Science of the Total Environment 899 (2023) 166306

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Developmental and neurobehavioral toxicity of 2,2′-methylenebis (6-tert-butyl-4-methylphenol) (antioxidant AO2246) during the early life stage of zebrafish

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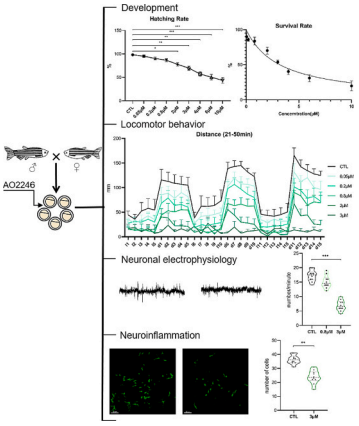
HIGHLIGHTS

- First report of neurobehavioral toxicity of AO2246 in zebrafish larvae
- AO2246 exposures induced defective locomotor activity in zebrafish larvae.
- AO2246 exposures induced abnormal neuronal electrophysiology in zebrafish larvae.
- AO2246 exposures induced neurological disorders in zebrafish larvae.
- AO2246 exposures induced altered transcriptional profile in zebrafish larvae.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Henner Hollert

Keywords:
Antioxidant AO2246
Fluorescence-labeled transgenic zebrafish
Locomotor behavior

ABSTRACT

Background: 2,2′-Methylenebis (4-methyl-6-tert-butylphenol) (AO2246) is a synthetic phenolic antioxidant extensively used in food packaging bags and cosmetics. Recently, AO2246 was detected with unexpectedly high concentrations in plasma and breast milk samples from pregnant and lactating women. Hence, it is essential to conduct a thorough investigation to evaluate the detrimental effects of AO2246 on biota.

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https://doi.org/10.1016/j.scitotenv.2023.166306
Received 27 December 2022; Received in revised form 30 July 2023; Accepted 12 August 2023
Available online 14 August 2023

Electrophysiology
Neurobehavioral toxicity
Gene expression profiles

Objective: To investigate the developmental and behavioral toxicity of AO2246 in zebrafish, as well as the molecular mechanisms underlying these effects.
Methods: Zebrafish embryos were exposed to AO2246 at concentrations ranging from 0.05 to 10 μM for up to 6 days postfertilization (dpf). Hatching rate, survival rate, heart rate, and body length were measured. Locomotor behavioral and electrophysiologal analyses were performed. Two fluorescence-labeled transgenic zebrafish lines (endothelium-Tg and macrophage/microglia-Tg) were employed. RNA sequencing was carried out.
Results: AO2246 has a 96-hour LC_{50} value of 3 μM. The exposure of AO2246 resulted in a significant reduction in both hatching rate and heart rate. Analysis of locomotor behavior demonstrated that larvae exposed to AO2246 doses exceeding 2 μM exhibited a significant decrease in both total distance and mean velocity. Electrophysiological recordings demonstrated a noteworthy reduction in spike activity at a concentration of 3 μM, relative to control conditions. The administration of AO2246 at 3 μM elicited morphological reactivity and immune alteration of the midbrain microglia in the macrophage/microglia-transgenic zebrafish line, indicating a potential contribution of neurological disorders to behavioral defects. RNA sequencing analysis revealed altered gene expression profiles at high AO2246 concentrations, particularly the dysregulation of pathways associated with neuronal function.
Conclusions: The present study demonstrates that AO2246 exposure elicits developmental and neurobehavioral toxicity in zebrafish larvae. Specifically, exposure to AO2246 was found to cause disturbances in neuronal electrophysiological activity and neurological disorders, which ultimately led to the impairment of locomotor behavior in zebrafish larvae.

1. Introduction

Antioxidants, both natural (such as vitamin A, C, and E) and synthetic, provide protection against the deleterious effects of oxidation and decay. While natural antioxidants are challenging to extract and preserve, synthetic antioxidants, particularly synthetic phenolic antioxidants (SPAs), are widely employed in various fields, including pharmaceuticals, dental materials, and everyday items, due to their established technology, cost-effectiveness, and efficacy (Wang et al., 2016; X. Xu et al., 2021). In recent years, there has been a growing concern regarding the environmental pollution and bioaccumulation toxicity to animals resulting from the widespread utilization of SPAs (Liu et al., 2015a; Liu and Mabury, 2018; Wang et al., 2018; Wang and Kannan, 2019; Du et al., 2019). 2,2′-Methylenebis (4-methyl-6-tert-butylphenol) (AO2246) is an important low molecular weight SPA. The total production volume of AO2246 reached 454–4536 tons per year in the United States (Liu and Mabury, 2020). Several studies reported the occurrence of AO2246, which has not received much attention previously, in different environmental matrices, such as 0.00068 μg/L in sewage effluent (China), 0.39 ng/g dry weight (dw) in indoor dust (North America), and 1.7–3.53 ng/g dw in sludge (China) (Liu et al., 2015a; Liu et al., 2015b; Wu et al., 2020). Recently, AO2246 was detected with unexpectedly high concentrations in maternal plasma (up to 80.8 ng/mL, equivalent to 0.24 μM), cord plasma (up to 69.7 ng/mL, equivalent to 0.21 μM), and breast milk samples (up to 3.14 ng/mL, equivalent to 0.01 μM) from pregnant and lactating women (Du et al., 2019; Zhang et al., 2020). This may be because of its generally used in food packaging bags and cosmetics (Qian et al., 2018; Liu and Mabury, 2020; Zhang et al., 2020; Wang et al., 2021). The increased utilization of AO2246 with rapid economic growth, coupled with its bioaccumulation potential, may result in an upward trend of its concentration within biota. Hence, it is essential to conduct a thorough investigation to evaluate the detrimental effects of AO2246 on biota.

Over the last years, zebrafish (*Danio rerio*) has gained prominence as a robust biological model for toxicological research, owing to its remarkable features such as high fecundity, rapid embryonic development, optically transparent embryo, and significant genomic similarity with mammals (Beis and Stainier, 2006; Peterson et al., 2008; Lammer et al., 2009). The suitability of zebrafish for neurotoxicity research is primarily attributed to the homology of neurodevelopmental processes between zebrafish and humans, as well as the ability to visualize the nervous system in zebrafish (Tropepe and Sive, 2003; Selderslaghs et al., 2013). Extensive investigations utilizing this in vivo model have facilitated a comprehensive understanding of the biological mechanisms underlying neurotoxicant-induced effects (Schartl, 2014; Kalueff et al., 2014). Previous research has indicated a correlation between exposure to AO2246 and reproductive dysfunction in the testes of male rats (Takagi et al., 1994; Takahashi and Oishi, 2006) and gonads of zebrafish (Yang et al., 2018a). AO2246 has been found to cause high mortality rates and morphological abnormalities in zebrafish (Yang et al., 2018b). However, the available data is restricted solely to morphological parameters, thereby leaving the effects of AO2246 on locomotor behavior, neurological function, and the underlying molecular mechanisms inadequately comprehended.

2. Material and methods

2.1. Reagents

2,2′-Methylenebis (4-methyl-6-tert-butylphenol) (AO2246; 119-47-1) was purchased from Sigma-Aldrich. Dimethyl sulphoxide (DMSO; 67-68-5) was used to prepare stock solutions of 1 M AO2246. The exposure solutions were prepared in embryo rearing medium (E3 medium, pH 7.2) to obtain final concentrations of 10, 6, 4, 3, 2, 0.8, 0.2, and 0.05 μM AO2246 (equal to 3.41, 2.04, 1.36, 1.02, 0.68, 0.27, 0.07, and 0.02 mg/mL). The solvent control group was 0.001 % (v/v) DMSO. Studies have not shown any effect of 0.001 % DMSO on various endpoints in zebrafish embryos (Hallare et al., 2006; Xiong et al., 2017). Consistent with this assertion, our experimental findings substantiated the absence of any discernible distinction in the development and behavior of zebrafish embryos between the E3 media group and the 0.001 % DMSO in E3 media group (Supplementary figure, Fig. S1).

2.2. Selection of zebrafish strains

Danio rerio wild-type and transgenic zebrafish were maintained at 27 °C ± 1 °C with a light/dark cycle of 14:10 h in an automatic circulation system. Two fluorescence-labeled transgenic zebrafish lines, [Vasculature endothelium-Tg(kdrl:mCherry) and Macrophage/Microglia-Tg(mpeg1:EGFP)], expressing fluorescent proteins in specific cell types, were obtained from the China Zebrafish Resource Center (http://www.zfish.cn/). Fish embryo exposure experiments were conducted according to the OECD 236 guideline (OECD, 2013) and the methodology described by Yang et al. (2018b) and Forner-Piquer et al. (2021), with some adaptation. Briefly, adult zebrafishes were randomly selected and placed in a mating box (two males and females/box each). After spawning and fertilization, eggs were collected within 30 min and screened using a stereoscopic microscope (SMZ745T, Nikon Corporation) to exclude unfertilized eggs, injured embryos, embryos with irregularities during cleavage or opaque eggs. The healthy embryos at 2.5

h postfertilization (hpf) were transferred to 6-well culture plates, with 30 embryos and 6 mL of exposure solution in each well (varying concentrations of AO2246 in E3 medium). Five replicates of 30 embryos/well for each control or AO2246 exposure group. The exposure continued for 6 days by replacing 75 % of the medium with chemicals each day. Upon completion of experiments, all larvae were humanely euthanized by administering 300 mg/L MS222. All experimental and animal care procedures were approved by the Animal Research Ethics Committee of Hangzhou Normal University and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Ethical Clearance Code No. 2020083).

2.3. Developmental toxicity analysis

To obtain LC_{50} value for AO2246 in zebrafish embryos, embryos were randomly divided into nine groups (control [CTL]; 0.05, 0.2, 0.8, 2, 3, 4, 6, and 10 μM AO2246 groups). Dead embryos were removed from the wells and mortality was recorded daily. Hatching rate, survival rate, heart rate, and body length were measured to evaluate the developmental toxicity of AO2246. Approximately 120 larvae per group were considered to determine the hatching (72 hpf) and survival rates (96 hpf). Eighteen larvae at 96 hpf per group were randomly selected, and their heart beats at 20 s intervals were recorded. Eighteen larvae at 144 hpf per group were photographed using Stereoscopic Microscope and Imaging System.

2.4. Locomotor behavioral analysis

After exposure for 6 days in 6-well plates, the zebrafish larvae were individually transferred to 96-well plate and placed in DanioVision Observation Chamber (Noldus). The temperature in the chamber was maintained at 27 °C. Twenty-four fishes from each group were assessed for behavior during mid-afternoon. The experimental protocol for the dark and light exposure time was similar to that reported previously (Cao et al., 2019; Yu et al., 2021) with some minor modifications. Briefly, after a 1-h dark adaptation, larvae were tracked following 30 min of alternating 5-min light and dark periods. Fish activities were recorded and analyzed using EthoVision software. The excel files of distance moved and activity were exported for further analysis.

2.5. In vivo vasculature and neurological analysis

N-phenylthiourea (PTU, 0.002 mL of PTU/mL of rearing medium) was used once daily to prevent pigmentation in the fluorescence-labeled transgenic zebrafish larvae from 24 to 144 hpf. Tg(kdrl:mCherry) is for vasculature architecture analysis and Tg(mpeg1:EGFP) is for neurological analysis. These transgenic larvae from the CTL and AO2246 exposure groups (n = 12) were anesthetized with MS-222 (150 mg/L) and immobilized in agarose gel with a low melting point. The larvae were observed under a two-photon Zeiss LSM710 confocal laser scanning microscope. Fluorescence was simultaneously detected using two non-descanned detectors. In vivo Z-stack images of the whole head were acquired followed by generation of 3D images using IMARIS 9 (Oxford Instruments).

2.6. In vivo electrophysiological analysis

Electrophysiological recordings were obtained in vivo at room temperature (25 °C) (Baraban, 2013). Briefly, 144 hpf zebrafish larvae (n = 10 in the CTL, 0.2 μM AO2246, and 3 μM AO2246 groups, respectively) were immobilized in agarose gel with a low melting point after paralyzing the fish with MS-222 (150 mg/L) for 5 min. The larvae were maintained under a Leica Z16 microscope, and a glass microelectrode was fixed in the midbrain region. Electrophysiological field potential activity was recorded for 30 min using a MultiClamp 700B amplifier (Molecular Devices). Data were analyzed using Clampfit 10 by focusing on a 10 min period, which was selected after the initial recording for 10 min. Zebrafish preparations with inadequate signal/noise ratios were excluded. After performing automated spike detection, the number of events and frequency were automatically calculated.

2.7. Gene expression analysis

RNA extraction was performed for gene expression analysis. After a 6-day exposure, zebrafish larvae (CTL and 3 μM AO2246 groups) were collected. A pool of 25 zebrafish larvae constituted 1 sample. Three replicate samples for each group. Each sample was homogenized in TRIzol reagent (Invitrogen), and total RNA was extracted. The quantity and purity of the RNA were analyzed using Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent). Complementary DNA (cDNA) was synthesized using SuperScript™ II Reverse Transcriptase (Invitrogen). RNA sequencing was performed using Illumina Novaseq™ 6000 (LC-Bio Technology CO.), according to the manufacturer's protocol. Real-time polymerase chain reaction (PCR) was performed via 7300 Real-Time PCR System (Applied Biosystems) using 2 μL of cDNA/20 μL of SYBR reaction mixture. Primer sequences of the target genes are listed in Table 1.

2.8. Statistical analysis

All experiments conducted in triplicate independently. Data analyses were performed using GraphPad Prism 8.0 software. When the data fulfilled the criteria for a parametric test, one-way analysis of variance (ANOVA) was performed followed by Dunnett's multiple comparisons test. For other cases, Kruskal–Wallis test (nonparametric) followed by Dunn's multiple comparisons test was used. Cumulative hatching rates were analyzed using two-way ANOVA followed by Dunnett's multiple comparisons test. All quantitative data are expressed as mean ± standard error (SEM). Statistical significance was considered at the following p values compared with the CTL group: * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

3. Results

3.1. Effects of AO2246 on mortality and development

From 2.5 to 96 hpf, larvae were systematically exposed to a range of AO2246 doses (10, 6, 4, 3, 2, 0.8, 0.2, and 0.05 μM). Compared with the CTL group, the hatching rates were significantly decreased in all exposure groups at doses of ≥2 μM at 72 hpf (Fig. 1B). Similarly, all exposure groups at doses of ≥2 μM showed markedly decreased heart rates at 96 hpf (Fig. 1C); however, no significant differences were observed at lower doses (0.8, 0.2, and 0.05 μM AO2246 groups). Based on a 96-h exposure, the LC_{50} value (concentration of AO2246 that resulted in 50 % mortality in larvae) was 3 μM (or 1.02 mg/mL). Survival rate curves are shown in Fig. 1D. After 144 h of exposure, a significant difference in total body length was noted in the 3 μM group compared with that in the CTL group (*, $p < 0.05$) (Fig. 1E and F). At AO2246 doses of >3 μM, deformities were observed in larvae. The most common malformations were pericardial edema, spinal curvature, and uninflated swim bladder (Fig. 1G). These defects may be responsible for high death rates in the high dose groups. Collectively, these data suggest that the toxicity of AO2246 negatively affects the development of zebrafish larvae.

Table 1
The primer sequences of real-time RT-PCR.

Gene name	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
zgc:172128	GTGATTCCAGCACAGAGAGACA	AATCCTCTTGTGAACCGCCA
noto	TCTGCGCTCCCGCTTATTTA	GCGTTCAGATCCCACCATGT
lepb	TTCCCCGTCACCTCCAACTA	AGTCCTTGCATGTGCCATTG
pcdh2ab11	GCTTTGGAATATAGCGGGCG	GCCCACATCTGCATCAAACG

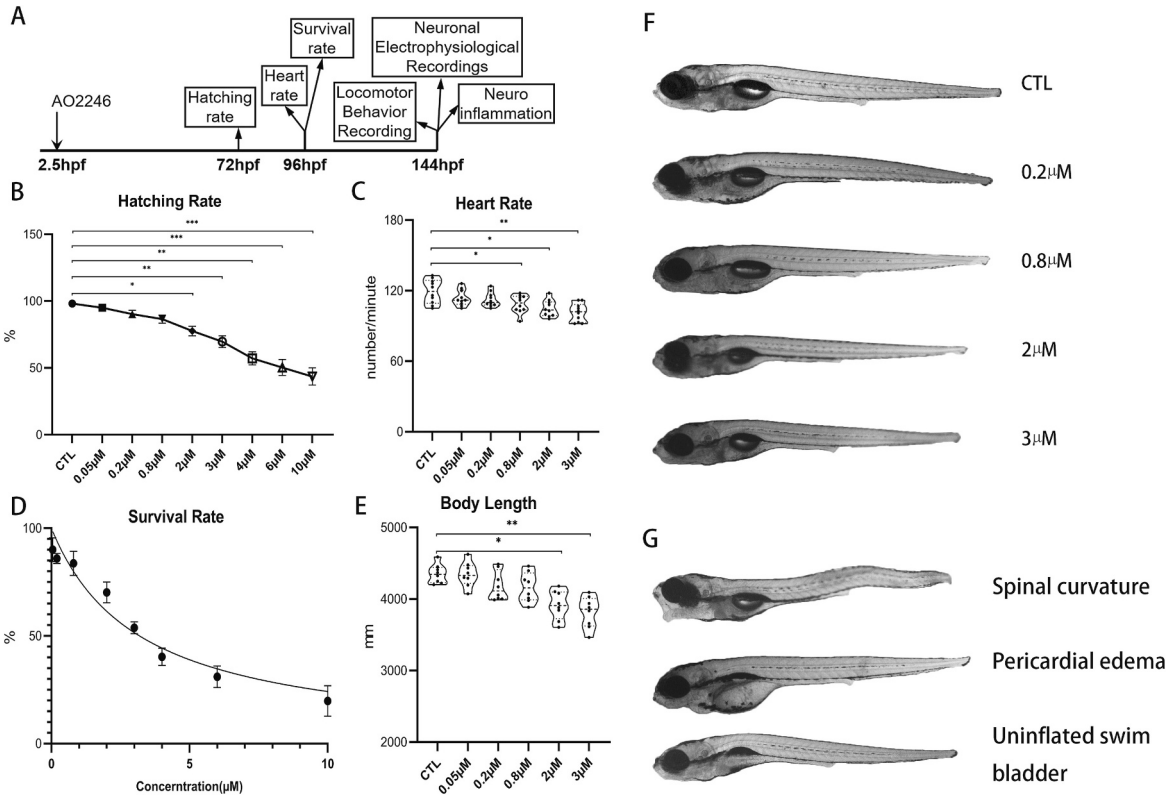

Fig. 1. Effects of AO2246 on survival and development of zebrafish embryos from 72 to 144 hpf. Experiments conducted in triplicate. A) The experiment setup diagram. B) Hatching rates at 72 hpf. C) Heart rates at 96 hpf. D) Survival rates at 96 hpf. E) Body lengths at 144 hpf. F) Photographs of zebrafish larvae at 144 hpf. G) Photographs of common malformations.

3.2. Effects of AO2246 on locomotor behavior

From the darkness adaptation period, behavioral analyses revealed defects in locomotor activities at AO2246 doses of ≥2 μM. Specifically, compared with the CTL group, total distance was significantly decreased in larvae exposed to AO2246 [$F(5, 138) = 45.26$, $p < 0.001$] (Fig. 2A). Similarly, the mean velocity was significantly decreased after exposure to 2 or 3 μM AO2246 [$F(5, 138) = 36.81$, $p < 0.001$] (Fig. 2B). Doses of <2 μM (0.05, 0.2, and 0.8 μM AO2246 groups) did not result in significant behavioral changes. An interesting observation was made during the following locomotor behavior recording. Based on a 5-min light/5-min dark alternation stimulation, the total distance at AO2246 doses of ≥0.8 μM was observably lower than that in CTL (**, $p < 0.01$). This not only occurred during the light period but also in the dark period (Fig. 2C). Similarly, significant differences in mean velocity were observed in larvae at AO2246 doses of ≥0.8 μM under the 5-min light/5-min dark alteration stimulation (**, $p < 0.01$) (Fig. 2D). Altogether, AO2246 exposure significantly impairs locomotor activity of zebrafish larvae.

3.3. Effects of AO2246 on neuronal electrophysiology

Behavioral modifications can function as highly responsive indicators for neurological conditions (Klüver et al., 2015; Fitzgerald et al., 2021). Thus, the investigation was carried out on the architecture and physiological function of the midbrain. The 3D images of Tg(kdrl: mCherry) larvae revealed that AO2246 exposure did not dramatically alter the vasculature architecture of the midbrain (Fig. 3A, B). Electrophysiological recordings revealed that a significant decrease in spike activity was observed at 3 μM, but not at 0.8 μM, compared with CTL conditions (Fig. 3C). Spike activity was highly variable at 3 μM AO2246 (Fig. 3D–F). Therefore, AO2246 induces disturbances in the neuronal physiology of larvae. These data indicate that the defective locomotor activity following exposure to high AO2246 doses is associated with disturbances in neuronal electrophysiology.

3.4. Effects of AO2246 on neurological disorders

To further explore the possible mechanism of locomotor behavioral abnormalities, the transgenic zebrafish expressing (mpeg1: EGFP) was employed to evaluate the status of macrophages/microglial cells, which have been associated with diverse neurological conditions, specifically neuroinflammation (Beumer et al., 2012; Hanamsagar and Bilbo, 2017). The macrophage/microglia was quantified in the Tg(mpeg1: mCherry) larvae midbrain Z-stack images (Fig. 4A, B). Compared with the CTL group, the total number of macrophage/microglia was notably different in the AO2246 exposure group (Fig. 4C). This revealed the signs of immune alteration in response to high AO2246 doses.

3.5. Effects of AO2246 on gene expression profiles

We performed RNA sequencing to identify pathways and potential molecular associations with the previously reported neurophysiological outcomes and cellular changes. We identified 128 up-regulated and 69 down-regulated differentially expressed genes (DEGs) in larvae exposed to AO2246 versus untreated larvae [$|\log2(FC)|$ value of >2 and p value of <0.01]. The 100 most significantly deregulated genes were selected. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed to determine the putative function of DEGs. GO analysis revealed that the genes were enriched in several factors representing three categories: molecular functions, biological processes, and cellular components (Fig. 5A). The top 20 enriched GO terms indicated that the five most significantly dysregulated biological processes were G protein-coupled receptor signaling

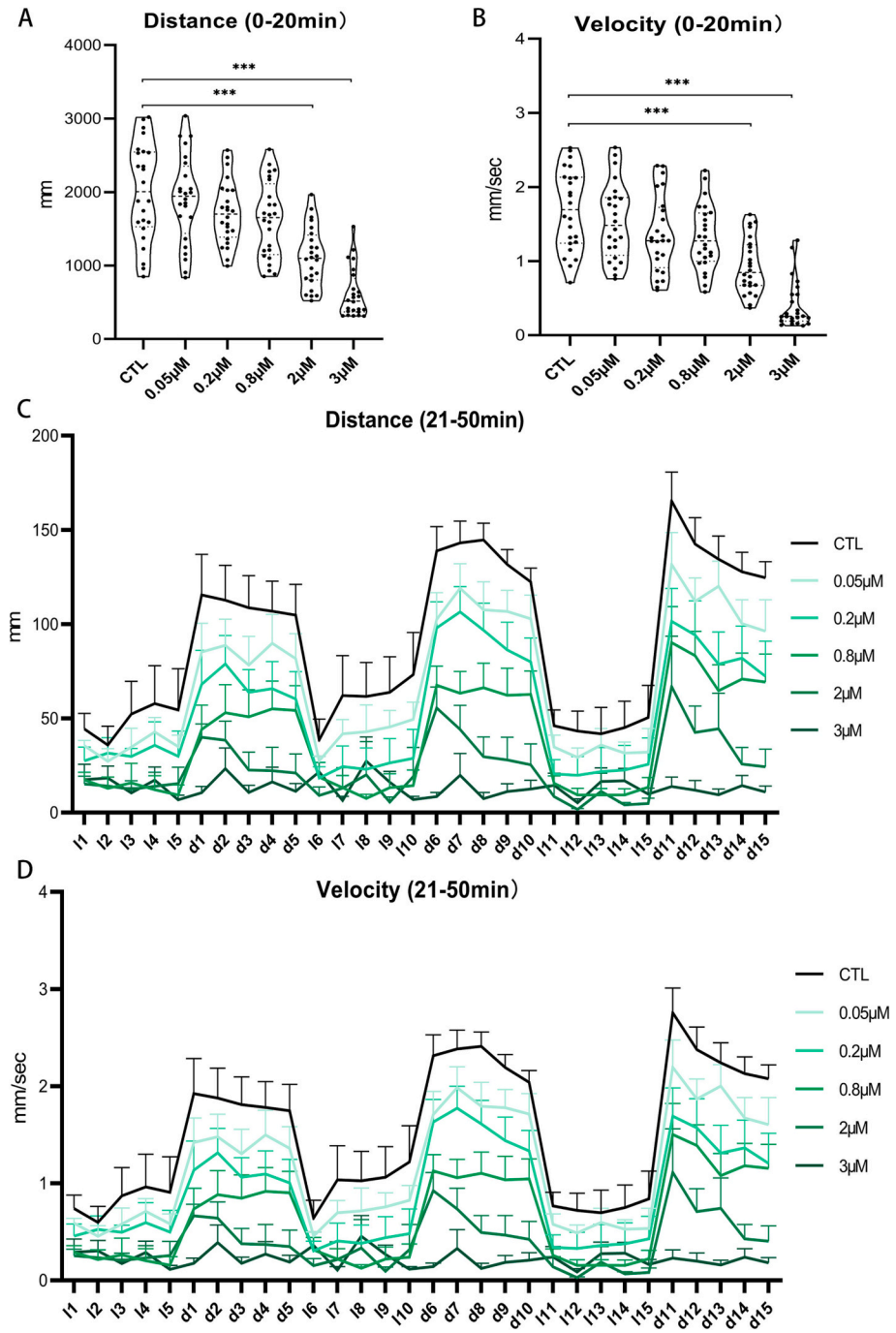

Fig. 2. Locomotor activity of zebrafish larvae after exposure to various doses of AO2246 (3, 2, 0.8, 0.2, or 0.05 μM) for 6 days. Experiments conducted in triplicate. A) Total distance during the 20-min dark adaptation stage. B) Mean velocity during the 20-min dark adaptation stage. C) Total distance during the 5-min light/5-min dark alternation stimulation stage. D) Mean velocity during the 5-min light/5-min dark alternation stimulation stage.

pathway (GO:0007186), olfactory receptor activity (GO:0004984), detection of chemical stimulus involved in sensory perception of smell (GO:0050911), G protein-coupled receptor activity (GO:0004930), and immune response (GO:0006955) (Fig. 5B). KEGG analysis revealed that the DEGs were also enriched in various pathways (Fig. 5C). The most significantly enriched KEGG terms were neuroactive ligand–receptor interaction and cytokine–cytokine receptor interaction (Fig. 5D). The expression patterns of the top four DEGs were confirmed by real-time PCR (Fig. 5E, F). Compared with the CTL group, the relative mRNA levels of two genes (zgc:172128, noto) were significantly up-regulated and those of two genes (lepb, pcdh2ab11) were significantly down-regulated in larvae treated with AO2246. These results indicate that AO2246 exposure results in changes in the transcriptional profile of zebrafish larvae.

4. Discussion

The present study conducted a comprehensive evaluation of the developmental and neurobehavioral toxicity of AO2246 in zebrafish larvae. The initial observation indicated that the exposure of AO2246 resulted in a notable decrease in both the hatching and survival rates of larvae (at doses of $\geq$2 μM), as well as a significant impact on their normal development and overall body length at doses of >3 μM (LC_{50} value). Subsequently, the presence of behavioral modifications (dark

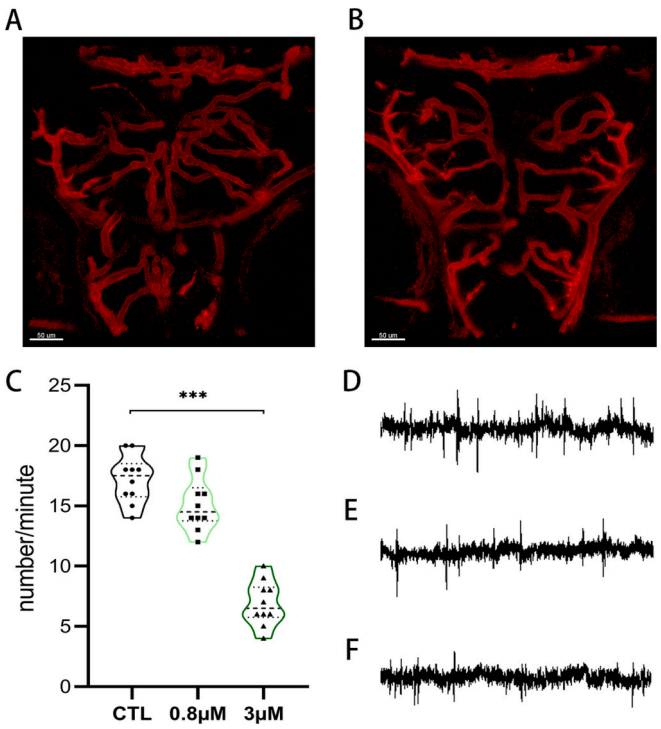

Fig. 3. Neurophysiological modifications of zebrafish larvae after exposure to AO2246 for 6 days. Experiments conducted in triplicate. A–B) Z-stack image of Tg(kdrl:mCherry) larvae in CTL (A) and AO2246 exposure group (B). Dorsal view with the caudal tail up. Scale bar: 50 mm. No significant changes were observed. C) Quantitation of spike frequency in the AO2246-treated and CTL groups. D–F) Examples of traces of CTL (D), 0.8 μM AO2246 (E), and 3 μM AO2246 (F).

stage) at doses of ≥2 μM was detected, concomitant with abnormal neuronal electrophysiology spike activity in the midbrain. Remarkably, administration of low doses (0.8 μM) of AO2246 elicited behavioral alterations during the light stimulation phase, while no discernible changes in neuronal electrophysiology were observed. Additionally, exposure to high doses of AO2246 resulted in a significant modification of the midbrain macrophage/microglia count, disclosing a hypothetical role for neurological disorders in contributing to behavioral impairments in these specific conditions. RNA sequencing analysis revealed a transcriptional profile that had been altered following exposure to AO2246.

In recent years, reports have indicated the neurotoxicity of SPAs. SPAs have been observed to interfere with the differentiation of neural crest cells in *Xenopus laevis* embryos (Y. Xu et al., 2021). Additionally, Yang et al. (2020) and Shi et al. (2022) have provided evidence that exposure to SPAs can induce disturbance in neurotransmitter systems in rats and zebrafish, respectively. Disturbance in nervous system of organism can adversely affect its morphological, physiological and behavioral characteristics (Knörr and Braunbeck, 2002; Reif et al., 2016; Yang et al., 2018b; Liang et al., 2020). Behavioral modifications can function as highly responsive indicators for neurological conditions (Klüver et al., 2015; Fitzgerald et al., 2021). Thus, the present study employed transgenic zebrafish expressing (mpeg1: EGFP) to evaluate the status of macrophages/microglial cells, which have been associated with diverse neurological conditions, specifically neuroinflammation (Beumer et al., 2012; Hanamsagar and Bilbo, 2017). Surprisingly, exposure to AO2246 led to a decline in immune cells in the brain, indicating the lack of neuroinflammation but the presence of disorders.

Recent research revealed that immune cells can exert an impact on neurological conditions not only through inflammation but also via a loss of normal microglial function, referred to as "dysfunctional microglia" (Mosher and Wyss-Coray, 2014; Ayyubova, 2022; Bianchin and Snow, 2022). Further studies identified that the depletion of functional microglia contributes to disease development (Bianchin and Snow, 2022; Neumann et al., 2023). In the context of our research, the experimental findings indicate a significant reduction in the quantity of macrophage/microglia following exposure to AO2246. Our hypothesis posits that the observed toxicity reactions may be attributed to either the dysfunction of microglia or the depletion of functional microglia.

Certain members of the G protein-coupled receptor (GPCR) family are implicated in neurological disorders. The agonists of Takeda G protein-coupled receptor 5 (TGR5) have demonstrated neuroprotective effects in various neurological disorders, as evidenced by recent studies (Zuo et al., 2019; Wu et al., 2019). Additionally, TGR5 activation can mitigate microglial cell activation (Jin et al., 2021). Conversely, GPRC151, another G protein-coupled receptor, has been shown to induce neurological disorders that are dependent on extracellular signal-regulated kinase (Jiang et al., 2021). GPCR 30 is involved in the anti-inflammatory effects in microglia (Du et al., 2018). Olfactory receptors, the largest subfamily of GPCRs, are also involved in microglial activation (Antunes and Simoes de Souza, 2016; Lee et al., 2020). The present study has identified significant dysregulation in the G protein-coupled receptor signaling pathway via transcriptional profile analysis. These findings suggested the potential implication of this pathway in neurological disorders arising from exposure to AO2246.

SPAs undergo metabolism in the human body, with the biological half-lives ranging from several hours to a few days (Verhagen et al., 1989). The emergence of transformation products (TPs) resulting from SPAs has been substantiated by various studies (Liu and Mabury, 2018;

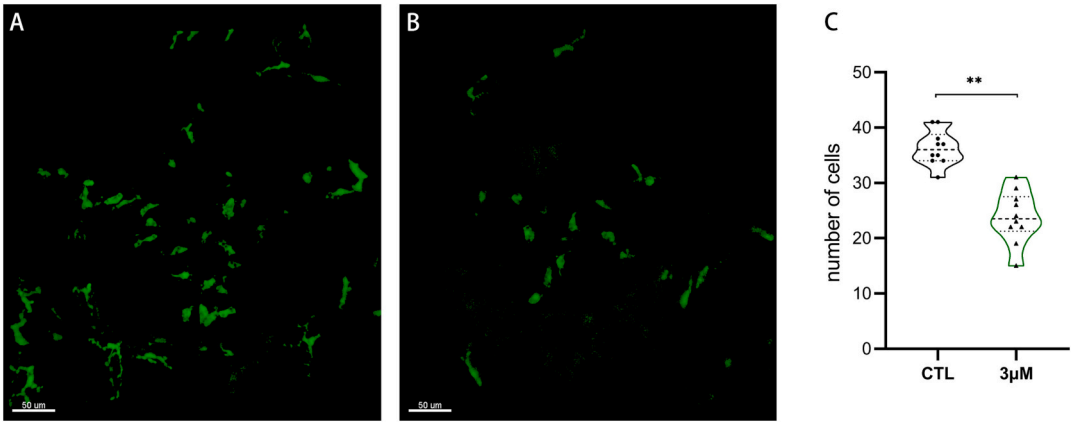

Fig. 4. Number of macrophage/microglia changed in response to AO2246 for 6 days. Experiments conducted in triplicate. A-B) Z-stack image of Tg(mpeg1:mCherry) zebrafish larvae in CTL (A) and 3 μM AO2246 groups (B). C) Number of macrophages/microglial cells.

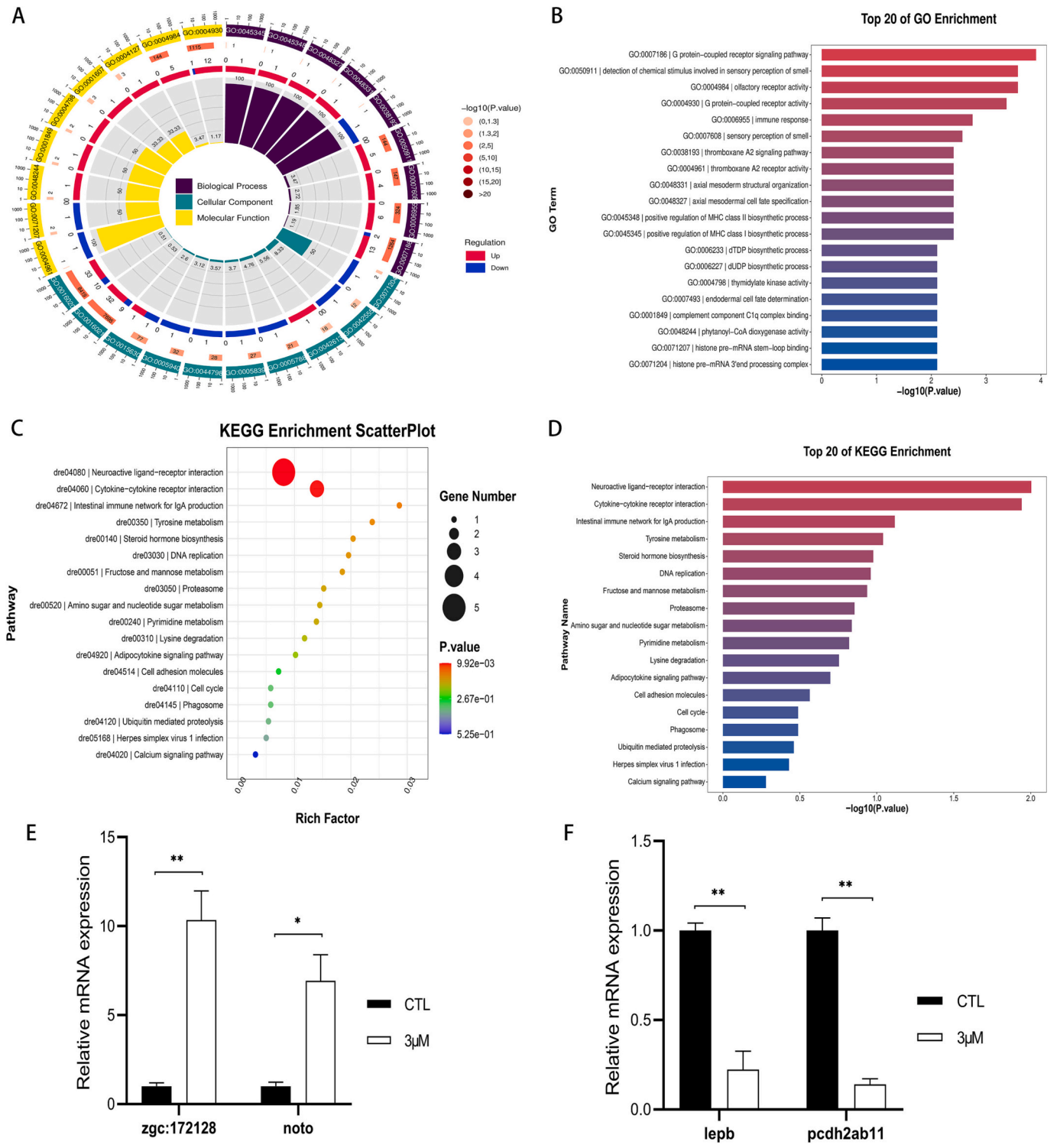

Fig. 5. Transcriptome analyses revealed differentially expressed genes (DEGs) in zebrafish larvae after AO2246 exposure for 6 days. Experiments conducted in triplicate. A) GO analysis of the 100 most significant DEGs. B) The top 20 GO enrichment terms. C) KEGG pathway analysis of the DEGs. D) The top enrichment KEGG pathways. E) Relative expression pattern of two up-regulated DEGs. F) Relative expression pattern of two down-regulated DEGs.

Wang et al., 2018; Li et al., 2019; Liu and Mabury, 2020; Castilloux et al., 2022). Some TPs of SPAs were reported to be more toxic than SPA itself, as demonstrated by their capacity to elicit cellular DNA damage, genotoxicity, and carcinogenicity in animal models (Nagai et al., 1993; Oikawa et al., 1998). According to recent research, the presence of 2,6-di-tert-butyl hydroxytoluene (BHT), a synthetic phenolic antioxidant, and its TPs has been observed in the maternal-placental-fetal unit. Additionally, the TPs have demonstrated notably higher transplacental transfer efficiencies than BHT itself, as reported by Wang and Kannan (2019). Several TPs of A02246, including 3,5-bis(1,1-dimethylethyl) phenol, 4-(1,5-dihydroxy-2,6,6-trimethylcyclohex-2-enyl) but-3-en-2-one, and other small polar compounds was identified by Ma et al. (2020). To date, no inquiries have been undertaken to assess the potential toxicological effects of the TPs of AO2246 on biota.

Consequently, further elucidation is required for the inquiry of whether the metabolites of AO2246 are responsible for eliciting toxic effects.

5. Conclusion

In summary, the present study offers valuable information regarding the developmental and neurobehavioral toxicity of AO2246 on zebrafish larvae in a dose-dependent manner. The observation of neuronal electrophysiological disturbances and neurological disorders implies a potential association with the emergence of locomotor behavior impairments upon exposure to AO226. Further investigation into the potential combined toxicities resulting from co-exposure to AO2246 and its TPs is warranted to enhance our comprehension of the associated human health risks in future research.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.166306.

CRediT authorship contribution statement

Yinan Chai: Data curation, Formal analysis, Investigation, Methodology. **Donglai Sheng:** Investigation, Methodology, Funding acquisition, Supervision, Validation. **Xiaowei Ji:** Data curation, Formal analysis, Writing – original draft. **Yanlong Meng:** Investigation, Methodology, Writing – review & editing. **Feihao Shen:** Data curation, Formal analysis. **Rui He:** Writing – original draft. **Runjia Ma:** Writing – original draft. **Yuying Wang:** Funding acquisition, Supervision, Validation, Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by the Natural Science Foundation of Zhejiang Province (grant number: LY20H060003, LY20H090007).

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