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Avobenzone and nanoplastics affect the development of zebrafish nervous system and retinal system and inhibit their locomotor behavior

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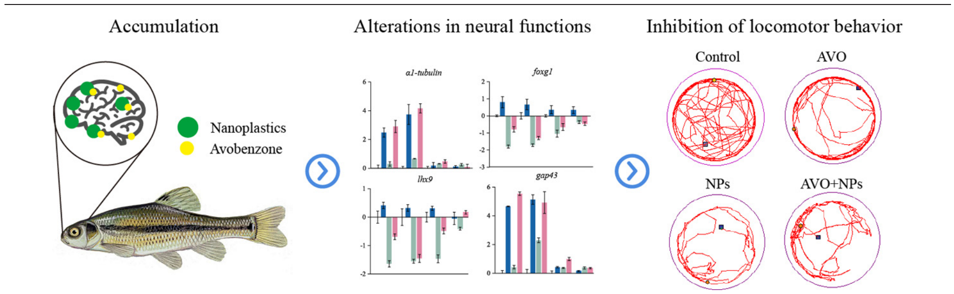
HIGHLIGHTS

- Avobenzone accumulation was enhanced by nanoplastics in zebrafish.
- Avobenzone affected the regulatory genes of nervous system development.
- Nanoplastics affected the regulatory genes of retinal system development.
- Avobenzone and nanoplastics inhibited zebrafish larvae locomotion.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of cosmetics is growing with each passing day, arousing widespread attention to their ingredients. Avobenzone (AVO) and nanoplastics (NPs) are typical ingredients in cosmetics, which coexist in the aquatic environment and have a combined effect on aquatic organisms. In this study, the accumulation of AVO and NPs in zebrafish larvae and effects on gene expression and enzymatic activity related to nervous functions, and locomotor behavior were investigated. AVO and NPs accumulated continuously in zebrafish, and the combined exposure enhanced AVO accumulation. After recovery, the accumulated concentrations of AVO and NPs in zebrafish remained unchanged, suggesting that AVO and NPs could not be eliminated in 72 h. The genes regulated nervous system development were affected mainly by AVO exposure, while the genes regulated retinal system development were affected by NPs exposure. Single and combined exposures of AVO and NPs affected the activities of acetylcholinesterase and antioxidant enzymes in zebrafish, and superoxide dismutase activity could not return to normal level after 72 h of recovery period. The locomotor activity of zebrafish larvae was significantly inhibited by AVO and NPs, which might be related to the alterations in functions of nervous system development and retinal system development as well as the interference of neurotransmitter system and antioxidant system.

1. Introduction

Cosmetics may contain 0.5% - 5% of microplastics, and may release around 4500 - 94,500 microbeads in a single use (Prata, 2018). Microplastics used in cosmetics such as scrubs and shampoos break down into nanoplastics (NPs) during mechanical processing (Hernandez et al., 2017). NPs are rinsed down household drains, and eventually released into the aquatic environment through sewage discharge (Carr et al., 2016). It was estimated that between 1.15 and 2.41 million tons of plastic waste flows from rivers into oceans every year (Lebreton et al., 2017). The fragmentation of microplastics in the aquatic environment could decompose to generate NPs by water shear forces (Enfrin et al., 2020). The abundance of microplastics in the surface water and sediments of the Three Gorges Reservoir, China ranged from 0.55×10^5 to 342×10^5 items/km^2 and 80 to 864 items/m^2, respectively (Zhang et al., 2017). He et al. (2020) detected microplastics in the sediment samples along Brisbane River in Australia, with abundance ranging from 0.18 to

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129.20 mg/kg. Microplastics abundance in the surface sediments from Atlantic Argentinean estuaries was investigated, reaching up to 1030 ± 657 items/kg dry weight (Díaz-Jaramillo et al., 2021). In the Antarctic Peninsula, microplastics abundance was 1.30 ± 0.51 items/g in sediments (Cunningham et al., 2020). Microplastics are widely detected around the world, while the limitations of current analytical methods result in few reports on the isolation and identification of NPs in the environment (Al-Sid-Cheikh et al., 2018).

Numerous experimental studies have highlighted that various organisms such as algae, mussel, scallop, and water flea could ingest or adsorb NPs to their surfaces (Besseling et al., 2014; Wang et al., 2021). The chemical stability of NPs allows them to accumulate in tissues, enter circulatory system, and induce toxic effects at cellular and molecular levels. Cellular energy homeostasis and oxidation of *Daphnia magna* were affected by NPs exposure (Liu et al., 2018). NPs induced oxidative stress and stimulated immune defense in juvenile *Macrobrachium nipponense* (Li et al., 2020). NPs exposure induced oxidative stress, liver inflammation, and lipid and energy metabolism disorders in red tilapia (Ding et al., 2020; Ding et al., 2018). Under NPs exposure, zebrafish exhibited oxidative stress, liver inflammation and lipid metabolism disorders (Lu et al., 2016). NPs have toxic effects on zebrafish and their offspring by disrupting thyroid hormone homeostasis and inducing reactive oxygen species generation (Zhou et al., 2021). In addition, NPs could be transferred to higher trophic level organisms, and eventually into the human food chain through aquatic organisms, thereby affecting human health (Lehner et al., 2019).

The organic sunscreen avobenzone (AVO) in cosmetics is designed to absorb ultraviolet radiation and protect skin from damage. AVO is widely detected in surface water, suspended particulate matter, sediment and various organisms in rivers. The concentrations of AVO were 24-721 ng/L in the surface water of Hong Kong, China, and 18-70 ng/L in the Arctic Ocean (Tsui et al., 2014). The average concentration of AVO in the suspended particulate matter of the Yangtze River, China was 692 ng/g dry weight (Yang et al., 2019). In the Yarlung Zangbo River, China, AVO concentrations ranged from 4.13 to 33.48 ng/g dry weight in sediment (Sun et al., 2021). AVO was detected in bigeye herring, bighead croaker, rice-paddy eel and Japanese stone crab, and the highest concentration was 21 ng/g lipid weight (Peng et al., 2017).

AVO could accumulate in the liver, brain, kidneys, gills, and muscle of crucian carp, inducing oxidative stress and inhibiting acetylcholinesterase (AChE) activity (Ma et al., 2017). Sublethal chronic exposure to AVO increased reproduction and decreased metabolism of *D. magna* (Boyd et al., 2021). The exposure to AVO affected human immune system function by increasing the production of various inflammatory cytokines in macrophages, especially tumor necrosis factor-α and interleukin-6 (Ao et al., 2018).

As typical ingredients in cosmetics, NPs and AVO have aroused widespread attention due to the ubiquitous occurrence of cosmetics in the aquatic environment (Sun et al., 2021). The increasing production of cosmetics along with their increasing occurrence in the aquatic environment makes the pollution of their ingredients become an important ecological problem. NPs have the characteristics of a small particle size, a large specific surface area, and strong hydrophobicity, which make them easier to adsorb and enrich coexisting pollutants in aquatic environment, thus affecting the environmental behavior and bioavailability of the coexisting pollutants (Alimi et al., 2018). However, the combined exposure of AVO and NPs is rarely reported, and the mechanism of the combined effects on aquatic organisms needs to be studied urgently. The early life stage (larvae and juveniles) is crucial to the development process of zebrafish as they are susceptible to chemical substances in the environment (Jacob et al., 2020). It is also a vulnerable period in the development of nervous system, when certain chemicals can disrupt developmental process of neural cells, alter biological functions, thereby leading to various disorders.

In this study, we focused on the effects of AVO and NPs on nervous system development of zebrafish larvae. The accumulation and elimination of AVO and NPs in zebrafish larvae were measured upon an exposure test followed by a recovery test. Based on the identified regulatory genes related to nervous functions in zebrafish larvae in our previous study, the nervous system development, stem cell differentiation and retinal system development regulatory genes were investigated. Biomarkers at the molecular level related to neurotoxicity and oxidative stress were determined, including AChE, catalase (CAT) and superoxide dismutase (SOD) activities. The locomotor behavior of zebrafish larvae was measured to reflect the influence of the nervous system disorders on individuals.

2. Materials and methods

2.1. Chemicals

AVO (CAS No. 70356-09-1, purity > 98%) was purchased from the Aladdin Biochemical Technology Corporation (Shanghai, China). Methanol and acetonitrile of HPLC grade were purchased from Merck (Darmstadt, Germany). The fluorescent polystyrene nanospheres were purchased from the Tianjin Baseline Chromtech Research Center (Tianjin, China) with a nominal diameter of 100 nm. The fluorescent dye used to label the NPs was green with 470 nm wavelength excitation and 526 nm wavelength emission. Before the experiment, the NPs suspension was dialyzed to remove additives and free dyes. The stock solutions of AVO and NPs were prepared in deionized water at a concentration of 1 g/L and stored in the dark at 4°C. The surface morphology of NPs was observed by transmission electron microscopy (TEM).

2.2. Zebrafish maintenance and embryo exposure

Zebrafish (*Danio rerio*) were obtained from the Institute of Hydrobiology of the Chinese Academy of Sciences (Wuhan, China). The zebrafish were acclimated in deionized water at 28 ± 1°C with a light/dark photoperiod of 14 h/10 h. The content of dissolved oxygen in water was kept above 6 mg/L by continuous aeration. The fish were fed twice per day with brine shrimp (*Artemia salina*). The zebrafish embryos were collected after 2 weeks of acclimatization. At 2 h post fertilization, healthy embryos were randomly selected and transferred into 24-well cell culture plates containing 2 mL of exposure solution per well. The solutions of AVO and NPs used in the co-exposure group were mixed 6 h in advance to reach adsorption equilibrium. The exposure concentration of AVO was chosen as 10 μg/L based on the environmentally relevant concentrations as previously reported (Tsui et al., 2014). The NPs exposure group contained 10 μg/L NPs, and the combined exposure group was set as 10 μg/L AVO + 10 μg/L NPs. For zebrafish accumulation measurements, the exposure lasted 144 h. For zebrafish recovery measurements, the zebrafish larvae were transferred to deionized water after 72 h of exposure, and the recovery period was 72 h. Based on the development stages of zebrafish, we selected segmentation stage (12 h) when nervous system begins to develop, larvae hatch out (72 h), as well as test ending (144 h) for sampling. Each treatment and control group was set in triplicate. All experiments were performed in accordance with the protocols approved by the Animal Experiments Ethical Committee of Hohai University.

2.3. Analysis of AVO and NPs accumulation

The zebrafish embryos or larvae were collected at 0, 12, 72 and 144 h of experiment, washed three times with deionized water, and used to detect AVO and NPs accumulation. AVO and NPs were extracted by ultrasonic method and KOH digestion method, respectively (Zhou et al., 2021). The concentration of AVO was detected by Agilent 1290 ultra-high-performance liquid chromatography (Eclipse Plus C_{18} chromatographic column: 30 × 2.1 mm, particle size 1.8 μm) with an Agilent 6460 triple quadrupole mass spectrometry equipped with an electrospray ionization source. The concentration of NPs was measured with a Hitachi F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

The zebrafish larvae were collected at 12, 72 and 144 h of experiment and used for qRT-PCR assay. The total RNA was extracted using the TaKaRa MiniBEST Universal RNA Extraction Kit (Takara Biomedical Technology, Kyoto, Japan). The purity and concentrations of the extracted RNA were analyzed based on the OD260/OD280 ratio using a NanoDrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington DE, USA). One microgram of RNA template was reverse-transcribed using the PrimeScript RT Reagent Kit (Takara Biomedical Technology, Kyoto, Japan). qRT-PCR was performed with 25 ng of template cDNA and 300 nM of forward and reverse primers per reaction using ChamQ SYBR qPCR Master Mix (Vazyme, Nanjing, China) in a final volume of 20 μL. The reaction conditions were as follows: pre-denaturation at 95°C for 30 s, denaturation at 95°C for 10 s, annealing at 60°C for 30 s. The specificity of the amplified product was confirmed by melting curve analysis. The primer sequences of genes related to zebrafish nervous functions were designed by NCBI Primer BLAST (Table 1). Relative levels of target mRNA normalized to β-actin were calculated based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.5. Biochemical analysis

AChE activity was measured to reflect neurotoxicity, and SOD and CAT activities were determined to reflect oxidative stress. The zebrafish larvae were collected at 12, 72 and 144 h of experiment. The homogenate (10%) of whole larvae was centrifuged at 4000 rpm at 4°C for 10 min, and the supernatant was collected for subsequent biochemical assays with a Synergy H4 microplate reader (BioTek, Winooski, VT, USA). The protein concentration of supernatant was determined by Coomassie Bright Blue method. The activities of AChE, CAT and SOD were measured by commercial kits from Jiancheng Bioengineering Institute (Nanjing, China). Detailed operation steps were carried out according to the manufacturer's instructions.

2.6. Embryo development and locomotor activity measurement

Locomotor activity measurement was conducted at 144 h. One larva was observed at a time and 24 replicates were performed for each treatment. The survival rate was measured based on whether the zebrafish had heartbeats. The swimming performance of morphologically normal zebrafish larvae was determined by the ZebraLab behavior monitoring station (Viewpoint Life Sciences, Civrieux, France). Larval swimming behavior ($n = 24$/group) was examined without any shock or noise interference. After 10 min of acclimation, a high-resolution camera was used to record the swimming trajectories of zebrafish larvae in a 24-well plate (1 larva/well) under continuous visible light for 15 min. The distance traveled by zebrafish larvae in 15 min was measured and the average speed was calculated.

Table 1
Primer sequences used in the present study.

Gene	Forward sequence (5′-3′)	Reverse sequence(5′-3′)
β-actin	AAAGCTGTGACCCACCTCAC	GCCAACCATCACTCCCTGAT
α1-tubulin	AAAAGTCGAGTGTGAGAGCG	GATGCACTCACGCATTTTCA
elavl3	CTGGTAAACCAGGTCACAGGT	TTATGGGGGAGAATCTGAAGCG
gap43	ACCAAATAGACAAACCAGACGC	CGCAAACCGCAGAAATCAGG
mbp	AATCAGCAGGTTCTTCGGAGGAGA	AAGAAATGCACGACAGGGTTGACG
shha	ACAGGCTCATGACACAGAGAT	CCAGTCAAATCCAGCCTCCA
syn2a	GTTCTGATCCGGCAACATGC	CAGACATGCAAATGCCCAGG
gfap	CTGGAATCTCTCCGTGGGTC	ACCGGAACAGTGATTCTGCTTT
pax6	GAAACTTGGAACCGTGCGTC	ACTCCACTGTGACTGTTTTGC
six6	CGAACTCGCGGTTTGTTGAG	CGTGATGCTGAAGCCTGTTTT
sox2	ACTCCATGACCAACTCGCAG	AATGAGACGACGACGTGACC
her5	AGACATGAGAAGGGTCCCCA	CTGTGCGTGCCCTTTTTACC
her6	CAAAACGGCTTCGGAACACA	CTGTGTTTAGGGCAGCGGTC
lfng	AGGAGGACCCATCCAGGTTC	CCCAATACCACACTGGTCCC
pax2	CCCGCGTTATTAAGTTCCCC	GATGTCCGCTGTTGCTTGAC
foxg1	CTGCTGCGTATGGCTATGGA	TGAGAGTTCCGGTGTTTCGG
lhx9	TCAGCTTCGCACCATGAAGT	CCCCAAGATTTGTTCTCCCTGA
olig2	TGCACCTGCTACCGGCAAT	GAAGGTCTGCTGGACACTCG
six3	CGCGGAGTCTGTTACGAGAG	ATTGCTTGATGCTGGAGCCTGT

2.7. Data analysis

Each sample used to measure chemical concentration, gene expression and enzyme activity contained 40 individuals with three replicates. The sample used to measure locomotor behavior consisted of one zebrafish larva with 24 replicates. The significance of differences among groups was evaluated by two-way analysis of variance (ANOVA) followed by the Tukey test using SPSS software (version 20.0, IBM, USA). All the data are shown as the mean ± standard deviation (SD) and were graphically illustrated with Origin (OriginLab Corporation, Northampton, USA). Differences were considered significant at $p < 0.05$.

3. Results

3.1. Characterization of NPs

The polystyrene nanospheres used in this study were regular and uniform in shape (Fig. S1). The diameter of NPs was 95.33 ± 3.28 nm, which was comparable to the nominal diameter.

3.2. Accumulation of AVO and NPs in zebrafish

No AVO or NPs was detected in the control group. AVO was not detected in the NPs alone exposure group, and NPs was not detected in the AVO alone exposure group. The concentrations of AVO in zebrafish increased continuously in the AVO alone and coexposure groups during the exposure period (Fig. 1A). The AVO concentration in the coexposure group was significantly higher than that in AVO group, which indicates that the addition of NPs promoted the accumulation of AVO in zebrafish. In the AVO alone group, there was no significant difference in AVO concentration in zebrafish before and after the recovery, indicating that it was difficult for zebrafish larvae to eliminate the accumulated AVO in a short period. However, a significant decrease of AVO concentration was observed in the coexposure group after the recovery compared to the 72 h coexposure without recovery period. Similar to the accumulation tendency of AVO, the concentration of NPs in both single and combined exposure groups increased gradually with exposure time (Fig. 1B). Compared to NPs alone, no significant variation in NPs concentration in zebrafish was observed when exposed to the mixture of AVO and NPs at all time points, which indicates that coexistent AVO has little effect on the accumulation of NPs in zebrafish. After 72 h of recovery, the concentration of NPs accumulated in zebrafish did not decrease significantly.

Compared to the control group, the survival rate of zebrafish larvae under single and combined exposures of AVO and NPs did not show significant difference (Table S1), which illustrated that the environmental relevant concentrations of AVO and NPs have no lethal effects on zebrafish. No morphological abnormalities were observed in zebrafish larvae after 144 h of treatment (Fig. S2). However, continuous exposure leads to the accumulation of AVO and NPs in zebrafish, which could lead to chronic toxicity.

3.3. Neural functions and regulatory genes

In our previous study, AVO and NPs were proved to affect neural mid cells (a subtype of neural cells) in zebrafish by regulating the expression

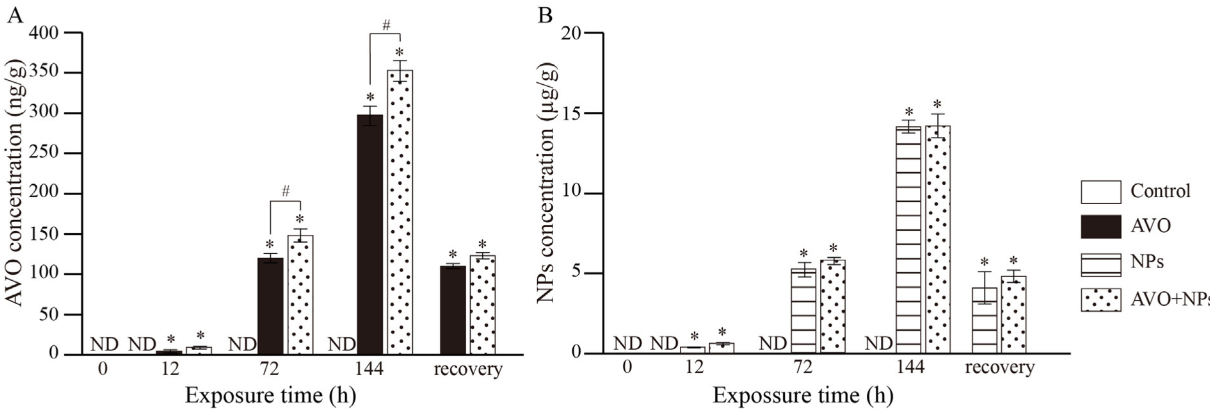

Fig. 1. Concentration changes of AVO (A) and NPs (B) in zebrafish during the experiment. ND represents not detected. Significant differences against the control group are shown as * ($p < 0.05$), while differences among AVO, NPs and AVO + NPs exposures are shown as # ($p < 0.05$).

of specific genes to affect cellular functions (Liu et al., 2021). These genes are expressed only in the central nervous system during early developmental stages (larvae and juveniles) and can be considered as biomarkers of developmental neurotoxicity. The main functions affected by AVO and NPs were nervous system development, stem cell differentiation, and retinal system development. The genes involved in the regulation of nervous system development were mainly altered by AVO (Fig. 2A). At 12 h and 72 h, the expressions of *α1-tubulin*, *elavl3*, *gap43*, *gfap*, *mbp* and *syn2a* were significantly upregulated by the AVO alone and combined exposures, while *lfng* expression was significantly downregulated. At 144 h, the expressions of *α1-tubulin*, *elavl3*, *gap43* and *mbp* in all the exposure groups were not significantly different from those in the control group. After recovery, the expressions of *α1-tubulin*, *elavl3*, *gap43*, *mbp*, *syn2a*, *lfng* and *olig2* in all the exposure groups were not significantly different from those in the control group, suggesting that these genes may susceptible to AVO in the early period of zebrafish development.

The changes in the expressions of genes related to stem cell differentiation are shown in Fig. 2B. The expression of *foxg1* was upregulated in the AVO group, while it was downregulated in the NPs and coexposure groups. The expressions of *her5*, *her6*, *shha* and *sox2* were altered significantly in the three exposure groups, indicating both AVO and NPs affected the differentiation of stem cell. After recovery, no significant difference was observed in the expressions of *foxg1*, *her6*, *shha* and *sox2* between the exposure groups and the control group. This suggested that the effects of AVO and NPs on stem cell differentiation can be eliminated through the recovery period.

Retinal system development was mainly affected by NPs exposure through the downregulation of *lhx9* and *six6*, and the upregulation of *pax2*, *pax6*, and *six3* (Fig. 2C). At 12 h, 72 h, and 144 h, the expression of *lhx9* was significantly downregulated by NPs (alone and in combination), while AVO upregulated *lhx9* expression, which means that NPs dominated the regulation of *lhx9* gene in the combined group. The alteration of *lhx9* in the combined exposure group was significantly lower than that in NPs group, suggesting that AVO attenuated the effect of NPs on the *lhx9* expression. The expressions of *pax2* and *pax6* were upregulated in three exposure groups. The expression of *six3* was significantly upregulated by NPs, and the combined exposure induced a stronger effect. Both AVO and NPs could obviously downregulate *six6* expression. There were significant differences in *pax2* and *six6* expressions between all the treatment groups and the control group after the recovery period, and the mediation of *lhx9* by NPs could not recover. It is suggested that the development of retinal system would be continually affected by the two pollutants, especially NPs.

3.4. Changes of enzymatic activities in zebrafish

AChE is an enzyme related to nerve conduction and is often used to reflect the neurotoxicity of chemicals on organisms. At 12 h and 72 h, there was no significant difference in the activity of AChE in all the exposure groups compared with the control group (Fig. 3A). At 144 h, AChE activity in the three exposure groups was significantly higher than the control group. The activity of AChE in the NPs group was significantly higher than that in the AVO group, while there was no significant difference between NPs and the combined group. There was no significant difference in AChE activity between the control group and the exposure groups after recovery.

The effects of AVO and NPs on antioxidant enzymatic activities in zebrafish are shown in Fig. 3B and C. At 12 h, there was no significant difference in CAT activity between the exposure groups and the contorl group. The SOD activity in the AVO alone and combined group was significantly increased. At 72 h, CAT activity in the AVO group was significantly increased, and SOD activity in the NPs alone and combined group was significantly increased. At 144 h, CAT activity in all three exposure groups was significantly higher than that in the control group, while SOD activity obviously increased in the AVO and coexposure groups. After recovery, CAT activity in the three coexposure groups returned to normal level, SOD activity in all the exposure groups was still significantly higher than that in the contorl group. The exposure of AVO and NPs induced oxidative stress in zebrafish, which could not recover in a short period.

3.5. Locomotor behavior of zebrafish larvae

Locomotor behavior is a sensitive indicator to evaluate the toxicity of target compounds on zebrafish. Compared with the control group, the locomotor trajectory of zebrafish larvae in all the exposure groups changed significantly (Fig. 4A). The zebrafish larvae in the three exposure groups tended to swim in a small space, with tracks mainly concentrating on one side of the well and turning in circles in a small range. The swimming behavior of zebrafish larvae in the recovery groups was significantly improved compared with the continued exposure groups, but it was still different from the control group. The single and combined exposure of AVO and NPs significantly reduced the swimming speed of zebrafish larvae (Fig. 4B). Compared with AVO exposure alone, the inhibitory effect of NPs alone and coexposure on the swimming speed was significantly greater. After recovery, although the swimming speed of zebrafish larvae increased in all the treatment groups, it was still significantly lower than that in the control group. The results of trajectory and speed indicated that the accumulation of AVO and NPs in zebrafish would continue to affect their locomotor behavior.

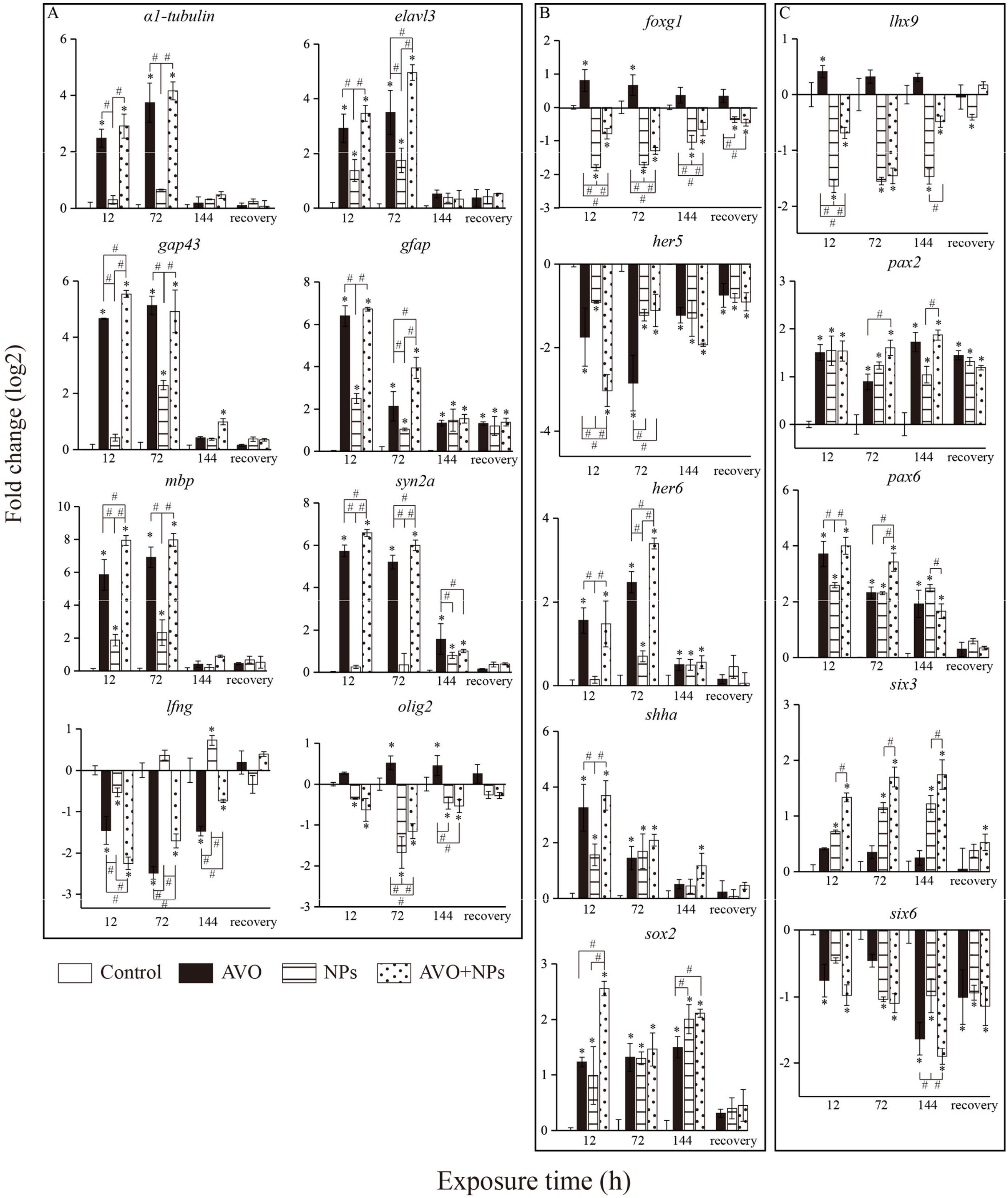

Fig. 2. Alterations in gene expression related to nervous system development (A), stem cell differentiation (B), and retinal system development (C). Significant differences against the control group are shown as * ($p < 0.05$), while differences among AVO, NPs and AVO + NPs exposures are shown as # ($p < 0.05$).

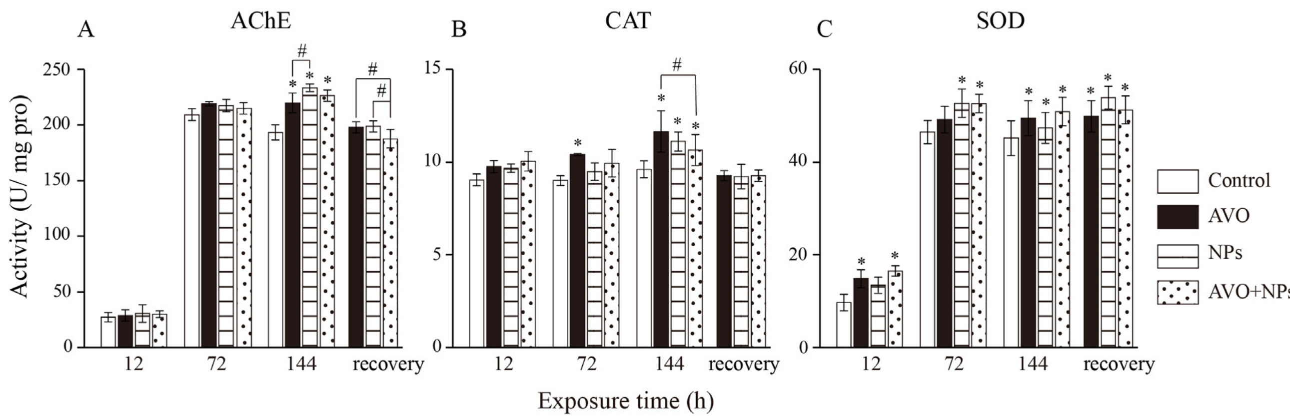

Fig. 3. Responses of AChE (A), CAT (B), and SOD (C) in zebrafish during the experiment. Significant differences against the control group are shown as * ($p < 0.05$), while differences among AVO, NPs and AVO + NPs exposures are shown as # ($p < 0.05$).

4. Discussion

The physical and chemical properties of large specific surface area and strong hydrophobicity enable NPs to adsorb coexisting pollutants and act as a carrier, thus affecting the bioavailability of coexisting pollutants (Ling et al., 2021). Previous research demonstrated that NPs increased phenanthrene concentration in zebrafish embryos (Zhang and Goss, 2020). NPs promoted the bioaccumulation and parental transfer of ethylhexyl salicylate (Zhou et al., 2021). In this study, the accumulation of AVO in zebrafish was promoted by NPs, suggesting that NPs exerted carrier effects. After recovery, the concentrations of AVO and NPs in zebrafish remained nearly constant, indicating that zebrafish larvae could not eliminate the pollutants accumulated in the body through metabolism or excretion in a short period. Long-term accumulation of pollutants in zebrafish would lead to chronic toxicity, and affect the growth and development of zebrafish. The main endocytic mechanism for the uptake of NPs was dynamin-dependent internalization. NPs degraded in lysosomes, resulting in alkalization and modification of cathepsin genes expression (Sendra et al., 2021).

The development of nervous system was mainly focused on the development of axons and dendrites. The gene *α1-tubulin* affects the development of axons and dendrites in the nervous system by encoding an intermediate filament protein that forms part of the microtubule cytoskeleton. The development of neurons, including the formation of axon branches, dendritic spines and synapses, is regulated by the interaction of the actin cytoskeleton and related proteins. Therefore, the upregulation of *α1-tubulin* in this study may affect the microtubule cytoskeleton and have an impact on the structure and function of brain. Neuronal Elav-like proteins are RNA-binding proteins that regulate RNA stability and alternative splicing, which are associated with axonal and synaptic structures (Ogawa et al., 2018). The gene *gap43* regulates the formation of synapses and the regeneration of axons after injury. The upregulation of *gap43* in this study represented an improvement in adaptability, which maintains the normal development of the brain to counteract the effects of pollutants. Glial fibrillary acidic protein is an intermediate filament protein highly expressed in the central nervous system. It is mainly found in astrocytes and radial glial cells, and is important in regulating the motility and shape of astrocytes. After injury to the central nervous system of vertebrates, astrocytes become reactive and respond in astrogliosis (Middeldorp and Hol, 2011). The increase of *gfap* expression in this study indicated central nervous system injury in zebrafish. In the central nervous system of zebrafish, *mbp* regulates the formation of axon myelin sheath. The gene *syn2a* is implicated in the formation of synapses and the release of neurotransmitters (Kao et al., 1998). The gene *lfng* plays a critical role in the synchronized oscillation of segmentation clock by regulating *Delta like 1*

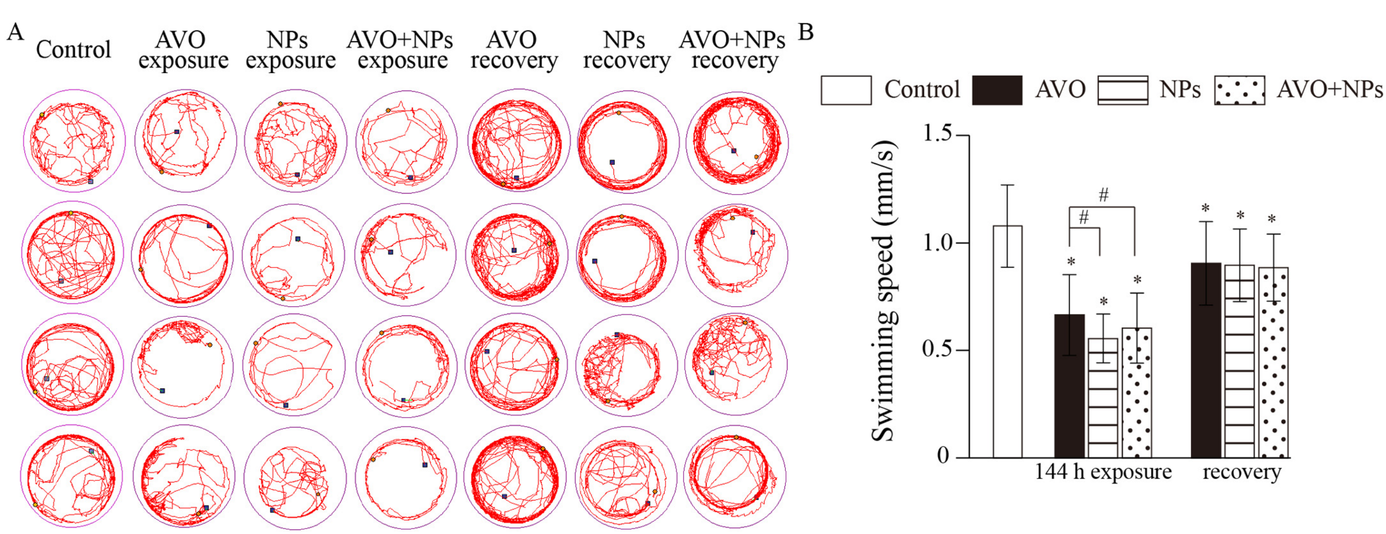

Fig. 4. Locomotor trajectory (A) and swimming speed (B, $n = 24$) of zebrafish larvae at 144 h. Significant differences against the control group are shown as * ($p < 0.05$), while differences among AVO, NPs and AVO + NPs exposures are shown as # ($p < 0.05$).

function to inhibit Notch activity (Okubo et al., 2012). The production of motor neurons and oligodendrocytes was controlled by *olig2* (Zhou and Anderson, 2002). In this study, most of the genes related to nervous system development were mediated by AVO, which suggests that AVO may affect the development of axons and dendrites.

Stem cell differentiation is regulated by *foxg1*, *her5*, *her6*, *shha* and *sox2*. Cell fate and neuronal differentiation are controlled by *foxg1*. The dysregulation of GABAergic neuronal differentiation caused by *foxg1* will lead to autism spectrum disorders (Mariani et al., 2015). The Notch signaling pathway was affected by DNA methylation and *foxg1* gene expression. Significant downregulation of the Notch signaling pathway was found in foxg1-knockout mice (He et al., 2019). The *her5* gene is a marker of embryonic neural stem cells and can maintain the number of neural progenitor cells (Chapouton et al., 2006). *Her5* also directs neuronal differentiation in a precise temporal and spatial manner in the midbrain, and its expression is controlled by fibroblast growth factor signaling (Dyer et al., 2014). The initiation of neuronal differentiation and the regulation of neuronal identity is controlled by *her6* (Scholpp et al., 2009). The proliferation of neural stem cells and the survival of nerve cells and glial cells during embryogenesis in zebrafish are regulated by *shha* (Müller et al., 1999). Overexpression of *shha* can enhance the survival of nerve cells, protect cells from the interference of neurotoxins, and inhibit the differentiation of nerve cells (Li et al., 2007). The expression of *sox2* is associated with embryonic stem cell differentiation, which inhibits neuronal differentiation and maintains progenitor cell properties (Tay et al., 2008). In this study, the expressions of *her5*, *her6*, *shha*, and *sox2* were significantly altered by AVO, while the expressions of *foxg1*, *her5*, *shha*, and *sox2* were significantly altered by NPs. AVO and NPs affected the differentiation of stem cell through the regulation of different gene expression. At 144 h and after recovery, the expressions of most genes related to stem cell differentiation returned to normal levels, suggesting that these genes may be expressed only in the early life of zebrafish.

The behavior of zebrafish relies on the mature retina, stabilized high-resolution imaging on the retina, and the eye movement reflexes in response to fast moving visual stimuli (Chhetri et al., 2014). The development of retinal system mainly includes the development of retina and the differentiation of retinal precursor cells. *Lhx9* is an important regulator of nitric oxide-synthesizing amacrine cell differentiation in the retina (Balasubramanian et al., 2018). *Pax2* is involved in the development of eyes, and regulates the establishment of visual system territories (Sanyanusin et al., 1995). In mice with *pax6* knockout, the number of V1 neurons was significantly reduced, suggesting that *pax6* controls the locomotion speed of vertebrates by regulating V1 neurons (Gosgnach et al., 2006). The *six3* gene controls the proliferation and differentiation of retinal precursor cells during early vertebrate eye development (Del Bene et al., 2004). The expression of *six6* regulates SWS2 and RH2 opsin, which plays an important physiological role in the vision of zebrafish (Ogawa et al., 2019). In this study, the expressions of *lhx9* and *six6* were downregulated, while the expressions of *pax2*, *pax6,* and *six3* were upregulated under NPs exposure. The expressions of *lhx9*, *pax2*, and *six6* in the NPs group showed significant difference with the control group after the recovery period. NPs could continuously affect the development of retinal system in zebrafish larvae. Similar phenomenon was observed in other researches. For example, bisphenol S exposure was proved to damage the optic nerve structure and lead to more empty areas in the zebrafish retina, affecting the normal vision of zebrafish (Gu et al., 2019). Another study showed that triphenyl phosphate exposure reduced the formation of stabilized high-resolution images on the retina and the tracking ability of the eyes of zebrafish larvae, representing a large threat to their survival (Shi et al., 2019).

AChE is a crucial enzyme in biological nerve conduction, which degrades acetylcholine in synaptic cleft, terminates the excitatory effect of neurotransmitter on postsynaptic membrane, and ensures the normal transmission of nerve signals in organisms (Behra et al., 2002). The increased AChE activity observed in the present study indicated that both AVO and NPs would have an impact on acetylcholine-mediated neurotransmission and even impair cholinergic system of zebrafish. After recovery, the activity of AChE in the three exposure groups returned to normal level, indicating that the accumulated pollutants at low concentrations in zebrafish larvae would not have a persistent neurotoxicity. Similarly, an increase of AChE activity observed in *Carassius auratus* induced by 200 μg/L of benzophenone-3, and the authors inferred that low concentrations of benzophenone-3 induced hormesis (Zhang et al., 2020). In addition, AChE induction was also observed in the clam (*Ruditapes philippinarum*) after exposure to 1000 μg/L ibuprofen for 3 days (Milan et al., 2013).

Oxidative stress is an imbalance between production and elimination of reactive oxygen species in organisms. CAT and SOD form the first defense line against oxidative stress in the antioxidant system, which combats reactive oxygen species and protects cells from oxidative stress (Chen et al., 2017a). NPs induced oxidative damage and inflammation in cells, which activated p38 MAPK signal pathway and ultimately induced cell apoptosis (Hu et al., 2021). A previous study demonstrated that reactive oxygen species generation was a major contributor to the developmental neurotoxicity exhibited by zebrafish larvae that have exposed to combined decabromodiphenyl ether and Pb, and the authors thought that detrimental changes in neurobehavior could be induced by a number of factors, including altered gene expression in the central nervous system, disruptions in motor neurons, and increases in oxidative damage (Zhu et al., 2016). In this study, the increase of CAT and SOD activities suggested the occurrence of oxidative stress, which may contribute to the neurotoxicity of zebrafish larvae. Furthermore, the regulation of the pollutants on the genes related to nervous functions in zebrafish larvae was accompanied by the responses of AChE and antioxidant enzymes, which confirmed the effects of AVO and NPs on the nervous system of zebrafish early stage.

With regard to the different life stages of zebrafish, the activities of AChE and SOD in the control group were higher in 72 and 144 h (larvae) than those in 12 h (embryos). Previous studies reported that zebrafish larvae showed higher AChE and SOD activities than embryos (Hu et al., 2016; Yen et al., 2011). In addition, we observed that the protein content in embryos was higher than that in larvae, which could be another reason for the higher activities of AChE and SOD in larvae in the control group.

Behavioral studies are becoming increasingly important in ecotoxicological research because they are an integration of biochemical and physiological processes that reflect higher level organizational changes related to ecology (Hellou, 2011). The locomotor behavior of fish is a comprehensive response controlled by various physiological and biochemical processes. The alteration of AChE activity in zebrafish larvae could lead to a significant increase in acetylcholine levels in the brain, which ultimately interferes with the function of the nervous system (Pereira et al., 2012). The central nervous system is vulnerable to oxidative stress, which leads to developmental brain defects, and subsequently leads to developmental neurotoxicity, and affects swimming behavior (Salminen and Paul, 2014). In the present study, by regulating the expression of genes related to nervous system development, AVO induced oxidative stress and neurotoxicity, which led to the decrease in locomotor activity of zebrafish larvae. The behavior of zebrafish is controlled by a variety of neural responses distributed in multiple brain regions that receive sensory signals from the eyes and other organs (Naumann et al., 2016). The effects of NPs on retinal functions observed in the present study could lead to a decrease in the swimming activity of zebrafish larvae. In addition, the inhibitory effects on zebrafish larvae locomotor behavior could resulted from oxidative stress and developmental neurotoxicity (Chen et al., 2017b). The decreased swimming activity of zebrafish would reduce their feeding activities, resulting in a decreased ability to grow and avoid predators, thus reducing their ability to adapt to natural ecosystems (Mattsson et al., 2015).

5. Conclusion

AVO and NPs accumulated continuously in zebrafish and could not be eliminated in a short recovery period. NPs can act as a carrier of AVO to promote the accumulation of AVO in zebrafish. Both AVO and NPs had effects on the stem cell differentiation of zebrafish embryos, which could be eliminated through the recovery period. AVO dominated the regulation of nervous system development genes in early life stage of zebrafish, while NPs dominated the regulation of retinal system development genes. In addition, AVO and NPs altered the activities of AChE and antioxidant enzymes and inhibited the swimming activity of zebrafish. The behavioral disturbances would influence feeding, growth and fitness of fish, and ultimately reduce their ability to adapt to natural ecosystems. Combining gene transcription and enzymatic activity analysis with locomotor behavior in this work could provide a comprehensive understanding of the effects of AVO and NPs on the nervous system of zebrafish early life stage.

CRediT authorship contribution statement

Yuxuan Liu: Methodology, Writing – original draft, Visualization. **Yonghua Wang:** Conceptualization, Resources, Writing – review & editing. **Na Li:** Investigation, Methodology. **Shengnan Jiang:** Investigation, Software.

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.

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Appendix A. Supplementary data

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