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RESEARCH ARTICLE

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Developmental neurotoxicity and toxic mechanisms induced by olaquindox in zebrafish

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Abstract

Olaquindox (OLA) has been widely used as an animal feed additive in China for decades; however, its toxicity and toxic mechanisms have not been well investigated. In this study, the developmental neurotoxicity and toxic mechanisms of OLA were evaluated in zebrafish. Zebrafish embryos were exposed to different concentrations of OLA (25–1,000 mg/L) from 6 to 120 hours post fertilization (hpf). OLA exposure resulted in many abnormal phenotypes in zebrafish, including shortened body length, notochord degeneration, spinal curvature, brain apoptosis, damage of axon and peripheral motor neuron, and hepatotoxicity. Interestingly, OLA increased zebrafish spontaneous tail coiling, while reduced locomotor capacity. Quantitative polymerase chain reaction (Q-PCR) showed that the expression levels of nine marker genes for nervous system functions or development, namely, α*1-tubulin*, glial fibrillary acidic protein (*gfap*), myelin basic protein (*mbp*), synapsinII a (*syn2a*), sonic hedgehog a (*shha*), encoding HuC (*elavl3*), mesencephalic astrocyte-derived neurotrophic factor (*manf*) growth associated protein 43 (*gap43*), and acetylcholinesterase (*ache*) were all down-regulated significantly in zebrafish after treated with OLA. Besides, the anti-apoptotic and pro-apoptotic genes *bcl-2/bax* ratio was reduced. These results show that OLA exposure could cause severe developmental neurotoxicity in the early stages of zebrafish life and OLA might induce neurotoxicity by inhibiting the expression of neuro-developmental genes and promoting apoptosis.

KEYWORDS
developmental neurotoxicity, OLA, olaquindox, zebrafish

1 | INTRODUCTION

Olaquindox (OLA), *N*-(2-hydroxyethyl)-3-methyl-2-quinoxalincarboxamide-1,4-dioxide, is a synthetic veterinary drug of quinoxalines that is used as an antibacterial agent and feed additive to treat bacterial infections and promote animal growth since the 1970s. However, due to its severe side effects in mutagenicity (Yoshimura, Nakamura, Koeda, & Yoshikawa, 1981), fertility (Gandalovicova & Sykora, 1986), allergy (Belhadjali et al., 2002), genotoxicity (Nunoshiba & Nishioka, 1989), and cytotoxicity (Cihak & Srb, 1983), the Commission of the European Community has forbidden the application of OLA as an animal growth promoter in 1999 (Song et al., 2011). However, OLA was widely used in China as an animal feed additive and an animal growth promoter until banned in 2018. Long-time abuse of OLA has polluted the animal-derived food products and water sources, and high dosages of OLA applied to livestock and poultry could result in residual problem in animal body, which eventually could pose a great threat to human health (Pei et al., 2016). According to the regulations of the Ministry of Agricultural and Rural Affairs of China, the maximum residue limit (MRL) of OLA in porcine

muscle is set at 4 μg/kg and in porcine liver at 50 μg/kg (Li, Dai, Yang, Wang, & Tang, 2017).

As a general rule, drugs administered by oral route are slowly absorbed and excreted with feces. Thus, animal wastes from intensive farming contain antibiotics in active form (Migliore et al., 1996). Wastes from terrestrial animals are often used for field manuring, and nearly all of the acquaculture animals excrete into water environment directly. As a result, 70%–80% of antibacterials end up in the environment (Wollenberger, Halling-Sørensen, & Kusk, 2000). OLA and OLA residues may therefore leach to surface water in the vicinity of fields and cause adverse effects to the ecosystem, and its toxicity on alga has been confirmed (Halling-Sørensen, 2000).

Some previous studies have reported that OLA had accumulative toxicity, hepatotoxicity, and nephrotoxicity (Wang et al., 2012; Yang et al., 2015; Zhou, Li, Wang, Ji, & Zhu, 2015). A long-term toxicity study of OLA in rats, pigs, and beagle dogs showed the toxic effects in liver, kidney, testes, ovaries, and endocrine glands (Fang et al., 2006). Chronic exposure could result in the accumulation of OLA in the liver and kidney of carp, leading to the liver and kidney lesions and thus metabolism and excretion disorders (Yang et al., 2018). Another study also confirmed that OLA would induce fatty or vacuolar degeneration of liver cells in common carp (Wang, Zhao, Yi, Huang, & Liu, 2004). In addition, several studies showed that exposure to OLA could induce apoptosis in HepG2 cells (Li, Dai, Yang, Wang, & Tang, 2017; Zou et al., 2011). However, no studies have been done to systematically evaluate the neurotoxicity of OLA in animals yet.

Though rodents that have been widely used have significant contribution to our understanding of developmental neurotoxicity (Aoyama, 2012; Crofton, Mundy, & Shafer, 2012; Kuwagata, 2012; Tsuji & Crofton, 2012), experiments using large numbers of rodents are time consuming, expensive, and raise ethical concerns. Using non-mammalian animals as alternative models may relieve some of these pressures by allowing for testing large numbers of subject while reducing expenses and minimizing the use of mammals (Bal-Price et al., 2012; Crofton, Mundy, & Shafer, 2012).

Zebrafish (*Danio rerio*) has become an important tool in developmental neurobiology (Bailey, Oliveri, & Levin, 2013; de Esch, Slieker, Wolterbeek, Woutersen, & de Groot, 2012; Nishimura et al., 2015). There are high similarities in the nervous system development between zebrafish and mammals (Appel, 2000; Guo, 2004; Guo, 2009; Hjorth & Key, 2002; Schmidt, Strahle, & Scholpp, 2013; Tropepe & Sive, 2003). Zebrafish has homologous basic processes of neurodevelopment with those occurring in humans (Tropepe & Sive, 2003), and visualization of several key players (neurons and axon tracts) and monitoring of processes (motoneuron activity) of the nervous system in zebrafish are possible (Sukardi, Chng, Chan, Gong, & Lam, 2011; Ton, Lin, & Willett, 2006). Many transgenic lines expressing fluorescent proteins in specific neuronal subpopulations have been developed and are available through public resources (Sato, Takahoko, & Okamoto, 2006). These transgenic zebrafish, combined with high content imaging technologies (Higashijima, Hotta, & Okamoto, 2000; Mikut et al., 2013), can provide relatively high-throughput developmental neurotoxicity testing. A number of laboratories have employed zebrafish to investigate the toxic effects of chemicals released into the environment on the development of nervous system, including nanoparticles, pesticides, and various organic pollutants (Guo et al., 2018; Li et al., 2018; Qian, Liu, Lu, & Sun, 2018; Shi et al., 2018). Furthermore, Zebrafish have been used for studying the mechanism of neurotoxicity by detection of neural developmental, neurochemical, and neurobehavioral changes (Bailey, Oliveri, & Levin, 2013; Eddins, Cerutti, Williams, Linney, & Levin, 2010).

The present study was aimed to evaluate the developmental neurotoxicity and toxic mechanisms of OLA in zebrafish systematically and comprehensively. We found that OLA was highly toxic to zebrafish embryos; it could induce concentration-dependent adverse effects in embryonic development, such as shortened body length, notochord degeneration, spinal curvature, brain apoptosis, damage of axon and peripheral motor neuron, and hepatotoxicity, it also increased the spontaneous tail coiling in zebrafish embryos while decreased locomotor speed in zebrafish larvae. In addition, the expression levels of nine marker genes for nervous system functions or development, namely, *α1-tubulin*, *gfap*, *mbp*, *syn2a*, *shha*, *elavl3*, *manf*, *gapd43*, and *ache* were all down-regulated significantly after OLA exposure.

2 | MATERIALS AND METHODS

2.1 | Zebrafish care and maintenance

Three lines of zebrafish were used in this study: wild-type AB strain zebrafish (*D. rerio*), Tg (NBT:MAPT-GFP)zc1, and Tg (Isl1:CMICP-GFP) transgenic zebrafish. All the zebrafish strains used in this study were obtained from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). 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. To obtain zebrafish larvae, four to five pairs of zebrafish were set up for natural mating every time. On average, 200–300 embryos were generated per pair. Embryos were maintained at 28°C in fish water (0.02% Instant Ocean salt in deionized water, pH 6.9–7.2, conductivity 480–510 μS/cm, and hardness 53.7–71.6 mg/L $CaCO_3$). The embryos were washed and staged at 6 and 24 hpf. 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., 2016) and by the China National Accreditation Service for Conformity Assessment (CNAS) and China Inspection Body and Laboratory Mandatory Approval (CMA).

2.2 | Chemicals and reagents

OLA ($C_{12}H_{13}N_3O_4$, MW 263.25, CAS NO.23696-28-8, purity ≥98%) was supplied by the China Institute of Veterinary Drug Control

(Beijing, China). OLA was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA), and stock solutions were prepared in fish water with a final DMSO concentration of 0.1% (v/v). Stocks were stored at −20°C in the dark. Acridinium chloride hemi-(zinc chloride) (AO) were purchased from Sigma-Aldrich. TRIzol reagent, reverse transcriptase kit and the SYBR Green system were purchased from Takara (Dalian, China).

2.3 | Developmental toxicity assessment

Thirty wild-type AB strain zebrafish embryos were distributed into 6-well microplates (Nest Biotechnology Co., Ltd, Shanghai, China) in 3ml fresh fish water per well. Zebrafish embryos were exposed to OLA continuously at nominal concentrations of 25, 50, 100, 200, 300, 500, and 1,000 mg/L from 6 to 120 hpf, respectively. Zebrafish treated with fish water were used as untreated control. exposure of OLA, zebrafish were observed under the stereomicroscope (SZX7, OLYMPUS, Japan) at 24, 28, 72, 96, and 120 h posttreatment (hpt). The effect of OLA on embryonic survival, hatching, and morphological development was assessed. Dead zebrafish were recorded and promptly removed from the solution during observations. The embryonic hatching rate was counted at 48 and 72 hpf. The abnormal phenotypes were determined by examining 5-day postfertilization (dpf) larvae under a stereomicroscope, including the following endpoints: pericardial and body edema, abnormalpigmentation, size of eye, body length, heart, head, tail, otoliths and muscle deformation, absence of liver and intestine, and bleeding. At the end of 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).

2.4 | Developmental neurotoxicity assessment

2.4.1 | Brain apoptosis

Thirty wild-type AB strain zebrafish embryos were treated with OLA at concentrations of 25, 50, 100, 200, and 300 mg/L from 6 to 24 hpf. After treatment, zebrafish from each group were washed with fish water three times, then stained with 2.5 mg/L acridine orange (AO) in fish water for 30 min. Next, zebrafish were rinsed thoroughly in fish water three times and observed for apoptotic cells that would display yellow-green fluorescent spots in the brain and spinal cord under a stereo fluorescence microscope (AZ100, Nikon, Japan). In order to determine the fluorescence intensity in brain regions, fluorescence signal (S) of apoptotic cells in the brain was measured with Nikon NIS-Elements D 3.10 Advanced image processing software. The induction percentage of brain apoptosis in zebrafish treated with OLA was calculated based on the following formula: induction percentage of apoptosis = [S (OLA) − S (Control)]/[S (Control)] × 100%.

2.4.2 | Axon damage

Thirty Tg (Isl1:CMICP-GFP) transgenic zebrafish embryos that express green fluorescent protein (GFP) in the cranial motor neurons and cranial sensory neurons (Higashijima, Hotta, & Okamoto, 2000) were treated with OLA at concentrations of 25, 50, and 100 mg/L from 6 to 72 hpf. After treatment, 10 zebrafish from each group were randomly selected, and images of the central nerve were acquired using a stereo fluorescence microscope. Qualitative analysis of image was performed using image-based morphometric analysis.

2.4.3 | Peripheral motor neurons damage

Thirty Tg (NBT:MAPT-GFP)zc1 transgenic zebrafish embryos in which the GFP was integrated into the gene sequence of beta tubulin were treated with OLA at concentrations of 25, 50, and 100 mg/L from 6 to 72 hpf. After treatment, 10 zebrafish from each group were randomly selected, and images were acquired under fluorescent stereomicroscope. The length (L) of motor neuron in zebrafish was calculated by NIS-Elements D3.10. The reduction percentage of peripheral motor neuron length in zebrafish treated with OLA was calculated based on the following formula: reduction percentage of peripheral motor neuron length = [L (Control) − L (OLA)]/[L (Control)] × 100%.

2.4.4 | Spontaneous tail coiling evaluation

Thirty wild-type AB strain zebrafish embryos were treated with OLA at concentrations of 25, 50, 100, 200, 300, 500, and 1,000 mg/L from 24 to 25 hpf. After treatment, tail coilings of embryos during 1 min were detected with a high-resolution camera mounted on a stereomicroscope. Ten embryos in each group were selected and detected randomly.

2.4.5 | Behavioral toxicity

Thirty wild-type AB strain embryos were treated with OLA at concentrations of 25, 50, and 100 mg/L from 6 to 120 hpf. The locomotor activity was quantified by a Video-Track system (Viewpoint Life Science, Lyon, France). Living larvae was selected and loaded into a 96-well microplate with a single animal in each well. Before monitoring, zebrafish acclimated in the 96-wells at 28°C for 10 min. The swimming patterns of zebrafish under continuous dark (60 min) were first recorded, and then under light-to-dark transition (5-min dark, 5-min light, 5-min dark, and 5-min light). Shortly after behavior recording, larvae were inspected under a stereomicroscope; any possible dead or malformed individuals were identified and their data would be excluded from further statistical analyses. Data were analyzed following the method described in our previous article (Huang et al., 2016). The reduction percentage of total distance (D) of

zebrafish treated with OLA under continuous dark was calculated based on the following formula: reduction percentage of total distance = [D (Control) − D (OLA)]/[D (Control)] × 100%. Based on total distance and time spent, the average swimming speed (mm/s) was calculated and was used to analyze if the swimming activity of zebrafish would be different between the dark and light.

2.4.6 | Gene expression analysis

Based on the previous tests, Zebrafish embryos at 6 hpf were exposed to 25, 50, and 100 mg/L OLA until 120 hpf, 30 embryos in each group. After OLA treatment, the total RNA of zebrafish in every group was extracted using TRIzol reagent (Invitrogen Life Technologies). The quality of RNA in each sample was verified by measuring the 260/280 nm ratio. A 2 μg of RNA from each sample was used to synthesize using FastQuant RT Kit (with gDNase) (Tiangen). Quantitative polymerase chain reaction (Q-PCR) amplifications were carried out with a CFX Connect detection system (Bio-Rad) using iTaq Universal SYBR Green Supermix (Bio-Rad) in triplicate. The PCR protocol used was 2 min at 95°C, 40 cycles of 5 s at 95°C, and 30 s at 60°C. Key marker genes of *α1-tubulin*, glial fibrillary acidic protein (*gfap*), myelin basic protein (*mbp*), synapsinII a (*syn2a*), sonic hedgehog a (*shha*), encoding HuC (*elavl3*), mesencephalic astrocyte-derived neurotrophic factor (*manf*) growth associated protein 43 (*gap43*), and *ache* were determined of the embryos at 120 hpf. These genes are of importance to the developmental neurotoxicity and are believed to be responsive to chemical exposure as previously reported (Wang, Lam, et al., 2015; Wang, Yang, et al., 2015). The mRNA levels of the *bcl-2* and *bax* were also evaluated of the embryos at 24 hpf, These genes are involved in apoptosis pathway. The primer sequences of genes were obtained as previously described (Table 1) (Guo et al., 2018). Melt curve analysis was used to confirm primer specificity. Melt curve analysis was used to confirm primer specificity. Expression data were normalized against the expression of β-actin, having a stable expression in all treatments.

TABLE 1 Sequences of primer pairs used in the real-time quantitative polymerase chain reaction (PCR) reactions

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

The relative expression levels of genes among groups were calculated using the 2-ΔΔCt method (Sharif, Steenbergen, Metz, & Champagne, 2015). Three biological replicates were performed for each sample.

2.5 | Statistical analysis

Statistical analyses were performed with one-way ANOVA and Dunnett's test using the SPSS 16.0 software (SPSS, USA). All data were shown as the mean ± standard error (SE), and $p < 0.05$ was considered statistically significant. All figures were generated by GraphPad Prism 5.0 Software (GraphPad, Inc., San Diego, CA, USA).

3 | RESULTS

3.1 | Developmental toxicity

The hatching rate was decreased as the OLA concentration increases. A total of 40% of embryos hatched in the normal control whereas no one hatched in 200, 300, 500, and 1,000 mg/L OLA at 48 hpf, respectively (Figure 1A). Embryo death occurred in 200, 300, 500, and 1,000 mg/L treatment groups, with mortality of 100 at 72 hpf of all these groups (Figure 1B). Morphologic abnormalities increased in a dose-dependent manner. No morphological abnormality was observed in embryos in 25 mg/L group. However, body length reduction, notochord degeneration, severe spinal curvature, and hepatotoxicity were observed in the 50 and 100 mg/L groups. Compared with the control group, the body length declined (9.92 ± 2.25)% and (18.61 ± 3.04)% in 50 and 100 mg/L groups ($p < 0.05$, $p < 0.001$), respectively (Figure 1C). Besides, notochord degeneration was obviously increased as OLA concentration increased; the incidence was 40% in 50 mg/L and 100% in 100 mg/L. Liver degeneration and yolk sac retention rate were 100% in 100 mg/L groups. The representative phenotype figures of zebrafish treated with OLA at various concentrations in different stages were presented in Figure 1D.

3.2 | Developmental neurotoxicity

3.2.1 | Brain apoptosis

Apoptotic cells in the brain of zebrafish embryos in each group were revealed by AO staining, which could reveal chemical-induced apoptosis in nervous system. No obvious apoptotic cells were observed in zebrafish larvae of the control group at 24 hpf, whereas apoptotic cells in higher concentrations of OLA-treated groups were apparent, mainly around the head area and spinal cord, with bright green fluorescent spots (Figure 2A,B). The induction percentages of apoptosis were (2.85 ± 2.59)%, (4.57 ± 3.99)%, (30.03 ± 8.97)%, (64.53 ± 8.96)%, and (69.40 ± 11.48)% at 25, 50, 100, 200, and 300 mg/L of OLA, respectively, and statistically significant difference

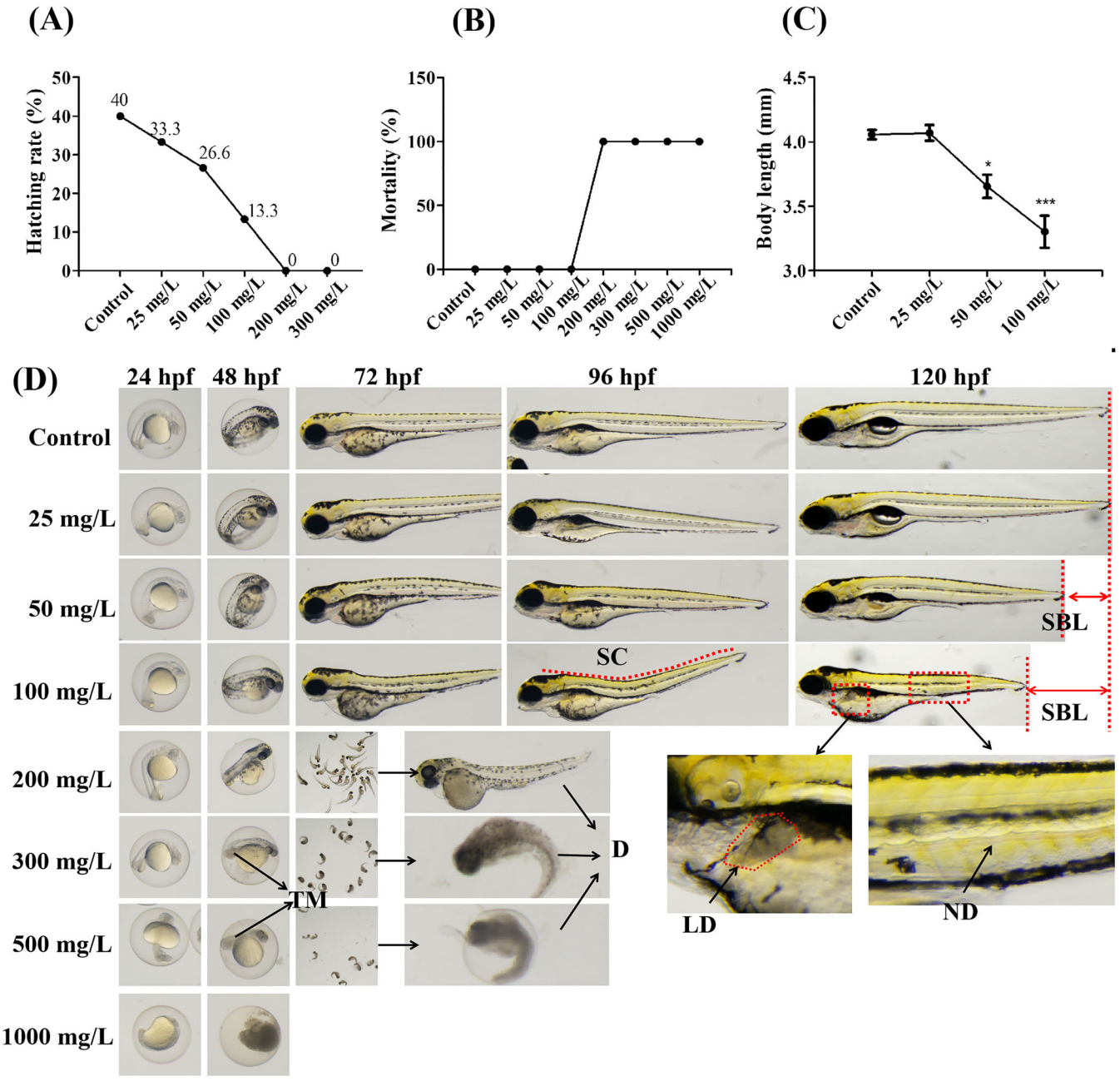

FIGURE 1 Effects of different concentrations of olaquindox (OLA) on developmental parameters. (A) Hatching rate of embryos at 48 h post fertilization (hpf), (B) mortality of larvae at 120 hpf, (C) body length of larvae at 120 hpf. (D) Morphological effect of OLA on zebrafish during the exposure at 24–120 hpf. D, death; LD, liver degeneration; ND, notochord degeneration; SBL, short body length; SC, spinal curvature; TM, tail malformation. Data are expressed as mean ± standard error (SE). Compared with control group: $^{*}p < 0.05$, $^{***}p < 0.001$

was observed at 100, 200, and 300 mg/L ($p < 0.05$ or 0.001) as compared with the control group, respectively (Figure 2C). We also assessed the apoptosis-related gene (*bax* and *bcl-2*) expression level in OLA-exposed embryos. The expression of pro-apoptotic gene *bax* was significantly up-regulated in zebrafish exposed to 200 and 300 mg/L OLA at 24 hpf ($p < 0.001$ for both).(Figure 2D). Meanwhile, the expression of anti-apoptotic gene *bcl-2* in the 100, 200, and 300 mg/L OLA-treated group was down-regulated compared with control ($p < 0.001$ for all) (Figure 2E). Thus, the *bcl-2/bax* ratio decreased after OLA exposure, especially in the higher OLA concentrations (100, 200, and 300 mg/L) (Figure 2F). These results strongly suggested that OLA induced acute cell apoptosis.

3.2.2 | Axon and peripheral motor neuron damage

No morphological abnormality of axon or peripheral motor neuron was observed in embryos from control groups. As shown in Figure 3A,

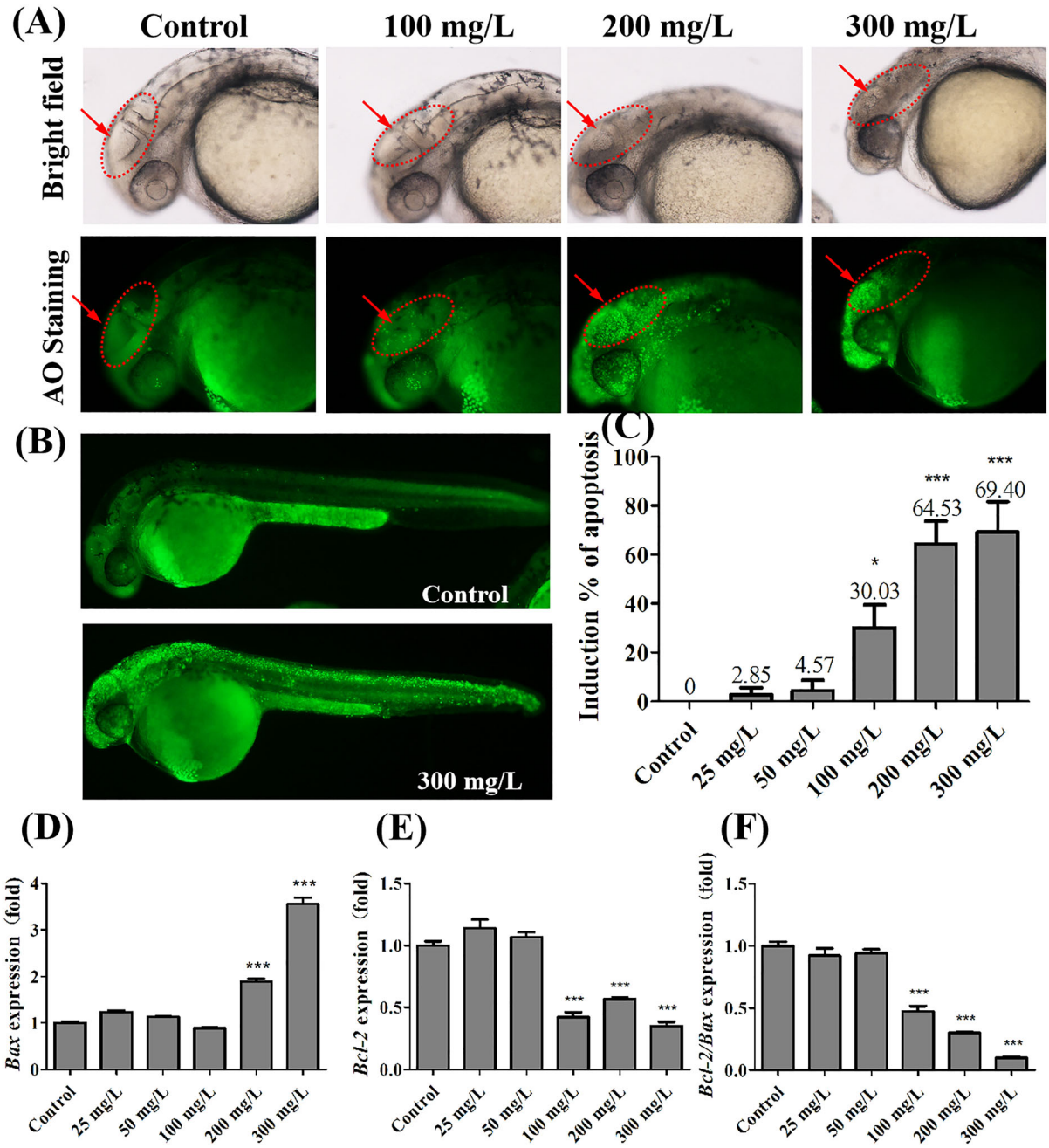

FIGURE 2 (A) Apoptosis in the brain of zebrafish larvae induced by olaquindox (OLA)-treated groups at concentrations of 100, 200, and 300 mg/L at 24 h post fertilization (hpf). (B) Apoptotic cells appeared around the head area and spinal cord. (C) The induction percentage of apoptosis in the brain regions. Relative expression levels of *bax* (D), *bcl-2* (E), *bcl-2*/*bax* ratio (F) in zebrafish after exposure to various concentrations of OLA for 24 h. Data are expressed as mean ± standard error (SE). Compared with control group: $^{*}p < 0.05$, $^{***}p < 0.001$

severe degeneration of peripherally projecting axons from the facial sensory ganglion cells was found in Tg (Isl1:CMICP-GFP) transgenic zebrafish at 100 mg/L OLA.

Also, peripheral motor nerves were observed to be bent and shorter in Tg (NBT:MAPT-GFP)zc1 transgenic zebrafish (Figure 3B) exposed to OLA in a dose-dependent manner; the reduction percentages of peripheral motor neuron length were (0.64 ± 2.50)%, (7.74 ± 1.60)%, and (18.82 ± 1.13)% at 25, 50, and 100 mg/L OLA, respectively. Statistically significant reduction was demonstrated in zebrafish treated with OLA at 50 and 100 mg/L ($p < 0.01$, $p < 0.001$) (Figure 3C).

3.2.3 | Spontaneous tail coiling

The spontaneous tail coilings of embryos were significantly increased in a dose-dependent manner after exposed to OLA (Figure 4A). The spontaneous tail coilings were (9.30 ± 0.58), (10.90 ± 1.11),

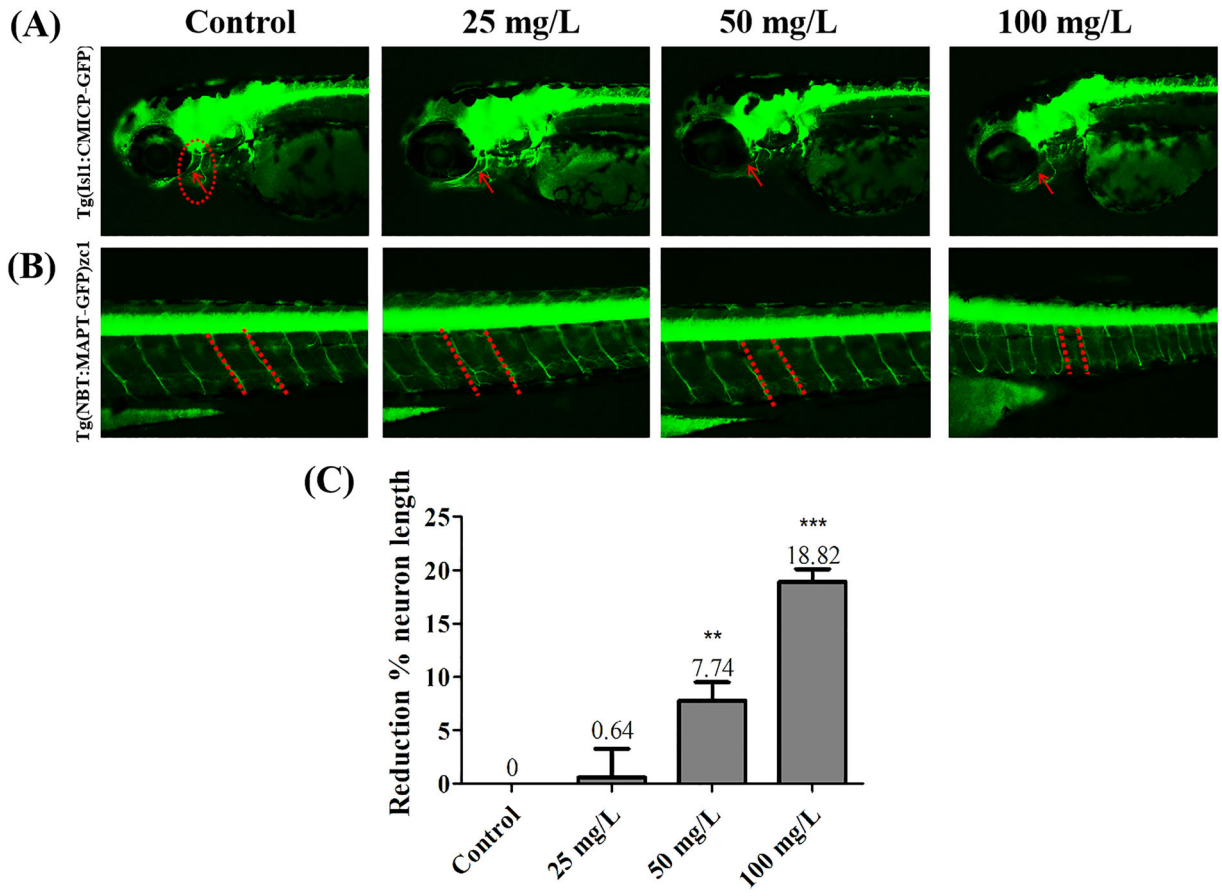

FIGURE 3 Effect of olaquindox (OLA) on Tg(Isl1:CMICP-GFP) and Tg(NBT:MAPT-GFP)zc1 transgenic zebrafish after exposed for 72 h. (A) Axons from facial sensory ganglion cells were severely damaged at 100 mg/L in Tg(Isl1:CMICP-GFP) transgenic zebrafish treated with OLA; (B) peripheral motor neurons: the peripheral nerves were bent and shorter in Tg(NBT:MAPT- GFP)zc1 transgenic zebrafish treated with OLA; (C) the reduction percentage of peripheral motor neuron length in larvae after exposed to 0, 25, 50, and 100 mg/L of OLA at 72 h post fertilization (hpf). Data are expressed as mean ± standard error (SE). Compared with control group: $^{**}p < 0.01$, $^{***}p < 0.001$

(13.40 ± 0.67), and (22.40 ± 1.49) times per minute in 200, 300, 500, and 1,000 mg/L of OLA-treated groups, respectively, compared with (4.60 ± 0.54) times per minute in the control group ($p < 0.001$ for all). OLA bellow 200 mg/L had no significant effect on zebrafish spontaneous tail coiling.

3.2.4 | Behavioral toxicity

Locomotor activity was evaluated in larvae at 120 hpf. Larvae treated with OLA (25, 50, and 100 mg/L) under continuous dark showed significant decrease in total movement distance compared with control, the reduction percentages of total distance were (43.49 ± 7.27)%, (52.32 ± 10.67)%, and (89.09 ± 5.68)% ($p < 0.01$ or 0.001), respectively (Figure 5A,5B). Zebrafish exposed to OLA also exhibited a significant decrease in average swimming speed and would speed down when the photoperiod shifted from dark to light (Figure 5C). Statistically significant deceleration was obeserved in zebrafish treated with OLA during the first and second dark phase (25, 50, and 100 mg/L: $p < 0.001$ for all the three), the first light period (50 and 100 mg/L: $p < 0.001$ for both) and the second light period (100 mg/L: $p < 0.001$), relative to control (Figure 5D).

3.2.5 | Mechanisms of developmental neurotoxicity

The mRNA level of genes related to the neuro-development was examined by q-PCR in zebrafish after OLA exposure at 120 hpf, including the expression of *α1-tubulin*, *gfap*, *mbp*, *syn2a*, *shha*, *elavl3*, *manf*, *gapd43*, and *ache*. Down-regulation of *elavl3* (0.74-, 0.80-, and 0.53-fold, $p < 0.001$, respectively), *gapd43* (0.86-, 0.74-, and 0.52-fold, $p < 0.001$, respectively), and *gfap* (0.89-, 0.77-, and 0.45-fold, $p < 0.001$, respectively) was observed in both 25, 50, and 100 mg/L exposure groups. The transcription of *α1-tubulin* and *shha* was significantly down-regulated by 0.72- ($p < 0.05$) and 0.68-fold ($p < 0.001$) respectively in the 50 mg/L group and by 0.34- and 0.54-fold ($p < 0.001$ for both) respectively in the 100 mg/L group. And, *ache* (0.34-fold, $p < 0.001$) *mbp* (0.41-fold, $p < 0.001$), *manf* (0.61-fold, $p < 0.001$) and *syn2a* (0.52-fold, $p < 0.001$) were also down-regulated significantly in 100 mg/L OLA groups (Figure 6).

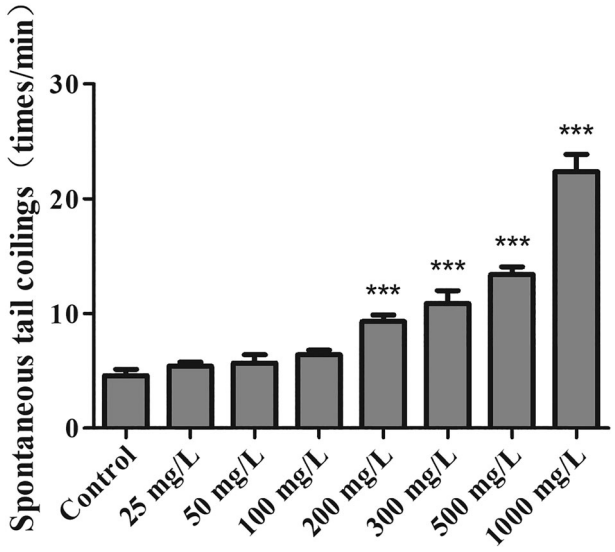

FIGURE 4 The numbers of spontaneous tail coilings were measured in zebrafish embryos exposed to 25, 50, 100, 200, 300, 500, and 1,000 mg/L of olaquindox (OLA) at 25 h. Data are expressed as mean ± standard error (SE). Compared with control group: ***$p < 0.001$

4 | DISCUSSION

Zebrafish has been widely used in chemical safety assessment and ecotoxicity evaluation. In this study, we investigated developmental toxicity of OLA in zebrafish, mainly focusing on developmental neurotoxicity, expected to understand more deeply about OLAs potential effects on human health and ecosystem. We found that OLA exposure to zebrafish induced brain and central nerve apoptosis, induced degeneration of axon and peripheral motor neuron, increased the spontaneous tail coiling but reduced locomotor activity. Pro-apoptotic gene *bax* was up-regulated, whereas an array of neuro-development and functions related genes were down-regulated. As supported by many earlier studies, the abnormal development of the central nervous system (CNS) and/or peripheral nervous system (PNS) was an important contributing factor to the toxic substance-induced neurotoxicity in the early life stage of zebrafish (Wang, Lam, et al., 2015; Wang, Yang, et al., 2015; Wu et al., 2016). To the best of our knowledge, As far as we know, this was the first study to report the developmental neurotoxicity and the possible mechanisms of OLA in an animal model.

OLA is known as a potent synthetic antibacterial agent commonly used in animal feed as a growth promoter. The abuse of OLA as animal feed additive has polluted the animal-derived food products as well as soil and water sources. Human health can be affected either directly through residues of an antibiotic in meat, which may cause side-effects, or indirectly through selection of antibiotic resistance determinants that may spread to a human pathogen (Marshall & Levy, 2011). OLA was found to be cytotoxic and genotoxic to Vero cells. Reduction in the viability of Vero cells by OLA was significant and resulted in 78% at 5 μg/ml and 14% at high dose of 80 μg/ml and OLA-induced DNA damage at 5–15 μg/ml (Chen et al., 2009). In the current study, OLA induced developmental neutral toxicity as low as

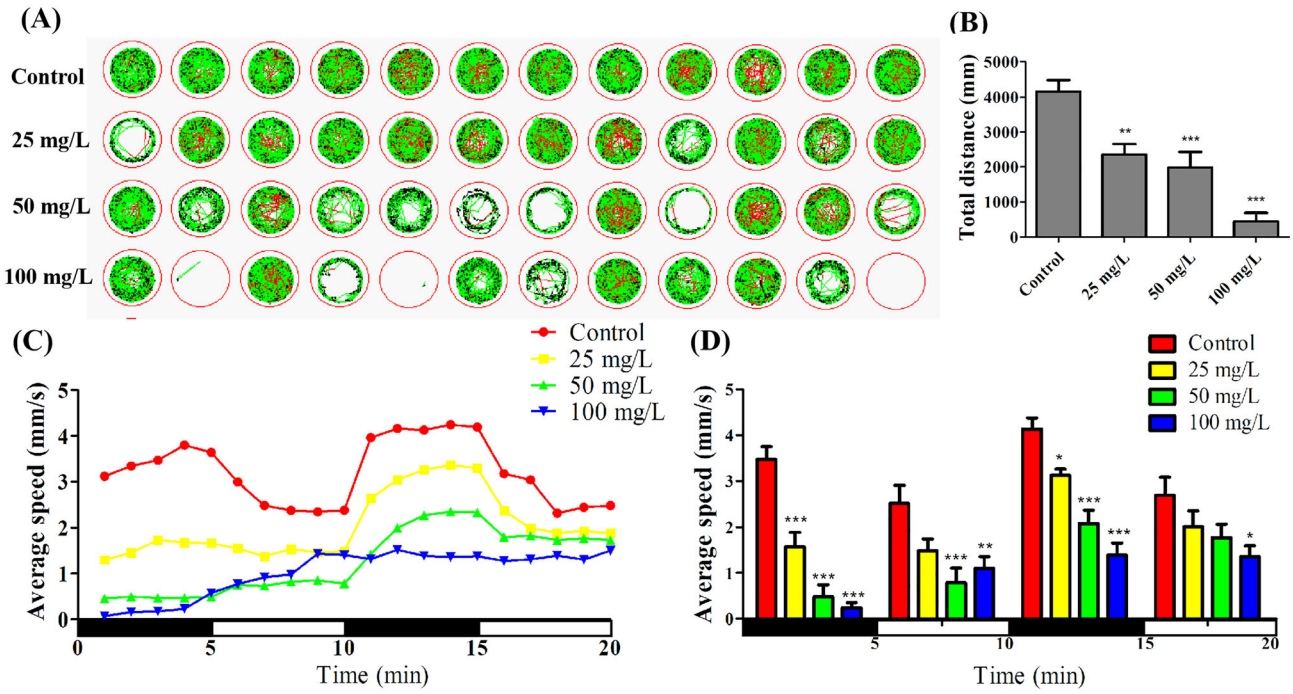

FIGURE 5 Locomotor behavior of zebrafish larvae at 120 h post fertilization (hpf) post exposure to olaquindox (OLA) was assessed. (A) Zebrafish movement distance decreased significantly in a dose-dependent manner; (B) Quantitative analysis of total movement distance after zebrafish exposed to OLA at concentrations of 25, 50, and 100 mg/L; locomotor patterns (C); and average swimming speed (D) during the dark–light–dark–light photoperiod stimulation test of the zebrafish from the control group and exposure groups were measured. Data are expressed as mean ± standard error (SE). Compared with control group: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

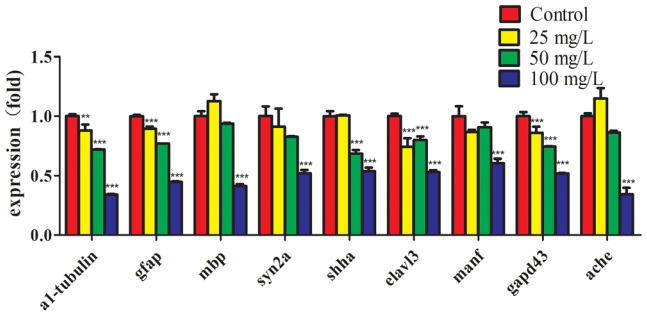

FIGURE 6 Relative expression levels of *α1-tubulin*, *gfap*, *mbp*, *syn2a*, *shha*, *elavl3*, *manf*, *gap43*, and *ache* in zebrafish after exposure to various concentrations of olaquindox (OLA) at 120 h post fertilization (hpf). Data are expressed as mean ± standard error (SE). Compared with control group: $^{**}p < 0.01$, $^{***}p < 0.001$

25 mg/L (μg/ml); the exposure levels were similar to the vitro cell study.

The developing embryonic brain is more sensitive to perturbation by toxins than the adult brain in most research of zebrafish (Rice & Barone, 2000) and mammalians (Costa, Steardo, & Cuomo, 2004; Crofton, Mundy, & Shafer, 2012; Thompson, Levitt, & Stanwood, 2009). In this study, apoptotic cells were found in the brain and spinal cord of zebrafish larvae at lower concentrations of OLA without other abnormal phenotypes, suggesting that the developing brain was very likely to be a major toxic target. The ratio of *bcl-2/bax* genes was decreased upon the treatment with OLA, implying that OLA exposure might reduce the expression of anti-apoptotic gene *bcl-2* and up-regulate pro-apoptotic gene *bax*, leading to apoptosis (Li et al., 2016; Zhu et al., 2015).

Behavior represents the sum of activities controlled by the nervous system, which can reflect the consequences of disruption of neuronal communications (Moser, 2011). Behavior test is widely used in neurotoxicity testing of pharmaceutical and environmental chemicals (Li et al., 2015). The locomotor behavior of zebrafish is considered to be an important index of neural development and elucidates basic mechanisms underlying neurobehavioral toxicology, especially since the introduction of automated video tracking systems (Bailey, Oliveri, & Levin, 2013; Drapeau et al., 2002). Here, we found that zebrafish embryos exposed to OLA just for 1 hour remarkably increased the spontaneous tail coiling without abnormal phenotype, indicating that OLA might cause neural excitation in a short time at high dose. However, in the locomotor activity tests, we found that zebrafish movement was significantly reduced after exposure to OLA for about 120 h at lower concentrations, even at a concentration that had no adverse effect on morphological development, hinting that OLA may have subacute or chronic neural inhibitory effect if exposed at a long time period. The abnormal behavior observed in this study was considered to represent developmental neurotoxicity caused by OLA as well as an adaptive response for defensing toxic stress (Chen, Yu, et al., 2012; Jin et al., 2016). The results were entirely consistent with previous reports on zebrafish exposed to other toxicants (Guo et al., 2018; Li et al., 2018; Qian, Liu, Lu, & Sun, 2018; Shi et al., 2018).

Exposure to OLA caused several other developmental abnormalities at higher exposure levels, such as increased mortality, reduced hatching rates, and reduced body length. The malformation percentages increased in a time- and concentration-dependent manner in zebrafish embryos. The most pronounced abnormalities were spinal curvature, notochord degeneration, and serious hepatotoxicity. These results suggest that high levels of OLA could induce more extensive damages on the developing zebrafish, in addition to the developmental neural toxicity.

Alteration in the expression of a variety of genes related to the CNS was another important factor that might contribute to developmental neurotoxicity (Chen, Sundvik, Rozov, Priyadarshini, & Panula, 2012). In this study, we measured and quantified nine marker genes in the zebrafish exposed to OLA via Q-PCR, that is, *α1-tubulin*, *gfap*, *mbp*, *syn2a*, *shha*, *elavl3*, *manf*, *gapd43*, and *ache*. The expression of two cytoskeleton-related genes (*α1-tubulin* and *gfap*) was observed to be significantly decreased. *α1-Tubulin* encodes an intermediate filament protein that is of importance in the microtubule cytoskeleton in the process of developing or regenerating dendrites and axons (Baas, 1997; Muller et al., 1999). *Gfap* is a sensitive and reliable astrocyte marker and a regulator of the astrocyte cytoskeleton differentiation and is important for many neuronal processes (Nielsen & Jorgensen, 2003). *Mbp* and *syn2a* gene play an important role in myelination and synapse development (Baumann & Pham-Dinh, 2001; Brosamle & Halpern, 2002; Muller, Bauer, Schafer, & White, 2013). The lower expression level of *mbp* gene suggested that exposure to OLA might affect the function of oligodendrocytes, and further affect the formation of myelin sheath. On the other hand, *syn2a* is a neuronal phosphoprotein that binds small synaptic vesicles to induce further synaptogenesis in mammals, playing an important role in both synaptogenesis and neurotransmitter release (Garbarino, Costa, Pestarino, & Candiani, 2014; Kao et al., 1998). Thus, the down-regulation of *syn2a* observed in zebrafish may affect synaptogenesis, neuronal differentiation, and neurotransmitter release and ultimately lead to neurobehavioral impairments. *Shha* is a signaling molecule that affects the form of 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 (Charron, Stein, Jeong, McMahon, & Tessier-Lavigne, 2003; Ingham & McMahon, 2001; Kolpak, Zhang, & Bao, 2005). The down-regulated transcription of *shha* indicated that OLA might have the potentiality to disturb the organization of the brain and the formation of organs in other organ systems (Chen, Huang, et al., 2012; Sun et al., 2016). *Elavl3* plays an important role in the neuronal development and individual behavior (Okano & Darnell, 1997; Pascale et al., 2004). As *elavl3* is an early neuronal marker, its down-regulation is likely a clue that OLA has adverse effects on developing neurons. *Manf* can maintain and regulate dopaminergic neurons and to direct the dopaminergic precursor cells differentiating into mature neurons (Chen, Yu, et al., 2012). *Ache* is essential for the development of neurons and muscle in the early life stage of zebrafish (Behra et al., 2002). *Gap43* is integral when

organisms offset the direct damage of toxicants (Alm et al., 2008), and down-regulation of *gap43* mRNA level after OLA exposure might disrupt its function in the process of neurite formation, regeneration, and plasticity, leading to developmental neurotoxicity in zebrafish larvae. We found that the expression of all nine marker genes for nervous system function or development was significantly down-regulated following OLA exposure. These findings indicate that OLA might induce developmental neurotoxicity through pathways involved in cytoskeleton regulation, axon growth, neuron maturation, and nervous system differentiation.

OLA was reported to reduce the number of intestinal *Escherichia coli* and suppressed *E. coli*-induced immune activation, which might be responsible for the enhanced *E. coli* growth in pigs (Ding, Wang, Zhu, & Yuan, 2006). OLA increased pathogen susceptibility in fish by inducing gut microbiota dysbiosis (He et al., 2017) and damaged earthworms and reduced catalase activity (Gao, Sun, Sun, & Bao, 2007). Our findings derived from this investigation provide valuable information for the potential neural health risk of OLA on humans and animals and could facilitate environmental and ecological risk assessment of OLA pollution.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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