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Clethodim exposure induces developmental immunotoxicity and neurobehavioral dysfunction in zebrafish embryos

Guanghua Xiong[a,b,c], Lufang Zou[a], Yunyun Deng[b,c], Yunlong Meng[a], Xinjun Liao[a,b,c], Huiqiang Lu[a,b,c,*]

[a] College of Life Sciences, Jinggangshan University, Ji'an, Jiangxi, China
[b] Jiangxi Engineering Laboratory of Zebrafish Modeling and Drug Screening for Human Diseases, Ji'an, Jiangxi, China
[c] Jiangxi Key Laboratory of Developmental Biology of Organs, Ji'an, Jiangxi, China

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ABSTRACT

Clethodim is one of the most widely used herbicides in agriculture, but its potential negative effects on aquatic organisms are still poorly understood. This study examined the effects of clethodim on zebrafish at aspects of early stage embryonic development, immune toxicity, cell apoptosis and locomotor behavior. Firstly, clethodim exposure markedly decreased the survival rate, body length, and heart rate and resulted in a series of morphological abnormalities, primarily spinal deformities (SD) and yolk sac edema, in zebrafish larvae. Secondly, the number of immune cells was substantially reduced but the levels of apoptosis and oxidative stress were significantly increased in a dose-dependent manner upon clethodim exposure. Thirdly, we evaluated the expression of some key genes in TLR signaling including *TLR4*, *MyD88*, and *NF-κB p65* and they were all upregulated by exposure to 300 μg/L clethodim. Meanwhile, some proinflammatory cytokines such as *TNF-α*, *IL-1β*, *IL8,* and *IFN-γ* were also activated in both the mock and the *TLR4-KD* conditions. Moreover, the locomotor behaviors and the enzymatic activities of AChE were obviously inhibited but the levels of acetylated histone H3 were greatly increased by clethodim exposure. In addition, incubation of zebrafish larvae with acetylcholine receptor (AChR) agonist carbachol can partially rescue the clethodim-modulated locomotor behavior. Taken together, our results suggest that clethodim has the potential to induce developmental immunotoxicity and cause behavioral alterations in zebrafish larvae. The information presented in this study will help to elucidate the molecular mechanisms underlying clethodim exposure in aquatic ecosystems.

1. Introduction

Clethodim is a highly efficient, safe and selective post-emergence herbicide, which can be used to control annual and perennial grasses in a wide variety of broad leaf crops such as soybean, cotton, sunflower and most vegetables [1–3]. Because of its high effectiveness against target species and relatively low mammalian toxicity, clethodim is extensively used for agricultural and non-agricultural applications [4–6].

However, like other herbicides, clethodim is water-soluble and can be easily transferred into aquatic environments. Previous studies have shown that clethodim is moderately toxic to fish and aquatic invertebrate species [7,8]. The reported 96 h LC50 varied from 18 mg/L to 56 mg/L in *Rainbow trout* and was 33 mg/L in *Bluegill sunfish* [9,10]. A 48 h LC50 of 20.2 mg/L has been reported for *Daphnia species* in the commercial formulation of clethodim [11]. Nevertheless, these studies were not in depth and there is limited informations on the molecular mechanisms of herbicide toxicity, especially from immunological and neurobehavioral perspectives [12,13].

The zebrafish (*Danio rerio*) is an ideal vertebrate model for studying developmental immunotoxicity and neurotoxicity [14,15]. Zebrafish embryos have numerous advantages such as small size, transparency, external fertilization, rapid development, and extreme sensitivity to many pesticides and herbicides [16]. Moreover, the proportion of homologous genes between zebrafish and humans is as high as 87%; and more importantly, the conservation of disease-related genes between them is up to 99% [17,18]. Furthermore, there is a clear temporal separation between innate and adaptive immune responses in zebrafish, and this provides a convenient system for in vivo study of the vertebrate innate immunity [7,19].

The zebrafish innate immune system is primarily comprised of neutrophils and macrophages [20]. Neutrophils are the first immune cells to fight against infection, and subsequently macrophages are

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[*] Corresponding author. College of Life Sciences, Jinggangshan University, Ji'an, Jiangxi, China.
E-mail address: luhq2@126.com (H. Lu).

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recruited to inflammatory tissues, with their main functions being phagocytosis of pathogens and tissue debris [21]. The innate immunity of zebrafish relies heavily on toll-like receptor (TLR) signaling and the members of TLR family along with associated adaptor molecules are highly conserved with other vertebrates [22,23]. TLRs are an important class of pattern recognition receptors (PRRs) that recognize microbial and danger signals, and ligand binding of TLRs is vital for initiation of the innate immune response [24]. The adaptor myeloid differentiation factor 88 (MyD88) plays a central role in signal transduction, and can transmit TLR signaling and activate key downstream transcription factors such as NF-κB, eventually leading to the up-regulation of proinflammatory cytokines such as *TNF-α, IL-1β, IL8, IFN-*γ [25,26].

Previous studies have shown that pesticides can disrupt the defense pathways of exposed aquatic organisms [27]. For example, cyhalofop-butyl was highly toxic to embryonic development in zebrafish, and resulted in significant up-regulation of reactive oxygen species (ROS) and cell apoptosis [15]. Cypermethrin can induce immunotoxicity and activate apoptosis-related genes, including *Bax, Bcl-2* and *caspase-3* in zebrafish [14]. Acetochlor can result in malformations and significantly up-regulate the innate immune genes, such as *IL-1β*, *CXCL-C1c* and *IL8* [28]. In addition, phthalate esters (PAEs) can damage hemocytes and decrease the cellular immunity of prawns [29,30].

It should be noted that chemical pesticides can target the nervous system and disrupt neurotransmitter transmission [31,32]. For example, chlorpyrifos can induce neurobehavioral disorders and cause learning deficits and locomotor alterations [33]. In addition, neurotoxic pesticides can selectively inhibit the acetylcholinesterase (AChE) activity of exposed aquatic organisms [34], and numerous studies have shown that histone acetylation may play a crucial role in behavioral changes [35]. Dieldrin induced an increase in the levels of histone H3 and H4 acetylation, and paraquat can promote histone acetylation associated with decreasing total histone deacetylase (HDAC) activity [36,37].

In the present study, we investigated the developmental immunotoxicity and neurobehavioral alterations of clethodim exposure in zebrafish embryos. Our results suggested that clethodim has the potential to induce developmental malformations, activate TLR4/MyD88 signaling pathway and proinflammatory genes. Clethodim caused the locomotor behavior disorders in zebrafish larvae, and the AChE activity was inhibited and the histone acetylation was promoted. In summary, this information will be helpful to understand the mechanisms of clethodim toxicity and bring new insights into the environmental risks of clethodim in aquatic ecosystems.

2. Materials and methods

2.1. Chemical reagents

Clethodim (purity > 99%) was purchased from Sigma-Aldrich (CAS No. 99129-21-2, St. Louis, USA). The stock solution (10 mg/L) was dissolved in dimethyl sulfoxide (DMSO) and stored at 4 °C. A working solution was prepared by diluting of the stock solution immediately prior to experimental use. It is worth mentioning that clethodim is available in both technical form and an emulsifiable concentrate. Unless otherwise specified, data presented herein refer to the technical product. Trichostatin A (TSA) was purchased from MedChemExpress (CAS No. HY-15144, MCE) and carbachol was purchased from Sigma-Aldrich (CAS No. 1092009, St. Louis, USA). All other reagents utilized were of analytical grade.

2.2. Maintenance of zebrafish

The wild type AB zebrafish and the *Tg (lyz: DsRed)* and *Tg (rag2: DsRed)* transgenic lines were used in this study. Zebrafish breeders were maintained at 28 ± 1 °C with a photoperiod of 14/10 (light/dark), and kept in a recirculating system in our facilities according to the established protocols. The fishes were fed twice daily with live brine shrimp. Male and female adult fish (1:1 or 2:1 ratio) were separated in isolated regions within spawning boxes overnight. The next morning, spawning was triggered once the baffle was removed. Subsequently, embryos were collected in egg water ready for use. All experimental fish were approved by the Institutional Animal Care and Use Committee (IACUC) of Jinggangshan University.

2.3. Embryo acute toxicity test

The acute toxicity test was performed on zebrafish embryos using a previously described method (Mu et al., 2013; Zhao et al., 2018). Briefly, normal embryos at 6 h post-fertilization (hpf) were randomly distributed in 6-well culture plates (20 embryos per well with 5 ml solution, four replicates for each group) and exposed to clethodim with concentrations of 10, 50, 100, 300 and 500 μg/L, respectively. The hatched larvae were counted and the survival rate was analyzed. In addition, body lengths (at 120 hpf) and heart rates (at 72 hpf) were determined manually. The fresh solutions were replaced every 24 h and dead embryos/larvae were removed daily.

2.4. Quantification of innate and adpative immune cells

Neutrophils were selectively labelled in *Tg (lyz: DsRed)* transgenic lines by using live imaging, and the red fluorescence in the clethodim-treated group was compared with that in the carrier (DMSO) alone group. On the other hand, the clethodim exposed larvae were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C and were stained with Sudan Black to highlight neutrophils. To further determine the distribution of macrophages affected by clethodim exposure, the albino zebrafish embryos were stained with 2.5 mg/ml neutral red at 72 hpf, and dorsal images of heads were acquired using an inverted fluorescence microscope (Leica M205FA, Germany). Next, we specifically tracked differentiating thymocytes in the *Tg (rag2:DsRed)* transgenic larvae and the fluorescence of dsRed positive cells was compared between the clethodim group and the control group. Ultimately, all the images were analyzed for total fluorescent areas above a threshold, and particle counting and quantification were completed using the ImageJ software (NIH, Bethesda, MD).

2.5. RNA extraction and qRT-PCR

Total RNA was extracted from zebrafish embryos or larvae using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. And 1 μg of total RNA was reverse transcribed using M-MLV reverse transcriptase (Invitrogen) to synthesize the first-strand cDNA. qRT-PCR was performed using SYBR Green reagents on the ABI StepOne Plus system (Applied Biosystems, USA). The thermal cycle was as follows: denaturation for 2 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, annealing at 60 °C for 15 s and extension at 72 °C for 30 s. Relative gene expression was normalized to an endogenous control gene (β-actin) and was calculated using the comparative Ct method formula $2-\Delta\Delta Ct$ (triplicate for each treatment). The sequences of the primers used in this study are listed in Supplemental Table One.

2.6. Measurement of anti-oxidative content and activity

To determine the enzymatic activities of acetylcholinesterase (AChE), catalase (CAT), succinate dehydrogenase (SDH) and the contents of malondialdehyde (MDA) influenced by clethodim, approximately forty fertilized zebrafish embryos were selected and exposed to 50, 100 and 300 μg/L clethodim at 72 hpf. After the exposure, about 20 larvae in each group were collected and homogenized on ice with 200 μL ice-cold PBS. The homogenate was centrifuged at 4000 × *g* at 4 °C for 15 min to obtain the supernatant, and this extract was used to analyze the enzyme activities or contents according to the

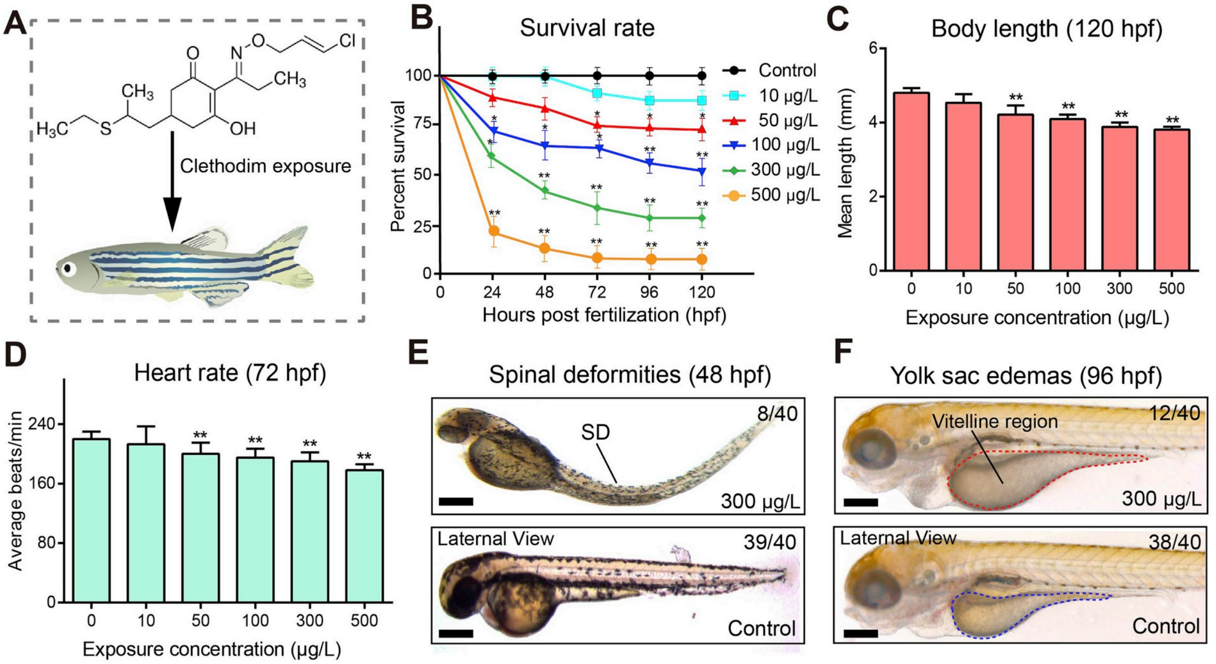

Fig. 1. Clethodim induces developmental toxicity in zebrafish embryos.
(A) Schematic diagram of acute toxicity test in zebrafish. The top panel shows the molecular formula of clethodim. (B) The survival rates of zebrafish embryos that exposed to various concentrations of clethodim, the data were evaluated from 24 hpf until to 120 hpf. The asterisks indicate significant differences compared with the control group (Dunnett post-hoc test, *, $p < 0.05$; **, $p < 0.01$). (C) The body lengths of zebrafish larvae that exposed to different concentrations of clethodim at 120 hpf (Student's *t*-test, **, $p < 0.01$). (D) The heart rates of zebrafish larvae that exposed to different concentrations of clethodim at 72 hpf (Student's *t*-test, **, $p < 0.01$). (E) Spinal deformities (SD) were observed in zebrafish larvae when exposed to 300 µg/L clethodim at 48 hpf. The representative photographs from each group are shown. Scale bar = 50 µm (F) Yolk sac edemas were observed in zebrafish larvae when exposed to 300 µg/L clethodim at 96 hpf. The representative photographs from each group are shown. Scale bar = 100 µm.

manufacturer's protocols (Nanjing Jiancheng Bioengineering Institute, China). The specific enzyme activity was expressed as units per milligram of protein. Besides, the levels of reactive oxygen species (ROS) were measured using dichlorofluorescein-diacetate (DCFH-DA) and the fluorescence intensities were quantified using a microplate reader at 485 nm (SpectraMax iD3, MD, USA).

2.7. Whole-mount TUNEL staining of cell apoptosis

Whole-mount terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of apoptotic cells in embryos was performed using "Click-iT™ Plus TUNEL Assay" (Invitrogen) according to manufacturer's instructions. Briefly, 40 embryos at 24 hpf in each group were collected and the chorion was stripped off. The embryos were washed with PBS and fixed with 4% paraformaldehyde (PFA) at 4 °C for overnight, followed by incubation with 50 µL of TdT reaction mixture at 37 °C for 1 h in a hybridization chamber. After PBS wash, Click-iT Plus reaction was performed at 37 °C for 30 min, then the fluorescence was recorded through Alexa Fluor 647 dyes with the laser-scanning confocal microscope (Leica TCS SP8, Germany).

2.8. Morpholino knockdown experiments in zebrafish

The morpholino knockdown approach was used to interfere with *TLR4* gene function in zebrafish embryos. Briefly, two antisense morpholino oligonucleotides (GeneTools, OR, USA) (5′-AGTAAAGAAATT CATCTTGTGTGAT-3′, translational blocking) and (5′-GACGGAAACAC AAATGTGCTTACCT-3′, splice blocking) were designed, to specifically target the *TLR4* mRNA (GenBank accession no. NM_001328605). The standard vivo-morpholino from Gene Tools (5′-CCTCTTACCTCAGTTA CAATTTATA-3′) was used as negative control. For microinjection, morpholinos were diluted to desired concentrations in 1 × Danieau's solution containing 1% phenol red, and approximately 1 nl was injected into the yolk region of the 1- to 2- cell stage embryo.

2.9. Determination of locomotor behavior in larvae

All locomotor behavior detection was performed in 24-well plates during the light phase between 9 a.m. and 4 p.m. After exposure to 100 µg/L clethodim or carrier control at 96 hpf, the free-swimming activities of zebrafish larvae in response to a 10-min light-to-dark photoperiod stimulation were recorded. The tested larvae were monitored using the DanioVision Observation Chamber System (Noldus IT, Netherlands). The videos were analyzed using EthoVision XT software, and the total distances moved together with mean velocity were used for statistical analysis (one-way ANOVA).

2.10. Western blot analysis

Embryos or larvae were harvested at the indicated time and homogenized in ice-cold RIPA buffer (strong) supplemented with a proteinase inhibitor cocktail (Sigma-Aldrich). Protein concentrations were determined using the BCA protein assay kit (Thermo Scientific, USA). Equal amounts of protein were fractionated by SDS-PAGE gels, and transferred onto PVDF membranes. The membranes were blocked in Tris-buffered saline containing 5% defat milk for 2 h at room temperature. Next, the membranes were hybridized with the following primary antibodies at 4 °C overnight: anti-Bax (ab44718, Abcam), anti-Bcl-2 (ab94720, Abcam), anti-caspase-3 (ab13585, Abcam), anti-TLR4 (ab13556, Abcam), anti-MyD88 (ab2064, Abcam), anti−NF−κB p65 (ab16502, Abcam), anti-Acetyl-Histone H3 (ab47915, Abcam), anti-beta-actin (ab8227, Abcam) and anti-GAPDH (#2118, Cell Signaling). Next day, the membranes were incubated with peroxidase-conjugated secondary antibody, and specific bands were detected by the ChemiDoc Touch Imaging System (Bio-Rad).

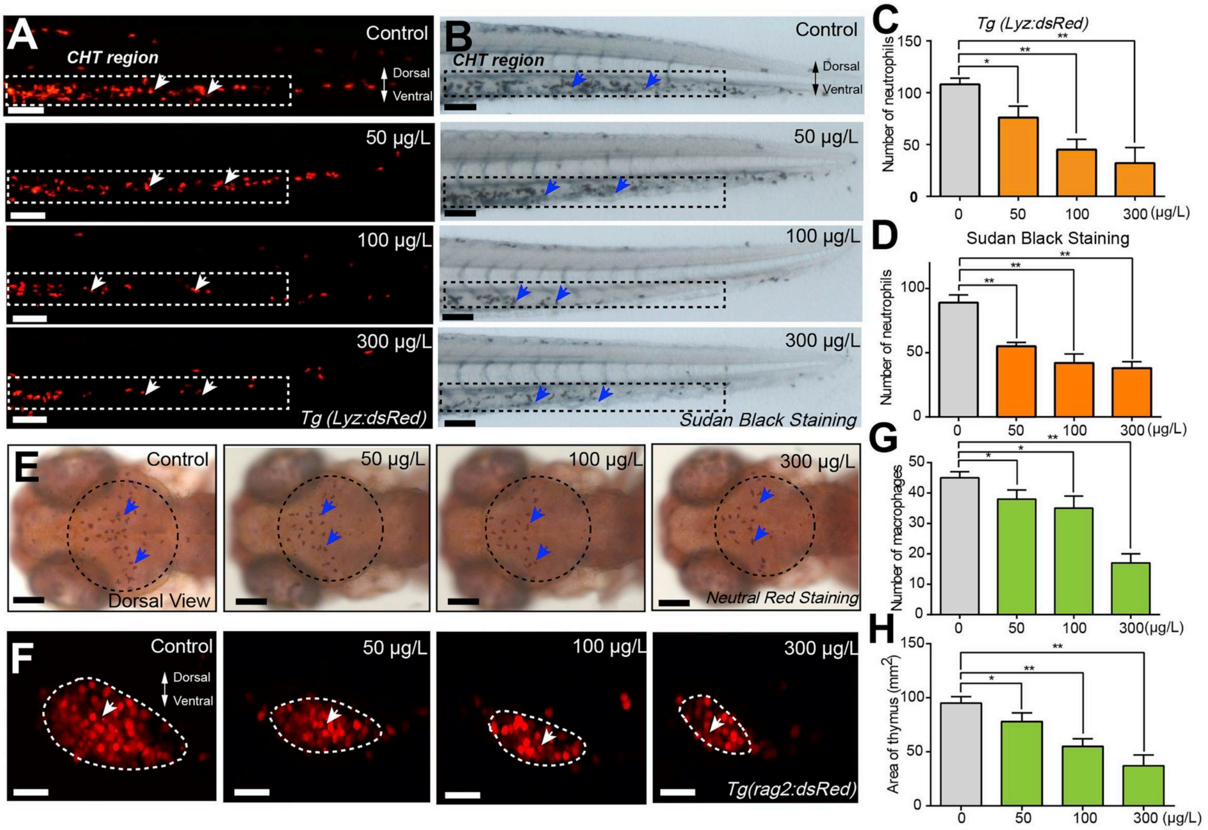

Fig. 2. Clethodim reduces the formation of neutrophil, macrophage and thymocyte in zebrafish larvae.
(A) Neutrophils were shown in *Tg (lyz: DsRed)* larvae at 48 hpf when exposed to different concentrations of clethodim. The white rectangle displays the CHT region of zebrafish tail and white arrow marks the RFP positive cells. (B) Neutrophils formation were shown in the CHT region of larval tail by Sudan Black Staining. The black rectangle displays the CHT region and the blue arrow marks the positive cells. (C,D) Quantification of neutrophil were performed in the selected areas of A and B. (E) Dorsal views of zebrafish larvae that exposed to different concentrations of clethodim at 72 hpf, then followed by fixating and staining with neutral red to mark macrophage. (F) Thymocytes were shown in *Tg (rag2: DsRed)* larvae that exposed to different concentrations of clethodim. The size of the thymus is marked by the white dash box. (G,F) Quantification of macrophages and thymocytes in the selected areas of E and F, respectively. For all experiments, at least 30 larvae were used for each treatment. *, $p < 0.05$; **, $p < 0.01$. Scale bar = 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.11. Statistical analysis

All experimental results were analyzed using the Prism 6 statistical package (GraphPad software). Morphological and behavioral differences were compared using the images captured by the microscope. Statistical significance was determined using the unpaired student's t-test or one-way ANOVA followed by Tukey's multiple comparison test. And the results were considered statistically significant when the *p* value was less than 0.05 or 0.01.

3. Results

3.1. The developmental toxicity of clethodim in zebrafish embryos

Clethodim is a widely used herbicide and the molecular formula is illustrated in Fig. 1A. To investigate the developmental toxicity of clethodim in aquatic organisms, zebrafish embryos were exposed to different concentrations of clethodim and the survival rate was calculated at multiple time points. The results showed that clethodim induced high mortality among embryos and caused a significant decrease of survival rate in a concentration-dependent manner (Fig. 1B). At a dosage of 500 µg/L clethodim, the survival rate dropped to 20% (8/40) at 24 hpf and almost all the exposed embryos were dead at 96 hpf. Based on the survival rates in tested concentrations, the lethal concentration 50 (LC50) of clethodim on zebrafish at 120 hpf was 100 µg/L.

In addition, clethodim exposure resulted in a series of morphological abnormalities in zebrafish embryos. The body lengths were significantly decreased as the body lengths at the exposed concentrations (50, 100, 300, and 500 µg/L) at 120 hpf were 6.8%, 7.4%, 10.6% and 12.8% less than that of the control group, respectively (Fig. 1C). Besides, the heart rate of zebrafish larvae at 72 hpf was obviously inhibited by 50 µg/L or higher dosage of clethodim exposure (Fig. 1D). Furthermore, other malformations such as spinal deformities (SD) were observed at 48 hpf in zebrafish larvae when exposed to 300 µg/L clethodim (Fig. 1E). Meanwhile, yolk sac edemas were also observed at 96 hpf when exposed to 300 µg/L clethodim (Fig. 1F). In summary, the above results suggested that clethodim can induce developmental toxicity in zebrafish embryos in a concentration-dependent manner.

3.2. Clethodim reduces the number of innate and adaptive immune cells

To evaluate the toxic effects of clethodim on the formation of innate immune cells, we firstly utilized the *Tg (lyz: DsRed)* transgenic line, which selectively expressed red fluorescent protein (RFP) and allowed us to track the individual neutrophils in zebrafish larvae. We focused on the larval tail because in this region neutrophils were normally restricted to the caudal hematopoietic tissue (CHT). As shown in Fig. 2A, the number of neutrophils was significantly decreased in the CHT region when exposed to different concentrations of clethodim at 72 hpf. To further validate these results, we specifically labelled neutrophils using Sudan Black Staining and obtained the similar results (Fig. 2B).

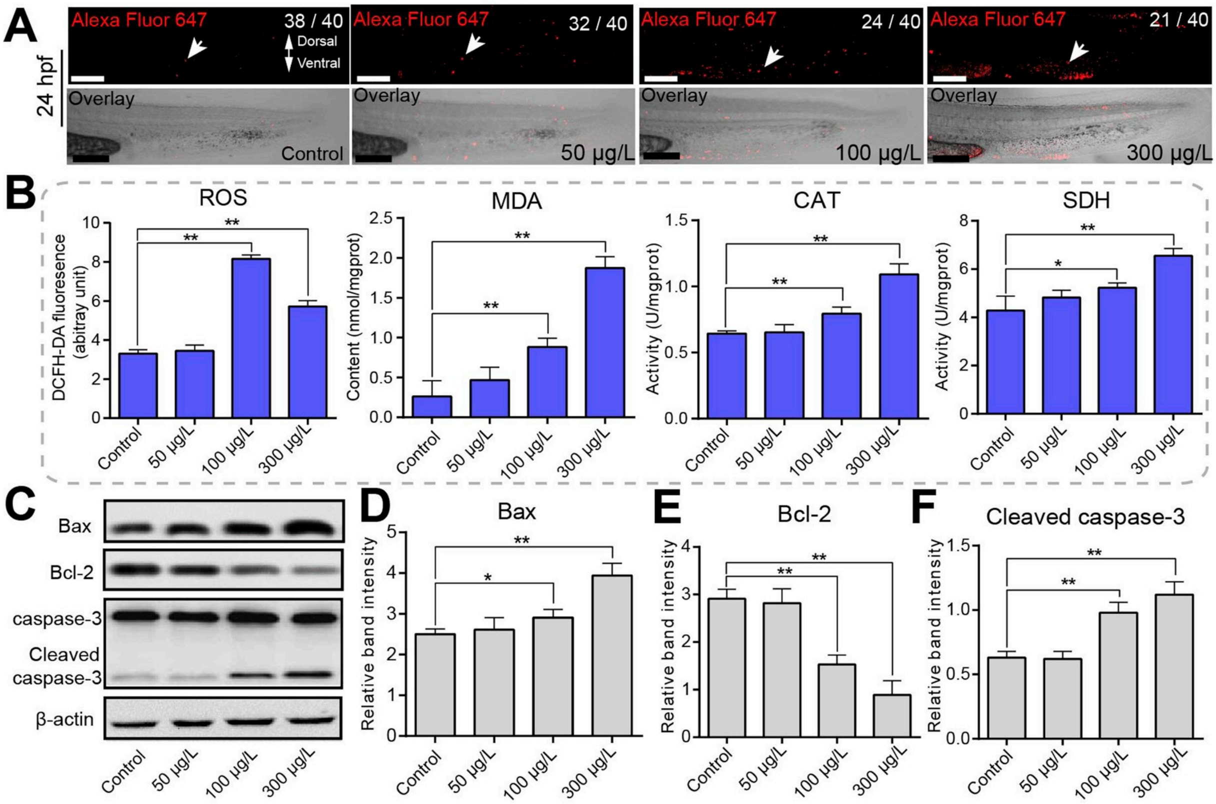

Fig. 3. Clethodim induces cell apoptosis and oxidative stress in zebrafish larvae.
(A) TUNEL staining of zebrafish larvae that treated with different concentrations of clethodim at 24 hpf. The white arrow indicates the apoptosis cells mainly appeared in caudal vascular region. The bottom images showed the overlay of red fluorescence with bright-field. (B) Detection the contents of ROS and MDA, along with enzymatic activities of CAT and SDH in larval zebrafish after exposure to different concentrations of clethodim. The values are presented as means ± SD (n = 4) from three biological replicates. (C) Detection the expression of Bax, Bcl-2 and cleaved caspase-3 protein by western blotting. The β-actin served as loading control. (D, E, F) Quantitation the expression levels of Bax, Bcl-2 and cleaved caspase-3 by ImageJ software. Data are expressed as fold-changes of clethodim-treated groups compared with the control group (*, $p < 0.05$; **, $p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

And quantification of neutrophils that presented in Fig. 2A and B demonstrated that clethodim exposure dramatically reduced the number of neutrophils in zebrafish larvae (Fig. 2C and D).

Next, zebrafish macrophages were stained with neutral red and the dorsal images of heads were acquired. As shown in Fig. 2E, clethodim exposure caused an obvious reduction in macrophage formation in a concentration-dependent manner. The number of macrophages was greatly down-regulated in both 100 μg/L and 300 μg/L clethodim-treated groups (Fig. 2G). To examine the toxic effects of clethodim on adaptive immune cells in zebrafish larvae, we used the *Tg (rag2: DsRed)* transgenic line to display thymocytes, which can differentiate into mature T lymphocytes in the thymus. Our results suggested that thymic dysplasia was significantly increased and the area of the thymus was greatly decreased after clethodim exposure in a dose-dependent manner (Fig. 2F and H).

3.3. *Clethodim increases the cell apoptosis and oxidative stress*

To test whether clethodim affects cell apoptosis in zebrafish, newly collected embryos exposed to different concentrations of clethodim were stained with TUNEL assays to detect apoptotic cells. The results suggested that clethodim significantly increased the levels of cell apoptosis, which mainly appeared in the caudal vascular region, in a dose-dependent manner (Fig. 3A). Besides, the change of oxidative stress is another important event in aquatic toxicology. Exposure to chemical toxicants may disturb the endogenous balance of ROS, and the elevated levels of ROS can subsequently be eliminated by antioxidant enzymes. Our results showed that the contents of ROS and MDA and enzymatic activities of CAT and SDH were markedly increased in the clethodim-treated groups (Fig. 3B). Furthermore, Bax is a pro-apoptotic Bcl-2 family protein that resides in the cytosol and translocates to mitochondria upon induction of apoptosis. As shown in Fig. 3C–F, the protein levels of Bax were significantly increased while the expression of Bcl-2 was correspondingly reduced in clethodim-treated groups. The increased Bax/Bcl-2 ratio may modulate apoptosis-associated processes as associated increase of caspase-3 activation was observed. Overall, the above results suggested that clethodim exposure can induce cell apoptosis and oxidative stress in zebrafish larvae.

3.4. *Clethodim induces MyD88-dependent TLR signaling activation*

TLR immune signaling plays an essential role in innate immunity, but its role in aquatic toxicology is largely unknown. The TLR pathway is triggered by toll-like receptors (TLRs) and the adaptor MyD88 is important in the induction of inflammatory cytokines through the activation of downstream transcription factor nuclear factor-kappa B (NF-κB). Our data showed that the relative mRNA levels of *TLR4, MyD88* and *NF-κB p65* were significantly increased when the zebrafish embyros were exposed to 300 μg/L clethodim (Fig. 4A). We also examined the transcription level of TRIF, which is another adaptor responding to the activation of TLRs. Surprisingly, TRIF was sharply decreased in zebrafish larvae after clethodim exposure. Besides, the protein levels of *TLR4, MyD88* and *NF-κB p65* were also elevated with the increase of clethodim dosage (Fig. 4B). Taken altogether, these results demonstrated that

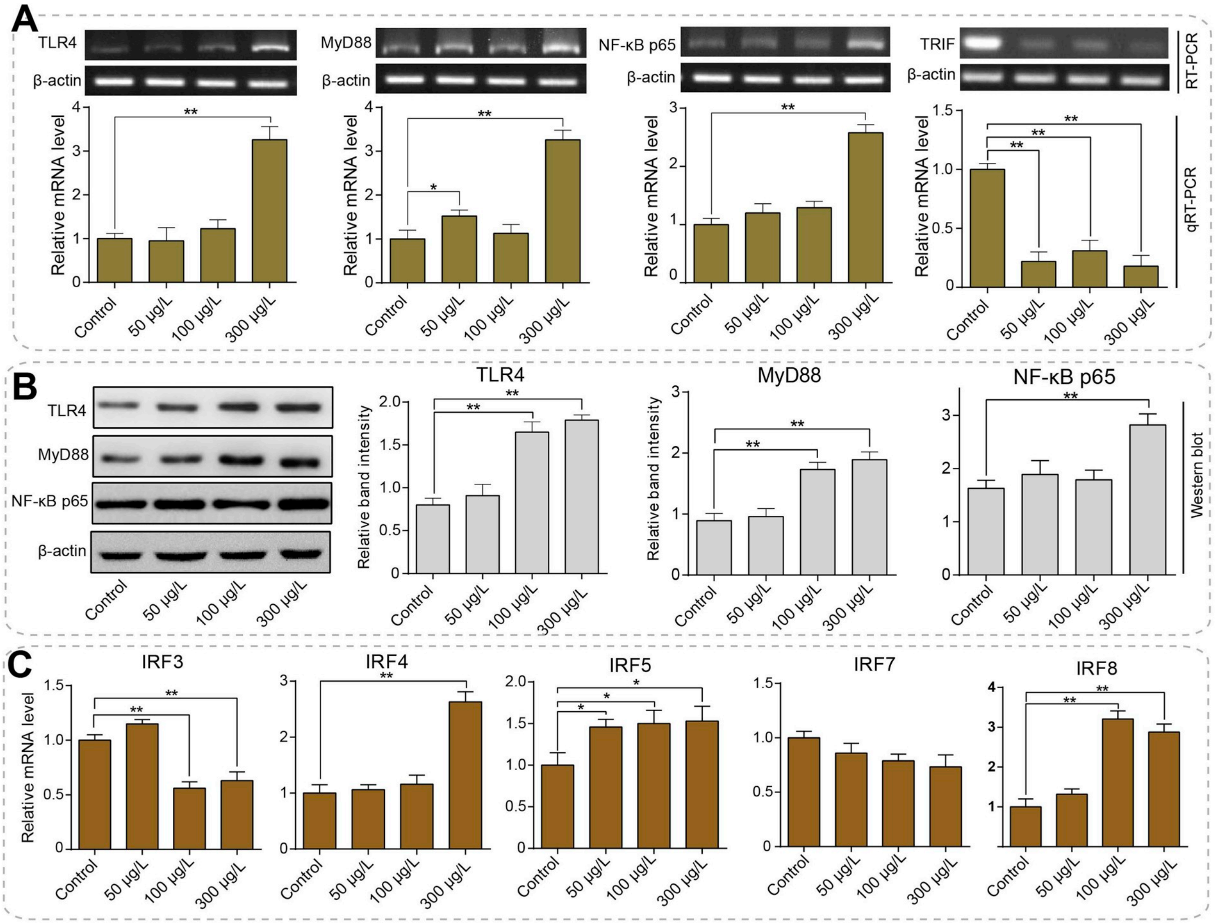

Fig. 4. Clethodim induces TLR4/MyD88-dependent immune signaling activation in zebrafish larvae.

(A) The mRNA expression patterns of *TLR4*, *MyD88*, *NF-κB p65*, and *TRIF* in zebrafish embryos exposed to different concentrations of clethodim, which analyzed by RT-PCR and qRT-PCR at 72 hpf. (B) The protein levels of *TLR4*, *MyD88*, *NF-κB p65* were analyzed by western blot and quantification of band intensity with ImageJ software. (C) The relative mRNA levels of interferon regulatory factor (IRF) −3, 4, 5, 7, and 8 in zebrafish embryos when exposed to different concentrations of clethodim. For all experiments, the values are represented as the mean ± SD (n = 4). *, $p < 0.05$; **, $p < 0.01$.

clethodim can induce TLR signaling activation through TLR4-mediated and MyD88-dependent signaling pathway.

On the other hand, interferon regulatory factors (IRFs) are transcription regulators which play an important role in the inflammatory activation. IRF5 has a pivotal role in regulating the expression of cytokines and apoptosis-related genes. IRF4 and IRF8 are highly homologous and are essential for the normal development of granulocyte/monocyte. We also tested the expression levels of these IRFs in zebrafish embryos after clethodim exposure. Our results showed that IRF4, IRF5 and IRF8 were significantly up-regulated with the increase of clethodim dosage (Fig. 4C). Besides, IRF3 and IRF7 are the main regulators of type I IFN expression. However, the mRNAs levels of IRF3 were markedly down-regulated and IRF7 seemed to have no obvious change in zebrafish embryos after exposed to different concentrations of clethodim.

3.5. *The differential expression of immune-related cytokines by TLR4 knock down*

Toll-like receptor 4 (TLR4) plays a critical role in innate immunity. We successfully knocked down the*TLR4* gene in zebrafish embryos using two morpholinos, and the oligonucleotide (TLR4_MO-TL) had a higher efficiency to block protein synthesis (Fig. 5A and B). Moreover, the survival rate of 300 μg/L clethodim-treated larvae had a significant improvement at different developmental stages in*TLR4-KD* conditions when compared with the control group (Fig. 5C). JAK/STAT is another immune signaling pathway that involved in cell proliferation, differentiation, apoptosis and inflammation. We also examined the expressions of janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3) in JAK/STAT signaling by qRT-PCR. It seems to be no obvious difference between the mock and *TLR4-KD* conditions (Fig. 5D). These results further suggested that clethodim affect developmental phenotypes of zebrafish embryos mainly through the TLR signaling pathway.

Next, to further elucidate the expression profiling of innate immune genes in clethodim-treated groups, we performed quantitative real-time PCR analysis of 8 cytokines in mock and *TLR4-KD* conditions. We found that nearly all the immune-related genes were significantly down-regulated in *TLR4-KD* conditions compared with the mock group (Fig. 5E). Clethodim exposure increased the expression levels of pro-inflammatory cytokines such as *TNF-α, IL-1β, IL6, IL8*, and *IFN-*γ in a dose-dependent manner both in the mock and *TLR4-KD* groups. However, some anti-inflammatory cytokines such as *TGF-β* and *IL10*, that were no obvious difference after clethodim exposure in *TLR4-KD* conditions, suggesting it have opposing roles in TLR signaling activation. We also tested another colony stimulating factor 1a (*CSF1a*), which causes hematopoietic stem cells to differentiate into macrophages. The *CSF1a* has similar expression profiles with some pro-inflammatory cytokines. The evidence further demonstrated that TLR signaling plays a vital role in the clethodim-mediated immunotoxicity in zebrafish embryos.

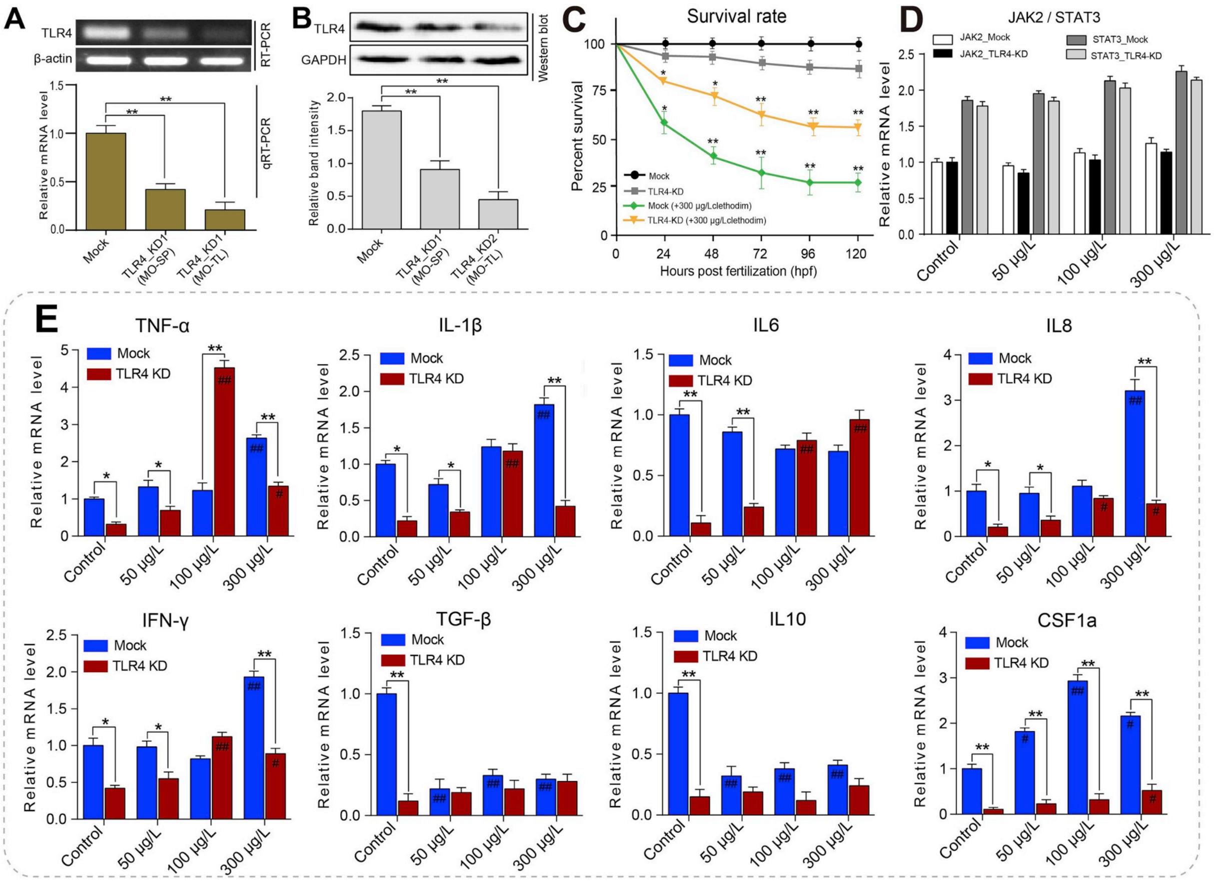

Fig. 5. The survival was greatly improved and the cytokines were significantly down-regulated by *TLR4* knock-down.

(A) Knock-down of *TLR4* in zebrafish embryos using two morpholinos. Morpholino-splice blocking (abbreviated for MO-SP) and morpholino-translational blocking (abbreviated for MO-TL). The relative mRNA levels of *TLR4* in each group were analyzed by RT-PCR and qRT-PCR, respectively. (B) Detection the *TLR4* protein levels in each group by western blotting. The relative band intensities were analyzed by ImageJ software. (C) The survival rates of larval zebrafish exposed to 300 μg/L clethodim in both the mock and *TLR4-KD* conditions. The asterisks indicate significant differences compared with the control group (Dunnett post-hoc test, *, $p < 0.05$; **, $p < 0.01$). (D) The relative mRNA levels of JAK2 and STAT3 after exposed to different concentrations of clethodim in both mock and *TLR4-KD* groups. (E) Quantitative real-time PCR analysis of pro-inflammatory and anti-inflammatory cytokines with different concentrations of clethodim exposure in mock and *TLR4-KD* groups. For all experiments, the values are represented as the mean ± SD (n = 4). *, $p < 0.05$, **, $p < 0.01$ (inter-group differences); #, $p < 0.05$, ##, $p < 0.01$(intra-group differences).

3.6. Neurobehavioral dysfunction after clethodim exposure in zebrafish embryos

In addition, we have also investigated the neurotoxicity of clethodim exposure in zebrafish embryos. We used the DanioVision Observation Chamber System to evaluate the locomotor behavior of zebrafish larvae affected by clethodim exposure. The results showed that 100 μg/L clethodim can significantly decrease the locomotor behavior of larval zebrafish at 5 dpf, as observed both from the locomotion traces and from the behavioral heatmaps (Fig. 6A and B). Besides, both the total distances moved and the mean velocity were significantly lower in the 100 μg/L clethodim-treated group than the corresponding values in the control group (Fig. 6C and D). The acetylcholinesterase (AChE) is an enzyme that catalyzes the hydrolysis of acetylcholine and has an important role in neural signal transduction. Our results suggested that AChE enzymatic activities were also extensively inhibited in the clethodim-treated group (Fig. 6E).

Previous studies indicated that histone acetylation is associated with neural plasticity and behavior. Our results demonstrated that the mRNA levels of histone deacetylase 1 (HDAC1) was sharply decreased with clethodim exposure (Fig. 6F). Besides, the level of acetylated histone H3 (AcH3), was significantly increased in a dose-dependent manner after clethodim exposure, which was similar to the effect of the HDAC inhibitor trichostatin A (TSA) treatment in zebrafish larvae (Fig. 6G). Moreover, incubation of acetylcholine receptor (AChR) agonist carbachol (10 μg/L) with the 300 μg/L clethodim-treated zebrafish larvae for 72 h can partially rescue the neurobehavioral disorders, by exhibiting slight increase in the locomotor behavior (Fig. 6H and I). Meanwhile, the total distance moved and mean velocity of larvae in the carbachol-treated groups were also improved (Fig. 6J and K). These results suggested that AChE and histone acetylation were involved in clethodim-induced neurotoxicity although the mechanism of which is not well understood.

4. Conclusion

In summary, our findings indicated that clethodim exposure induced the developmental immunotoxicity and neurobehavioral disorder in zebrafish larvae. Clethodim can reduce the number of immune cells and increase the levels of apoptosis and oxidative stress. Clethodim also activated the TLR4-mediated and MyD88-dependent immune signaling and induced the expression of inflammatory-related genes. In addition, clethodim can inhibit the locomotor behavior and modulate the AChE activities and histone acetylation. Given the observed immunotoxic and neuroprotective effects, further studies using various animal models to determine the environmental risks of clethodim are warranted.

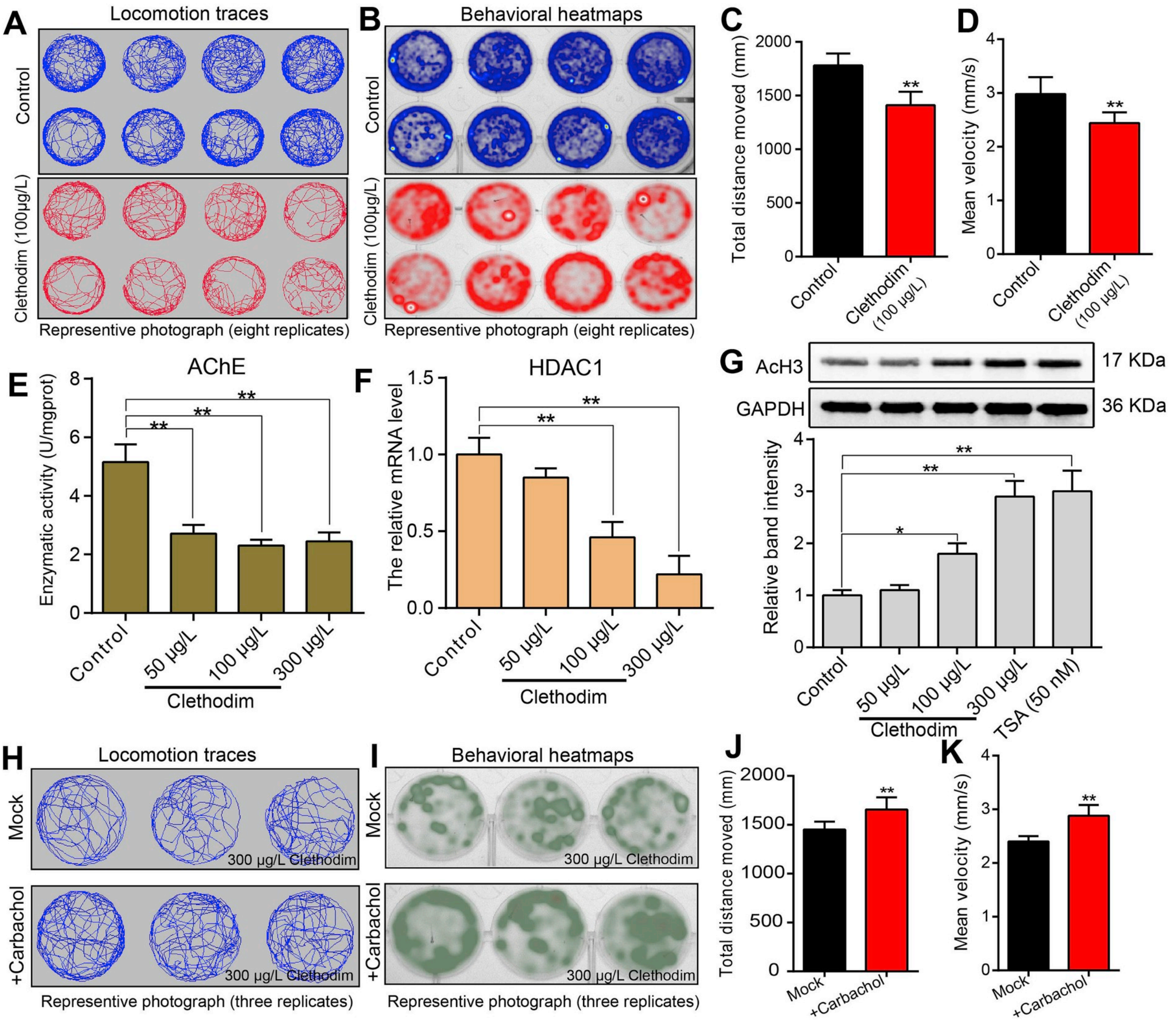

Fig. 6. Clethodim induces neurobehavioral dysfunction and modulates AChE activities and histone acetylation in zebrafish larvae.
(A) The locomotion traces of zebrafish larvae exposed to 100 μg/L clethodim at 5 dpf. Eight representative photographs are shown in each group. (B) The behavioral heatmaps of zebrafish larvae exposed to 100 μg/L clethodim at 5 dpf. (C, D) The total distance moved and average velocity of zebrafish larvae exposed to 100 μg/L clethodim at 5 dpf. The values are represented as the mean ± SD (n = 4). *, $p < 0.05$; **, $p < 0.01$. (E) The AChE enzymatic activities of larval zebrafish exposed to different concentrations of clethodim. (F) The relative mRNA levels of HDAC1 in different concentrations clethodim exposure. (G) The protein expression levels of acetylated histone H3 (AcH3) of clethodim exposed zebrafish embryos, the trichostatin A (TSA) -treated group serve as positive control. (H, I) The locomotion traces and behavioral heatmaps of clethodim-exposed larval zebrafish in mock and carbachol-incubated groups. Three representative photographs are shown in each group. (J, K) The total distance moved and average velocity of clethodim-exposed larval zebrafish in mock and carbachol-incubated groups. The values are represented as the mean ± SD (n = 4). *, $p < 0.05$; **, $p < 0.01$.

5. Discussion

In recent decades, the use of herbicides has extensively improved the labor efficiency in agricultural production. However, the adverse effects of herbicides in aquatic organisms, including zebrafish, have raised great concern allover the world. This study comprehensively evaluated the aquatic toxicity of clethodim exposure in the early stages of zebrafish development. Our data clearly indicated that clethodim was highly toxic to zebrafish embryos and larvae, with a LC_{50} value of 100 μg/L clethodim for 120 hpf larvae. Clethodim exposure also caused a series of developmental abnormalities, such as inhibited growth, reduced heart rate, and declined survival rate, together with morphological deformities during embryonic development. The most pronounced morphological alterations of zebrafish larvae exposed to 300 μg/L clethodim were spinal deformities and yolk sac edema, and these occurred in up to 20% (8/40) and 30% (12/40), respectively. The same malformation has been reported in zebrafish embryos exposed to environmental toxicants, such as cyhalofop-butyl [15] and chlorpyrifos [38].

To investigate whether clethodim exposure could modulate the immune cells, we analyzed the neutrophils and macrophages using transgenic zebrafish lines combined with staining techniques. We found that clethodim can induce a progressive reduction of neutrophils in the entire tail. In addition, clethodim caused a significant decrease in macrophage formation in a concentration-dependent manner, and thymic dysplasia was also observed with clethodim exposure. These results suggested that clethodim can affect the quantity and distribution of innate and adaptive immune cells in zebrafish larvae. Similar results have also been reported in a previous study. For example, it's demonstrated that dibutyl phthalate (DBP) can inhibit the neutrophil and macrophage formation and decrease the ability of macrophage phagocytosis [39].

Clethodim exposure can significantly increase the levels of cell apoptosis according to the TUNEL staining, and it should be noted that the majority of apoptosis cells accumulated in the caudal vascular region. Oxidative stress has become another important focus of aquatic toxicity. Our results showed that the expressions and activities of antioxidant proteins including ROS, MDA, CAT, and SDH were significantly up-regulated with clethodim exposure. In the current study, the ratio of Bcl-2/Bax was decreased and the cleaved caspase-3 was upregulated with treatments of different concentrations of clethodim, suggesting that high concentrations of clethodim might change the ratio between Bcl-2 and Bax and lead to the activation of apoptosis-associated pathways. Similar results were observed in cypermethrin and *cis*-bifenthrin induced apoptosis and immunotoxicity in larval zebrafish [14,40]. In brief, our data suggested that oxidative stress and cell apoptosis played important roles in the clethodim-induced immunotoxicity.

Previous studies showed that environmental toxicants could induce or inhibit the mRNA expression of innate immune-related genes [41,42]. TLR4 is a pattern recognition receptor that recognizes PAMPs and initiates inflammation in the membrane of immune cells. Chemical toxicants can recognize and bind to TLR4 and activate the nuclear transcription factor NF-κB through adaptor of MyD88, the most classical immune signaling, that results in the releasing the cytokines and a series of inflammatory proteins. In the present study, 300 μg/L clethodim exposure significantly activated TLR immune signaling and increased the expressions of *TLR4, NF-κB,* and *MyD88* at both the mRNA and protein levels. TNF-α is secreted by activated macrophages and Il-1β activates neutrophils and macrophages. Il-6 promotes the induction of acute phase proteins and IFN-γ belongs to the antiviral cytokines and mediates immune and inflammatory responses. Our results showed that these pro-inflammatory genes were all up-regulated in response to clethodim exposure, both in the mock and *TLR4-KD* conditons. It is surprised that the survival rate has significantly improved in the *TLR4-KD* groups, suggesting that TLR signaling are involved in clethodim-mediated developmental immunotoxicity in zebrafish.

Clethodim is an acetyl-coenzyme A carboxylase (ACCase) inhibitor, that is likely to interfere with lipid metabolism and even affect the neurobehavioral function of exposed organisms [37,38]. Our results strongly suggested that clethodim had the potential to induce neurotoxicity and locomotor behavior impairments in larval zebrafish. The moved distance and swimming velocity of zebrafish larvae exposed to 100 μg/L clethodim for 120 hpf were severely decreased compared with those in the control group. More importantly, the activities of AChE and HDAC1 were significantly inhibited by clethodim action, and this was thought to be involved in the observed swimming behavior deficits. However, the histone acetylation was up-regulated in a dose-dependent manner and incubation the zebrafish larvae with AChR agonist carbachol can partially reverse the locomotor behavior of clethodim-treated larvae.

In conclusion, the present study brings some new insights into the molecular mechanisms of clethodim toxicity including possible effects on development, immunotoxicity, apoptosis and locomotor behavior during the early stages of zebrafish. Future studies should focus on evaluating the toxic effects of clethodim from different perspectives.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.12.002.

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