic endogenous substances in the embryo treated

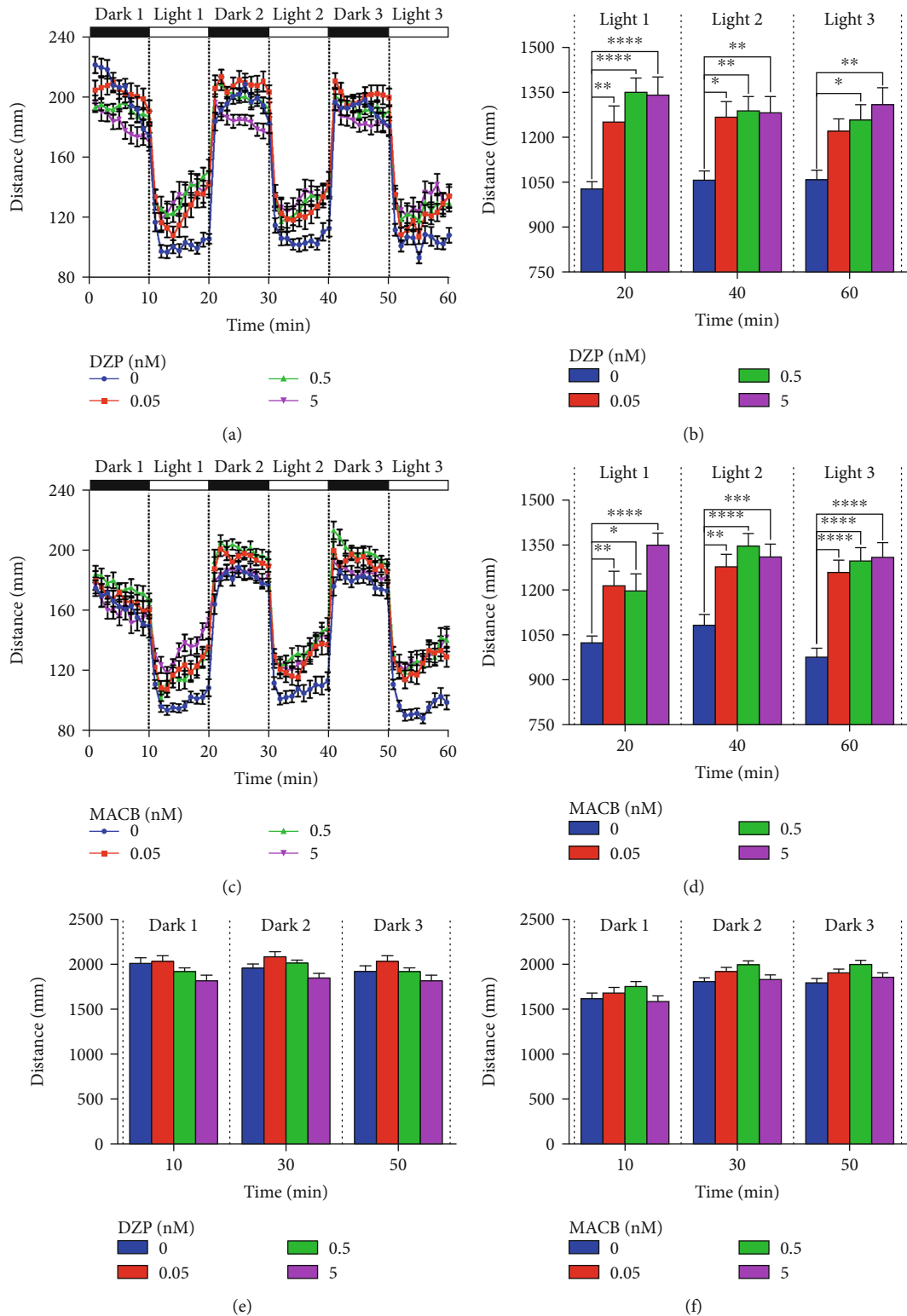


FIGURE 3: DZP and MACB enhance the locomotor behavior activity of larvae. (a) The distance of zebrafish larvae treated with DZP. Larvae moved every 1 min at 120 hpf exposed to alternating cycles of light and dark for 60 min each. (b) Total distance moved in each light period by larvae treated with DZP. (c) The distance of zebrafish larvae treated with MACB. Larvae moved every 1 min at 120 hpf exposed to alternating cycles of light and dark for 60 min each. (d) Total distance moved in each light period by larvae treated with MACB. Total distance moved in each dark period by larvae treated with (e) DZP and (f) MACB. Data was analyzed by nonparametric, one-way analysis of variance (ANOVA) test, $n = 20 \sim 24$ larvae per group, followed with Tukey's multiple comparisons test, expressed as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$; NS: not significant.

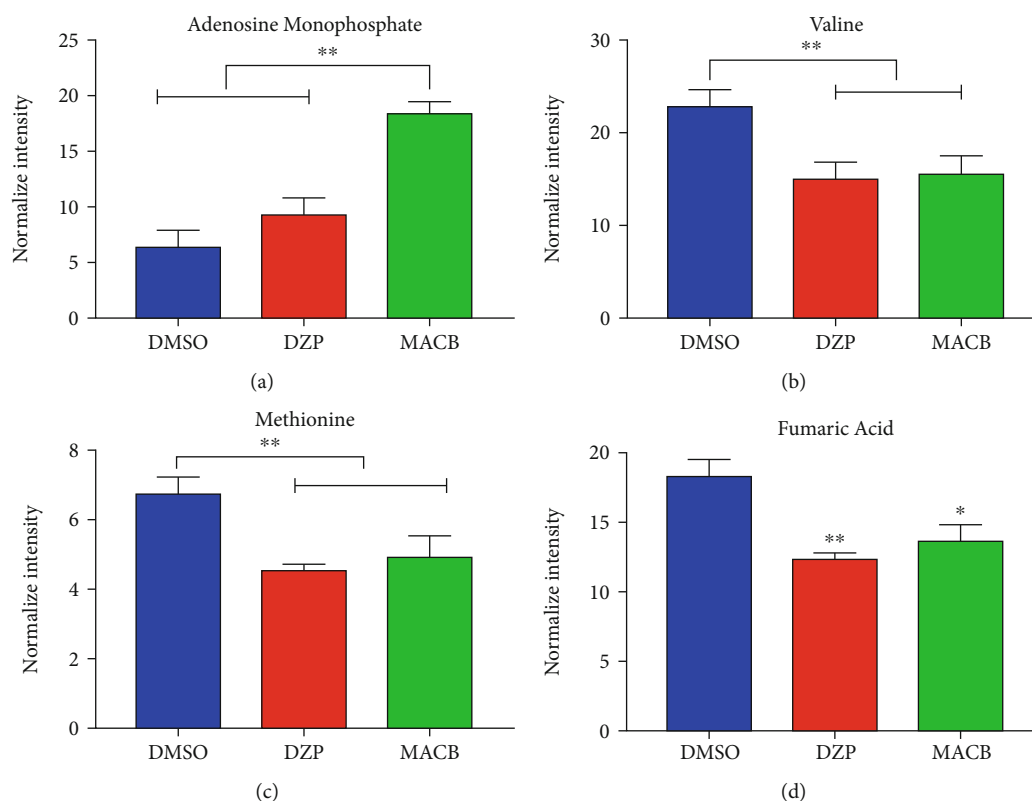


FIGURE 4: DZP and MACB affected the levels of nutrients in embryos. (a) MACB upregulated adenosine monophosphate. (b) DZP and MACB downregulated (b) valine, (c) methionine, and (d) fumaric acid. Data was analyzed by nonparametric, one-way analysis of variance (ANOVA) test, with 3 replicates ($n = 50$ larvae per group) followed with Tukey's multiple comparisons test, expressed as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$.

with DZP or MACB at 5 nM. Zebrafish embryo was entirely dependent on their own nutrients in yolk sac to sustain growth and development [36]. The results showed that MACB increased the level of adenosine monophosphate (AMP) (Figure 4(a)), which generated the cyclic adenosine 3',5'-monophosphate (cAMP). The cAMP was an important signalling molecular improving axonal regeneration [37]. The valine promoted cellular sensitization [38] and was downregulated by DZP and MACB (Figure 4(b)). Moreover, the levels of methionine were also decreased by DZP and MACB (Figure 4(c)). The valine and methionine could affect the activity of brain-derived neurotrophic factor (BDNF) [39]. In addition, the fumaric acid has neuroprotection role in multiple sclerosis (MS) therapy [40]. The metabolic of fumaric acid was disrupted (Figure 4(d)), indicating neural developmental disorders in DZP- and MACB-treated zebrafish embryos. These results indicated that DZP and MACB could disrupt neural metabolism at low doses.

3.5. Detection of Gene Expression Related with *oct4*, *sox2*, and *Nanog*. To explore molecular mechanism regulating the proliferation and differentiation, we analyzed the expression of transcription-related factors in 10 hpf zebrafish embryos. Octamer-binding transcription factor 4 (*oct4*), SRY- (sex-determining region Y-) box 2 (*sox2*), and homeobox protein *nanog* regulate the differentiation of embryonic stem cells into specific cells. Our results showed that DZP and MACB

upregulated expression levels of *oct4* and *sox2* at 5 nM (Figures 5(a) and 5(b)). This trend was consistent with the accelerated somite growth at 12 hpf and the enhanced sport ability at 120 hpf. In addition, the risk of MACB was higher than DZP, because MACB also upregulated the expression level of *nanog* (Figure 5(c)). Therefore, both DZP and MACB promoted the early development of nervous system in zebrafish embryos through increasing pluripotency and self-renewal rates (Figure 5(d)).

4. Discussion

The pharmaceutical pollution in drinking water posing a risk to human health has attracted great concern. A diverse set of chemical exposure has been shown to affect infant nervous development at low concentration due to the immature development of the blood-brain barrier (BBB) [30, 41]. In our previous study, we found that the widely used DZP has contaminated drinking water and generated disinfection byproduct MACB. Thus, we further investigated their neurotoxicology and metabolic effects in zebrafish. We demonstrated that DZP and MACB increased the number of somites and neurons and upregulated the expression of pluripotency and self-renewal transcription factors, promoting the early development of nervous system in zebrafish embryos.

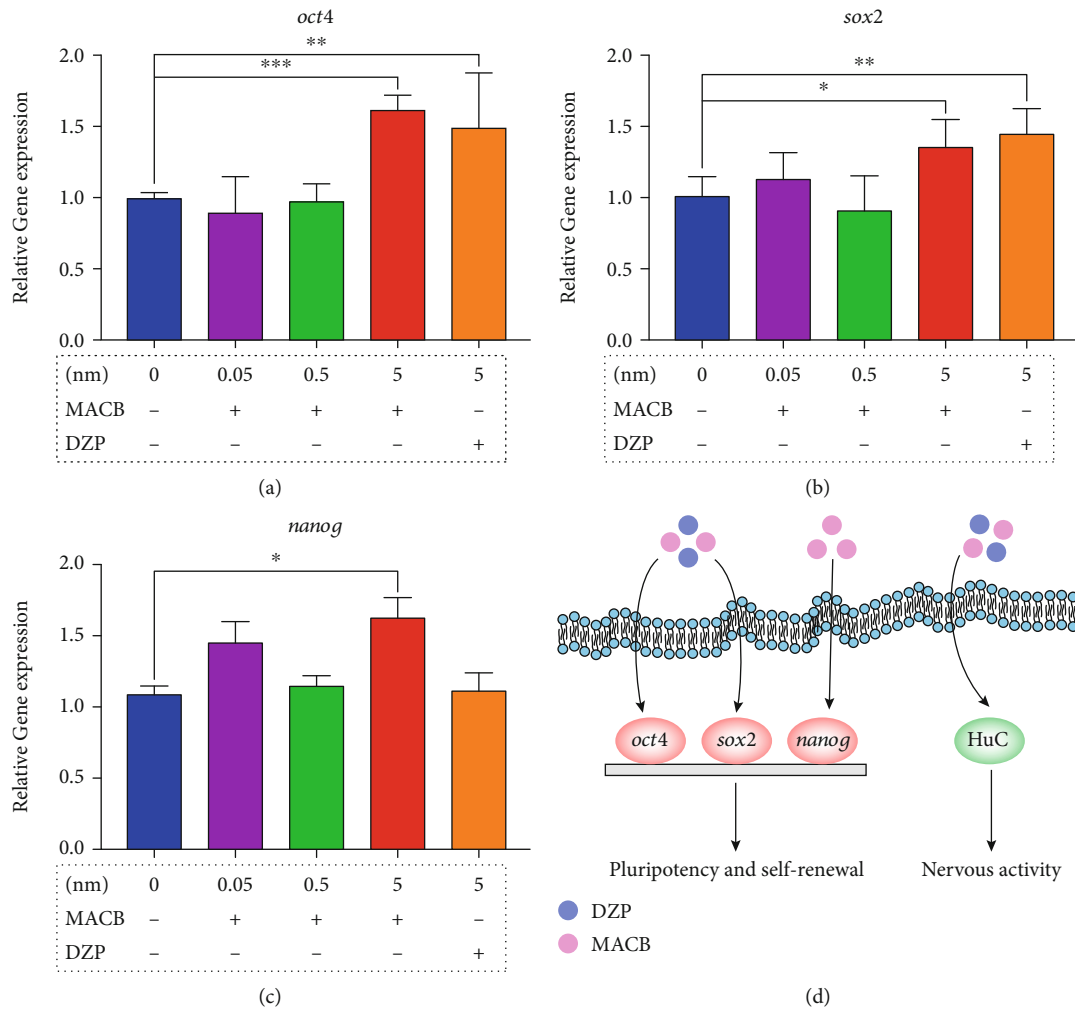


FIGURE 5: Expression level of transcription factors, *oct4*, *sox2*, and *nanog*, were upregulated. (a) The mRNA expression level of (a) *oct4* and (b) *sox2* were upregulated by DZP and MACB at 10 hpf. (c) The mRNA expression level of *nanog* was upregulated by MACB at 10 hpf. Data was analyzed by nonparametric, one-way analysis of variance (ANOVA) test, $n = 30$ larvae per group, followed with Tukey's multiple comparisons test, expressed as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$; NS: not significant. (d) Diagram showing how DZP and MACB affect early proliferation and differentiation and nervous activity.

The total number of somites formed during somitogenesis is tightly fixed within a given species. The somites are derived from the paraxial mesoderm and are located on either side of the neural tube. Somitogenesis is of vital developmental importance and controls the differentiation of peripheral spinal nerves, vertebrae, and skeletal muscle [42]. It suggests that DZP and MACB would affect early embryo development at the micropollutant dose. The rapid onset of somitogenesis in DZP- or MACB-treated group suggests that the nervous inhibitor can accelerate early development at low concentration (nM), which was similar with the effect of antidepressant venlafaxine (10 ng) stimulating neurogenesis in zebrafish embryo [43]. The larval behavioral phenotype observed in response to cycles of dark-light could assess swimming behavior at these early life stages. Although the behavioral test is relatively simple, it can be used to screen the early neurological dysfunction caused by exogenous compounds [44]. DZP and MACB treatment significantly increased the activity of larvae in the dark but not in the light,

which may be corresponded with an increase in primary neurogenesis in larval zebrafish. Therefore, DZP and MACB had neuroexcitatory effects and stimulated the spontaneous movement of juvenile fish at low doses.

AMP facilitates the cAMP level that improves axonal regeneration [37] that explains why DZP and MACB accelerated zebrafish motion. In addition, the AMP strictly controls the activity of AMP-activated protein kinase (AMPK), which maintains metabolic homeostasis of energy and regulates growth and proliferation through nutritional signals [45]. The increased AMP could impair protein balance and decrease the levels of valine and methionine [46]. In addition, fumaric acid could clean ROS during the process of inflammation to protect neurons and glial cells [40]. DZP and MACB decreased the levels of fumaric acid that implied the occurrence of inflammation in early embryos. Thus, DZP and MACB might disturb the energy balance and protein homeostasis.

Many transcription factors can regulate the differentiation of embryonic stem cells into specific cells. The *oct4*, a

member of the family of POU-domain transcription factors, was required for pluripotency and self-renewal in embryonic stem (ES) during embryo development. The *sox2* is a transcription factor that is essential for maintaining self-renewal or pluripotency of undifferentiated embryonic stem cells, including neural stem cells. Homeobox protein *nanog* is a transcriptional factor that helps ESCs maintain pluripotency by suppressing cell determination factors. DZP and MACB promote the expression level of *oct4* and *sox2* accelerating the early neurodevelopment of zebrafish embryo [47, 48]. In addition, MACB upregulated the expression of *oct4* and *nanog*. In previous study, antipsychotic lithium enhanced *oct4* and *nanog* expression to facilitate reprogramming depending on major target GSK3 β [49]. Thus, MACB enhanced proliferation and differentiation of somatic cells which might depend on GABAA receptors or other targets. GABAergic neurons are critical for early development of hippocampus and neocortex [50]. Previous study showed that DZP could downregulate GABAergic synapses which modulated neural behavior with compartmentalized Ca²⁺ dynamics [51]. In addition, intracellular calcium mobilization is required to modulate development and tissue patterning in zebrafish embryos [52]. Therefore, it is possible that MACB accelerated the developing embryos through modulating intracellular calcium.

5. Conclusion

In conclusion, DZP and MACB accelerated the development of embryo somites and neurons through upregulating the transcription factors at drinking water pollution levels. Meanwhile, DZP and MACB promoted energy metabolism and resulted in excited behavior. Our results highlighted that psychiatric drugs stimulated neural genesis at low doses.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Xiaole Zhao, Xiaoyong Huang, Kui Zhu, and Bing Shao designed the research. Xiaole Zhao and Xiaoyong Huang performed the research. Jiachen Shi and Xiaofei Jia contributed new analytic tools. Xiaole Zhao, Xiaoyong Huang, Kui Zhu, and Bing Shao analyzed and wrote the paper.

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