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Unraveling the mechanisms of perfluorooctanesulfonic acid-induced dopaminergic neurotoxicity and microglial activation in developing zebrafish

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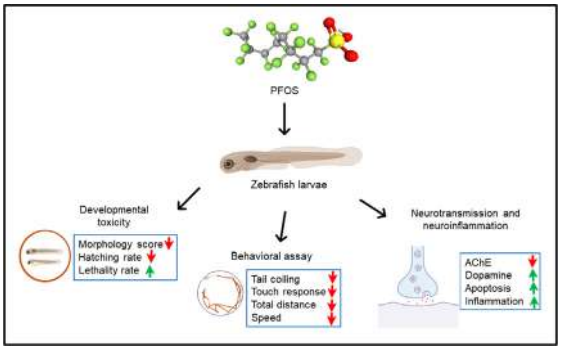
HIGHLIGHTS

- PFOS exposure induces developmental malformations and neurotoxicity.
- PFOS exposure exhibits reduced locomotor movement, affecting behavior.
- PFOS-mediated microglial activation leads to neuronal inflammation and apoptosis.
- Neurotransmission and dopamine signaling gene expressions were altered.

GRAPHICAL ABSTRACT



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ABSTRACT

Perfluorooctanesulfonic acid (PFOS) is a prevalent, persistent organic pollutant in environmental matrices, yet its precise mechanism of neurotoxicity remains unclear. This study investigated the developmental and neurobehavioral effects of PFOS exposure (0, 100, 500, and 1000 μg/L) on zebrafish. The findings indicated that PFOS exposure caused various developmental abnormalities, including increased mortality, delayed hatching, shortened body length, bent spine, and edema in the pericardial and yolk sac regions. Subsequently, larvae exhibited a significant decrease in spontaneous movement frequency, altered touch-evoked response, and locomotor behavior. In fact, aberrant cellular responses in the brain and cardiac regions were observed. Microglial activation is a critical component of the inflammatory immune responses related to neurotoxicity. Likewise, our findings indicated that PFOS-induced microglial activation might be responsible for neuronal inflammation and apoptosis. Furthermore, AChE activity and dopamine content at the neurotransmitter level were also disrupted after PFOS exposure. The gene expression of dopamine signaling pathways and neuroinflammation were also altered. Collectively, our findings highlight that

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PFOS exposure can induce dopaminergic neurotoxicity and neuroinflammation through microglial activation, thus ultimately affecting behavior. Taken together, this study will provide mechanistic effects underlying the pathophysiology of neurological disorders.

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are widespread and anthropogenic organic contaminants utilized in commercial and industrial applications, including surfactants, fire retardants, lubricants, paints, food packaging, adhesives, paper, and textile coatings (Kissa, 2001; Renner, 2001; Yuan et al., 2018; Araújo et al., 2022). PFAS are synthetic fluorinated chemicals with all hydrogen substituted with fluorine atoms in carbon-hydrogen bonds (Zhang et al., 2022). Their occurrence has been documented in almost all environmental matrices, wildlife, and humans worldwide (Giesy and Kannan, 2001; Kannan et al., 2005; Houde et al., 2006; Fromme et al., 2009; Butt et al., 2010). PFOS is a consequence of the metabolic breakdown or environmental degradation of other PFAS, which is stable because of its carbon fluoride bond. Moreover, due to its extensive dispersion, extraordinary persistence, bioaccumulation, and potential toxicological effects, PFOS poses significant hazards to humans and the environment (Quiñones and Snyder, 2009; Arias Espana et al., 2015; Prevedouros et al., 2006; Tsuda, 2016). Despite the discontinued production of PFOS in the United States in the early 2000s, its production and usage persist globally. While surface waters usually contain PFOS concentrations within the range of 0.1 to 100 ng/L, higher concentrations of up to 600 ng/L have been detected in rivers downstream of fluorochemical industrial sites (Chen et al., 2012; Oliaei et al., 2013; Banzhaf et al., 2017; Nakayama et al., 2010; Zareitalabad et al., 2013). The National Health and Nutrition Examination Survey (NHANES) reported PFOS in 98 % of human blood samples with a half-life of 4.8 years (M. et al., 2007; Olsen et al., 2007; Calafat et al., n.d.). PFOS accumulation has been found in various human organs, including the liver, pancreas, and, most notably, adipose tissues (Maestri et al., 2006; Mahapatra et al., 2021). It can permeate the placental barrier and inflict fetal exposure, as evidenced by its presence in fetal tissues and umbilical blood samples (Chen et al., 2012; Inoue et al., 2004). Regarding its neurological consequences, PFOS is reported to accumulate in mammalian brains and subsequently linked to abnormalities in neurotransmission (Dassuncao et al., 2019; Eggers Pedersen et al., 2015). Therefore, PFOS has aroused several health concerns in the context of neurotoxicity. As a result, a deeper knowledge of the molecular pathways is needed to understand the potential PFOS-induced neurotoxicity and the underpinning mechanisms by which PFOS causes microglial-mediated neuroinflammation.

Microglia are the native macrophage cells of the CNS and act as safeguards and emerging critical regulators against invading pathogens. Microglia are resilient cells that emerge from a transitory wave of erythromyeloid progenitor cells in the hematopoietic system (Muzio et al., 2021) and then infiltrate the developing brain before forming the blood-brain barrier. Several studies demonstrate that microglia are crucial in neuronal function and behavioral alterations linked with chronic stress. For instance, activated microglia produce inflammatory cytokines such as interleukins (IL) and tumor necrosis factors (TNF) during neurodegenerative processes, and many others (ROS and glutamate) (Woodburn et al., 2021). Upon secretion of these chemicals, microglia may exhibit either protective or harmful effects depending on the features of the microenvironment. Therefore, dysfunctional microglia can be linked with several neurodegenerative and neuropsychiatric disorders (Vidal and Pacheco, 2020).

Dopamine is a crucial neurotransmitter that significantly regulates neural functions, including behavior (Juárez Olguín et al., 2016). Dopamine signaling regulates physiological processes, including movement control, motivation, and reward. Dysregulation of this pathway has been implicated in several neurological and psychiatric disorders, such as Parkinson's disease, schizophrenia, and addiction (Mishra et al., 2018). Dopamine receptors, particularly drd2a, drd3, and drd4a, are critical components of this pathway and are the primary targets of several drugs used to treat these disorders (Mishra et al., 2018; Missale et al., 1998; Hasbi et al., 2011).

The zebrafish is an outstanding vertebrate model in developmental biology and neurotoxicity research (Félix et al., 2018). They help assess chemically induced malformations and developmental neurotoxicity due to their transparent bodies (Rafferty and Quinn, 2018) and rapid development of the brain and central nervous system within six h post-fertilization (hpf) to six days post-fertilization (dpf). The developing brain is sensitive to environmental stress because crucial neurodevelopment and neurotransmitter maturation stages, including neurogenesis, migration, differentiation, and synaptogenesis, occur throughout fetal and early postnatal life (Cowan et al., 2016). This makes zebrafish a well-established model for observing the neurobehavioral impacts of environmental pollutants.

To the best of our understanding, no previous research has endeavored to decipher the possible link between dopamine signaling, neuroinflammation, and locomotory behavior, with a particular emphasis on microglial activation. Consequently, this research aimed to evaluate developmental neurotoxicity and behavior and investigate the putative effects of PFOS on the expression of neuroinflammation-related genes and their underlying processes. Following PFOS exposure, behavioral changes, phenotypic changes, AChE activity, dopamine content, microglial cell population, and mRNA expressions of neuroinflammation and dopamine signaling genes were assessed in zebrafish larvae. This research attempts to address a gap in the literature by thoroughly analyzing the possible harmful effects of PFOS on developing organisms. The study's findings demonstrate a greater understanding of the molecular interaction between microglial cells and dopaminergic neurons in response to inflammation while also highlighting significant implications for further investigation of PFOS's neurotoxic effects on modulating the dopamine system.

2. Material and methods

2.1. Chemicals and reagents

An analytical standard of Potassium perfluorooctanesulfonate (CAS No. 2795-39-3, >98 % purity) was acquired from Sigma (US). The 2D and 3D chemical structure of PFOS-K, depicted in Fig. 1, was downloaded from PubChem CID 23669238 (https://pubchem.ncbi.nlm.nih.gov). All additional chemicals and reagents were of analytical quality and procured from Merck Corporation. The TRIzol reagent and cDNA synthesis kit utilized in the study were purchased from Thermo Fisher Scientific.

2.2. Zebrafish husbandry and egg collection

The adult wild-type zebrafish (*Danio rerio*, Tübingen strain) were procured from the Zebrafish Facility at METLab, Banaras Hindu University, India. The zebrafish were acclimated in glass aquaria at a well-regulated temperature (27 ± 1 °C) and photoperiod (14/10 h light/dark). A semi-static water circulatory system was used to rear the zebrafish, with 50 % of the water renewed daily. The water quality was routinely monitored throughout the study. The zebrafish were fed dry food twice and live feed (*Artemia* sp.) once daily. To initiate breeding, adult male and female zebrafish were randomly selected in a 2:1 ratio and placed in a breeding tank but kept separated until the following morning. After 30 min of the onset of light, fertilized eggs were collected and washed twice in E3 medium (58 mM NaCl, 0.7 mM KCl, 0.4 mM CaCl2, 0.6 mM MgSO4, 0.5 mM NaHCO3, pH 7.2–7.4), and then placed into 12 well plates with ten eggs per well. All animal experiments were performed in accordance

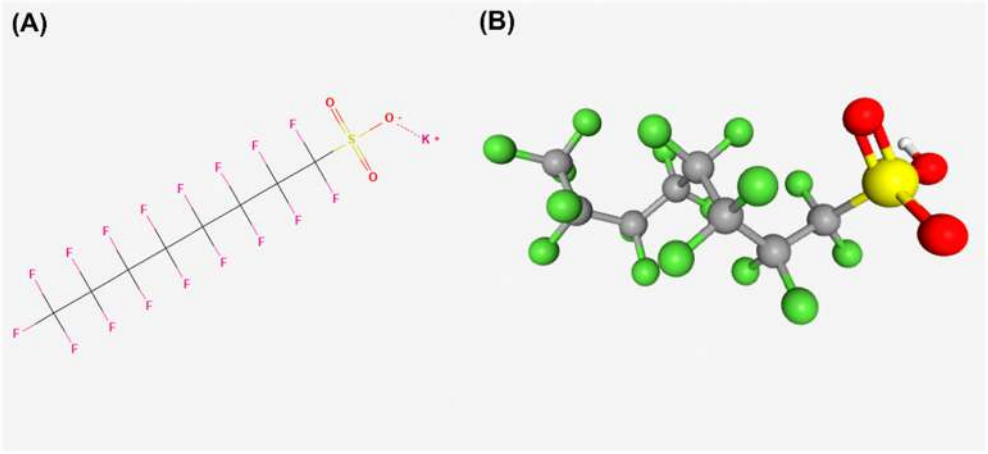

Fig. 1. (A) 2D and (B) 3D chemical structure of heptadecafluorooctanesulfonic acid potassium salt used in this study as obtained from PubChem (CID: 23669238).

with the guidelines and regulations set by the Institutional Animal Ethical Committee (IAEC), Banaras Hindu University, India (approval letter No. BHU/DoZ/IAEC/2019–20/020 Dated 27/08/2019).

2.3. PFOS exposure and experimental design

To conduct this study, healthy and fertilized 4-h postfertilization (hpf) embryos were utilized and divided into four distinct experimental groups. These groups included a solvent control (DMSO 0.01 % *v*/v) and three PFOS treatments (100, 500, and 1000 μg/L of PFOS), with exposure concentrations based on a prior study (Shi et al., 2008). The Organization for Economic Cooperation and Development (OECD) test guideline 236 was followed to perform toxicity testing (OECD, 2013). Specifically, embryos were randomly assigned to flat bottom 12 well plates (CLS3513, Corning®) containing 5 mL of test solutions (n = 10), with three wells allocated for each experimental group, and the experiment was conducted in triplicate. The exposure solutions were replaced entirely daily to ensure that the embryos remained exposed to the target concentrations throughout the study until 120 hpf. To ensure the quality of the medium, non-fertilized and deceased embryos were eliminated concurrently.

2.4. Developmental toxicity assessments

The developmental progress of the embryos in each treatment group was monitored at 24, 48, 72, and 96 hpf, with the hatching and mortality rates recorded for each time point as described previously (Gupta et al., 2023). Using a stereomicroscope, the embryos were examined for developmental abnormalities, such as pericardial edema, body axis curvature, tail and head malformations, and other visible deformities (Stemi 508, Zeiss) at each of the specified time points. An HD camera (Axiocam 208, Zeiss) attached to the same stereomicroscope was used for subsequent imaging. The malformation rate was calculated by dividing the number of defective zebrafish larvae at 96 hpf by the total zebrafish in each group. In addition, the morphology scoring of developing embryos was performed according to a previously described scoring protocol (Panzica-Kelly et al., 2010). Other phenotypic features, such as body length, eye size, yolk sac area, pericardial size, tail curvature, and pigmentation, were measured and quantified using Fishinspector software (v1.03) and R studio (Teixidó et al., 2019).

2.5. Behavioral assays

2.5.1. Spontaneous tail coiling and touch-evoked response

To evaluate spontaneous tail coiling (STC) behavior, embryos (n = 10) at 20–30 hpf were kept without stimulation for 1 min, and the number of coiling per minute was counted manually. For assessing the touch-evoke response (TER) behavior, five dpf embryos (n = 10) from each group were randomly selected, and the individual embryo was placed at the center of a plate (Ø 22 mm) containing E3 medium. Each embryo was gently poked at the tail with a needle, and the distance traveled was recorded with a video camera (60 frames per second) (Supplementary: Video_S1). We slightly modified the method described by Banset et al. (Basnet et al., 2017); instead of placing concentric circles on the microscope stage, we added them while post-processing the video. Briefly, the recorded videos were processed, and an overlay of four concentric zones (Ø 5 mm = zone 1, Ø 10 mm = zone 2, Ø 15 mm = zone 3, and Ø 20 mm = zone 4) was added to each of the clips (Fig. 5B). Upon touching with a needle, in which zone the larvae end up tabulated manually.

2.5.2. Locomotor assays

The zebrafish locomotor behavioral testing was conducted following the method described by Van Laar et al. but with some modifications (Van Laar et al., 2020). Five dpf zebrafish larvae were placed in 12-well plates with black walls and an optical glass bottom. Then the video was recorded in a self-made specialized behavioral chamber (DanioTrack, ©METLab, Fig. 6A), illumination was provided from the bottom, and a high-resolution camera (Sony) was placed on top to capture videos. After acclimatization in the chamber, the movements were captured for 10 min on light induction (200 Lux, White). As previously described, the video clips were analyzed using LSRtrack and LSRanalyze, freely available software tools for analyzing zebrafish neurobehavioral responses (Zhou et al., 2014). The recorded videos were interpreted following the principle of centroid tracking, which contains key behavioral metrics, including active velocity, total distance traveled, and percent time moving.

2.6. Acridine orange (AO) staining

Following exposure to PFOS for 96 h, 20 larvae from each group were washed with E3 medium and then transferred to a fresh medium that contained 10 μg/mL of AO (a1301, ThermoFisher Scientific), according to the procedures outlined in Tucker and Lardelli's study (2007) (Tucker and Lardelli, 2007). After keeping the larvae in the dark for 30 min, they were washed twice in E3 medium to wash out any excess stains. Before imaging, larvae were treated with 0.016 % MS – 222 to restrict movements. At least 20 images of each larva were captured across the z-plane using a confocal microscope system (SP8, Leica). Images were also acquired in the transmitted light (TL) filter for each larva. The brain and cardiac region were marked separately during image processing.

2.7. Neutral red (NR) staining

After the 96-h exposure, 20 larvae were selected from each group and immersed in media supplemented with 2.5 μg/mL of NR (72,210,

Sigma-Aldrich) for 2 h (Kuil et al., 2019). The larvae were washed with E3 medium, anesthetized using 0.016 % MS-222, and imaged using a confocal microscope (Leica Microsystem) to capture at least 20 serial optical sections across the z-plane. The imaging was done concurrently with the acquisition of brightfield images.

2.8. Quantification of NR and AO staining

Serially sectioned images acquired by confocal microscopy were stacked to get a 2D image of the 3D projections. The stacked images further merged with images captured using the TL filter channel. Subsequent quantification of NR-positive microglial cells and AO-positive apoptotic cells were performed for each image using ImageJ (ver. 1.53v) platform.

2.9. Neurotransmission and neuroinflammation assessments

Fifty larvae from each experimental group were collected and immediately snap-frozen in microcentrifuge tubes at a temperature of −80 °C. The brain tissues of the larvae were then homogenized and centrifuged at 5000*g* for 2 min in pre-cooled phosphate-buffered saline (PBS) to obtain the supernatant. The level of dopamine in the supernatant was assessed using the colorimetric method with 3,3,5,5-tetramethylbenzidine (TMB) as the substrate, as described previously (Liang et al., 2021). Acetylcholinesterase (AChE) activity was measured using acetylthiocholine (ASCh) as the substrate. The conjugation product between thiocholine (a byproduct of ASCh degradation) and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) was measured at 414 nm, every 20 s for 5 min, as per the protocol established previously (Ellman et al., 1961). Gene expression levels related to dopamine signaling (*th1*, *dat*, *drd2a*, *drd3*, and *drd4a*), neuroinflammation (*tnf-α*, *nF-κβ*, *il-1β*, and *il-6*), and apoptosis (*p53*, *bcl-2*, *bax*, and *caspase 3*) were also assessed in the zebrafish larvae. For this, total RNA was extracted from 96 hpf zebrafish larvae ($n = 30$) using a Trizol reagent, and the amount was measured using a spectrophotometer. The quantitative real-time PCR (qPCR) experiment used specific primers (Table S1). The qPCR was carried out using the Revert-Aid RT Reverse Transcription Kit and Maxima SYBR Green qPCR Master Mix (2×) according to the manufacturer's (Thermo fisher scientific) instructions. The relative mRNA expression was calculated using the $2-\Delta\Delta Ct$ method, as described (Livak and Schmittgen, 2001).

2.10. Statistical analysis

The experimental data underwent three replications, and the results were represented as mean ± SEM. One-way or two-way ANOVA tests were conducted on all data sets, followed by post hoc tests such as Dunnett's or Tukey's multiple comparisons as required. The generated graphs were plotted using GraphPad Prism software (version 9). A significance level of $P < 0.05$ was considered statistically significant for each experiment.

Table 1
Table showing the correlation between PFOS concentration and mortality rate of zebrafish embryos at different time points. The Pearson's correlation coefficient (r) and *p*-value are presented for each time point, with a p-value <0.05 considered statistically significant.

Time point	Pearson's r	p-value
24 Hours	0.287	0.697
48 Hours	0.984	0.016
72 Hours	0.990	0.010
96 Hours	0.989	0.011
120 Hours	0.996	0.004

3. Results

3.1. PFOS induced developmental toxicity and morphological abnormalities

To investigate the impact of PFOS on the embryonic development of zebrafish, we monitored the exposed embryos from 24 to 96 hpf. We observed a direct positive correlation between the concentration of PFOS and the mortality rate as time progressed in each group (Fig. 2A, Table 1). Additionally, we found a noteworthy decline in the hatching rate at 500 and 1000 μg/L at 72 and 96 h, respectively, in relation to the concentration of PFOS (Fig. 2B).

We investigated the impact of PFOS exposure on zebrafish embryos by examining typical characteristics at various developmental stages (24 to 96 hpf). As PFOS exposure increased, several morphological malformations were induced in embryos, including pericardial edema (PE), yolk sac edema (YSE), head and tail malformation, and spine deformation (SD) (Fig. 3A). The body length of the PFOS-treated group decreased significantly with increasing PFOS exposure concentration (Fig. 3B). While YSE and PE were the most pronounced morphological changes. Therefore, the yolk sac area (YSA) rate (Fig. 3C), pericardial area (Fig. 3D), and tail curvature (Fig. 3E) in the 500 and 1000 μg/L group increased significantly compared to the control following 96 h exposure. Additionally, a significant decrease in the eye area (Fig. 3F) and pigmentation (Fig. 3G) was observed in the 500 and 1000 μg/L groups. Overall, the malformation rate in the PFOS-treated group increased dose-dependent compared to the control (Fig. 3H). Furthermore, the morphology of zebrafish embryos from each group decreased with increasing PFOS exposure (Fig. 2I). These results demonstrate that PFOS induced concentration-dependent developmental toxicity.

3.2. Exposure to PFOS leads to altered spontaneous movement

The initial signs of movement in zebrafish embryos were observed as alternating side-to-side tail contractions, also known as spontaneous movement, triggered by growing neurons in the somite near the tail. We recorded the frequency of STC of treated embryos in all the exposed groups

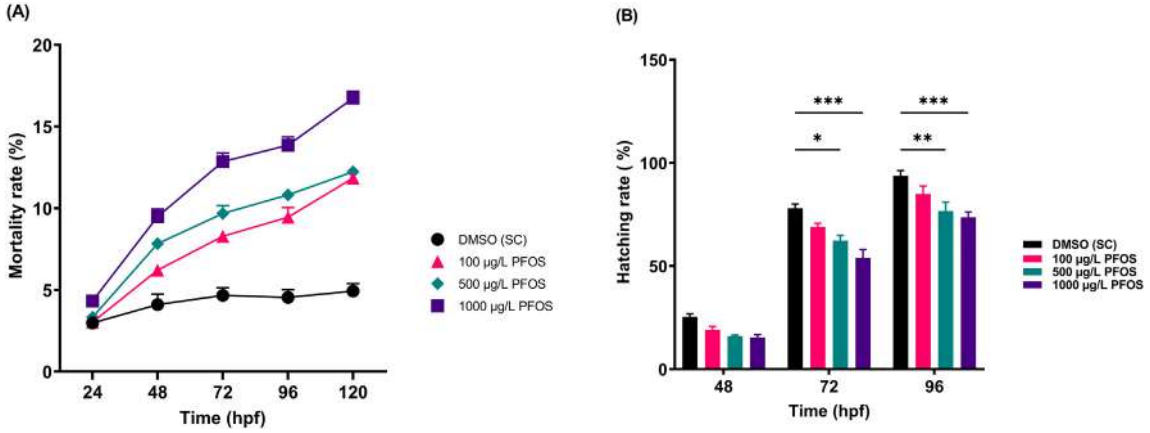

Fig. 2. The mortality rate (A) and hatching rate (B) of zebrafish embryos exposed to PFOS. No embryos were hatched at 24hpf (data not plotted). Values represented as mean ± SEM. Significance levels: [$P < 0.05$, $P < 0.01$, and $P < 0.001$ denoted by *, **, and ***, respectively].

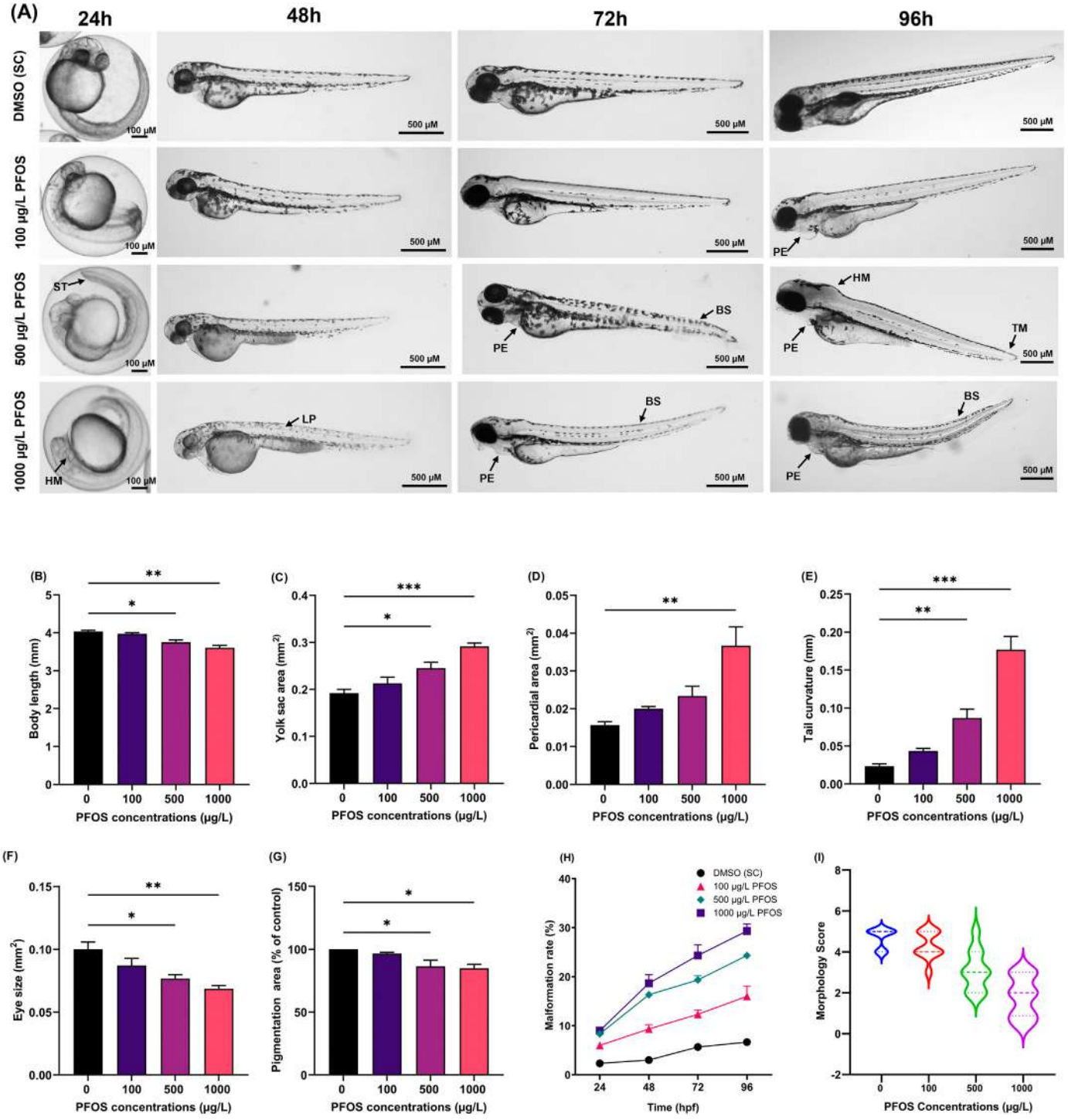

Fig. 3. (A) Morphological changes observed in zebrafish larvae exposed to different concentrations (100, 500, and 1000 µg/L) of PFOS. The morphological changes included short tail (ST), head malformation (HM), low pigmentation (LP), pericardial edema (PE), bent spine (BS), tail malformation (TM). (B) The body length, (C) yolk sac area, (D) pericardial area, (E) tail curvature, (F) eye size, (G) pigmentation area, (H) malformation rate, and (I) morphology score of embryos at 96 hpf. The values are presented as mean ± SEM. The statistical significance was represented as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

at different developmental stages (20–30 hpf) and analyzed the data statistically. The results showed that PFOS treatment inhibited STC embryonic movement in a concentration-dependent manner during the entire developmental period. In the higher PFOS concentration groups (500 and 1000 µg/L), STC embryonic movement was reduced compared to the control group, but no significant changes were observed (Fig. 4). These findings suggest that exposure to PFOS may lead to motor neuron dysfunction in zebrafish embryos.

3.3. PFOS exposure reduced embryonic touch-evoke response

To further study PFOS effects on zebrafish neural circuits, the larvae from each group were poked with a needle at 72 hpf, and their responses were recorded. Upon touch, larvae tend to escape from that point to a distant point to avoid danger. As shown in Fig. 5B, the arena was divided into four zones, from zone 1(no displacement) to zone 4 (maximum displacement). Our results showed that most of the larvae from the untreated

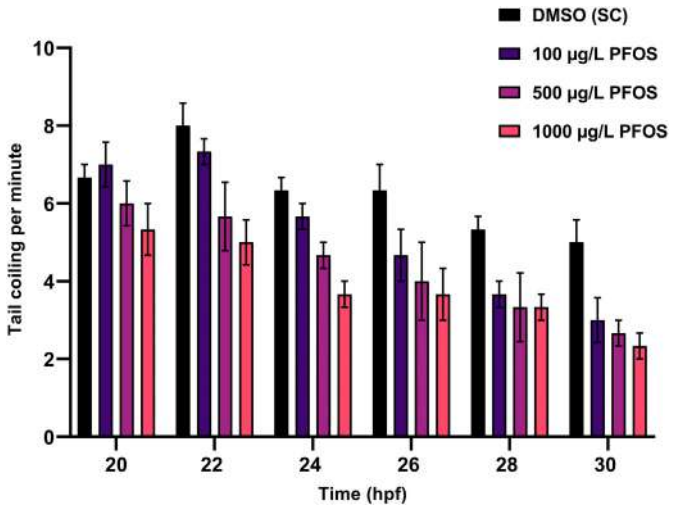

Fig. 4. Embryos were monitored for tail coiling per minute (or STC), hourly at 20 hpf until 30 hpf. Per replicate, ten embryos were measured. The data points represent the mean value of three replicates, and the error bars represent the standard error.

group traveled to zone 4 upon touching. At the same time, the response got weaker in the PFOS-treated embryos (Fig. 5C). In the 1000 µg/L PFOS group, most embryos showed no to minimal displacement, traveling to zone 1 and zone 2. The hindbrain controls this form of movement. The larvae can fully respond to environmental stimuli and govern locomotion, which is critical for survival in the natural world. Our results suggest that PFOS hinders hindbrain development in the developing zebrafish and thus decreases their chance of survival in the actual habitat.

3.4. PFOS exposure affected larval locomotory behavior

After being exposed to PFOS for 96 h, zebrafish larvae were found to have significantly affected motor abilities. Behavioral analysis of the larvae treated with different concentrations of PFOS showed abrupt swimming trajectories (Fig. 6E), and their activity index, such as active velocity (Fig. 6B) and percent time moving (Fig. 6D), were affected. However, the total distance traveled in the control and treatment groups (Fig. 6C) did not show significant changes. It was observed that exposure to lower concentrations of PFOS (100 µg/L) was less sufficient in inhibiting the behavioral activity. In contrast, zebrafish larvae exhibited reduced activity when exposed to higher concentrations of PFOS (500 and 1000 µg/L), indicating a potential link between PFOS exposure and disturbance in the motor nervous system of zebrafish larvae.

3.5. PFOS-induced aberrant cellular responses and gene expression

Cellular apoptosis was observed in zebrafish embryos exposed to PFOS using AO staining. The results showed that exposure to PFOS significantly increased the number of apoptotic cells in the brain and cardiac region of the PFOS exposure group (Fig. 7A). Furthermore, the fluorescence intensity was found to be markedly intensified in the brain and cardiac region of zebrafish treated with 500 or 1000 µg/L of PFOS exposure, respectively (Fig. 7B and C). The expression levels of apoptosis-related genes were determined after PFOS exposure to further confirm the apoptosis. The qPCR assays indicated that the expression level of *p53*, a well-known stress-responsive protein, and *bax*, a critical apoptotic regulator, was significantly upregulated in PFOS-treated zebrafish embryos. In contrast, the mRNA level of anti-apoptotic genes (*bcl-2*) was unchanged after PFOS exposure for 96 h. Next, we observed increased *caspase-3* gene expression following the exposure (Fig. 7D). Overall, these findings demonstrate that the developmental toxicity of PFOS exposure in embryonic zebrafish is accompanied by ameliorated apoptosis.

3.6. PFOS exposure resulted in increased microglial activation

We analyzed the formation of microglia, an essential embryonic macrophage derivative. A vital NR staining approach was used to mark microglia in the larval zebrafish brain as bright fluorescent red spots. An increased number of brain microglia was observed in PFOS-exposed groups in comparison to the untreated group (Fig. 8A). The number of NR + cells in the brain region was counted and found to be increased with treatment concentration (Fig. 8B). To further support the surge of microglial activity, the transcript levels of inflammatory markers (*tnf-α*, *nf-κβ*, *il-1β*, and *il-6*) were measured, and we observed a significant upregulation in PFOS-exposed groups (Fig. 8C). These findings support that PFOS may hinder the innate immune system in developing organisms.

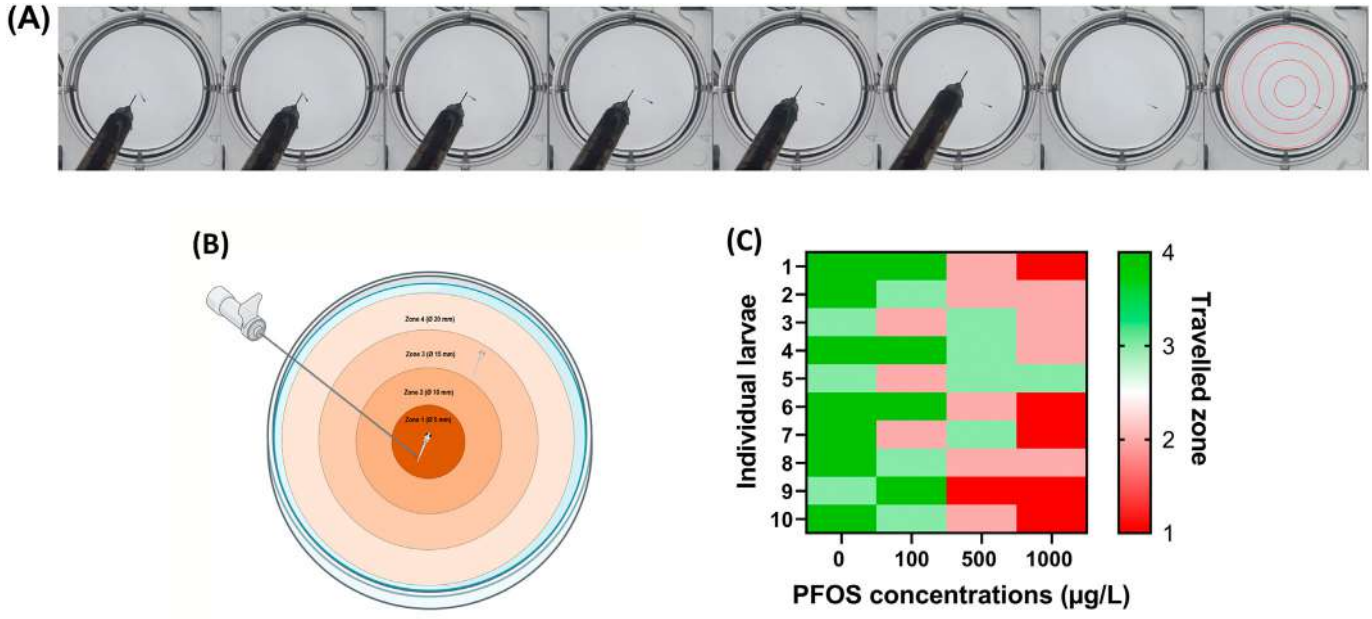

Fig. 5. (A) Frame by frame picture shows typical touch-evoke escape response in 5 dpf zebrafish larvae. (B) A schematic diagram shows divisions of virtual zones (Zone 1: Ø5 mm, Zone 2: Ø10 mm, Zone 3: Ø15 mm, and Zone 4: Ø20 mm. (C) Heatmap showing traveled zone by individual larvae from different experimental groups.

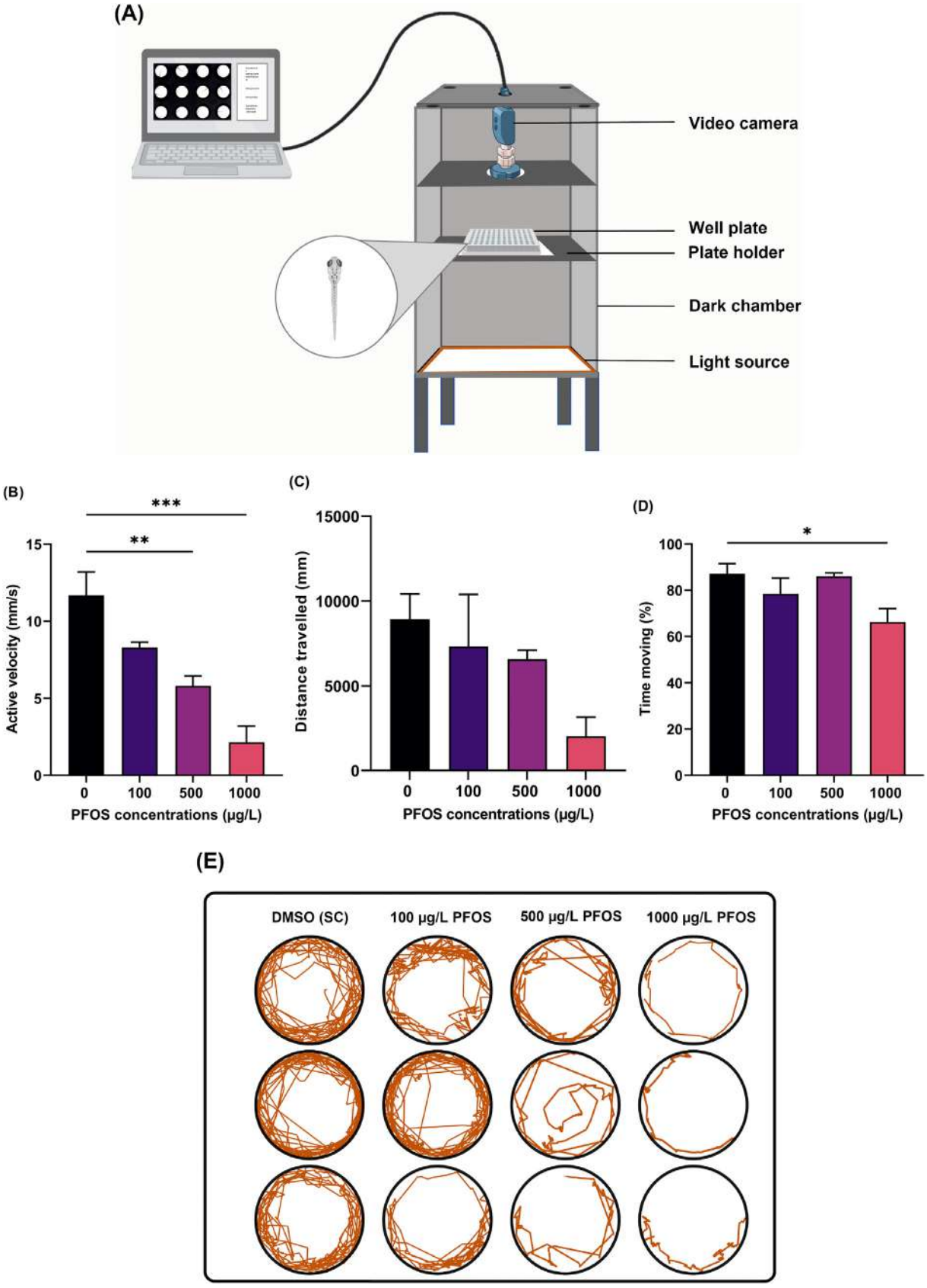

Fig. 6. (A) Schematic representation of our self-designed behavior observation chamber (DanioTrack, ©METLab) for zebrafish. Graphs showing (B) active velocity, (C) distance moved, and (D) percent time moving of 5 dpf larvae exposed to PFOS. Data is acquired and processed in the LSR track and LSR analysis. Data represented as the mean ± SEM. Significance level: * P < 0.05, ** $P < 0.01$ and *** $P < 0.001$. (E) Vector trajectories of the movements of 5 dpf larvae exposed to PFOS. Videos were captured in the DanioTrack observation chamber.

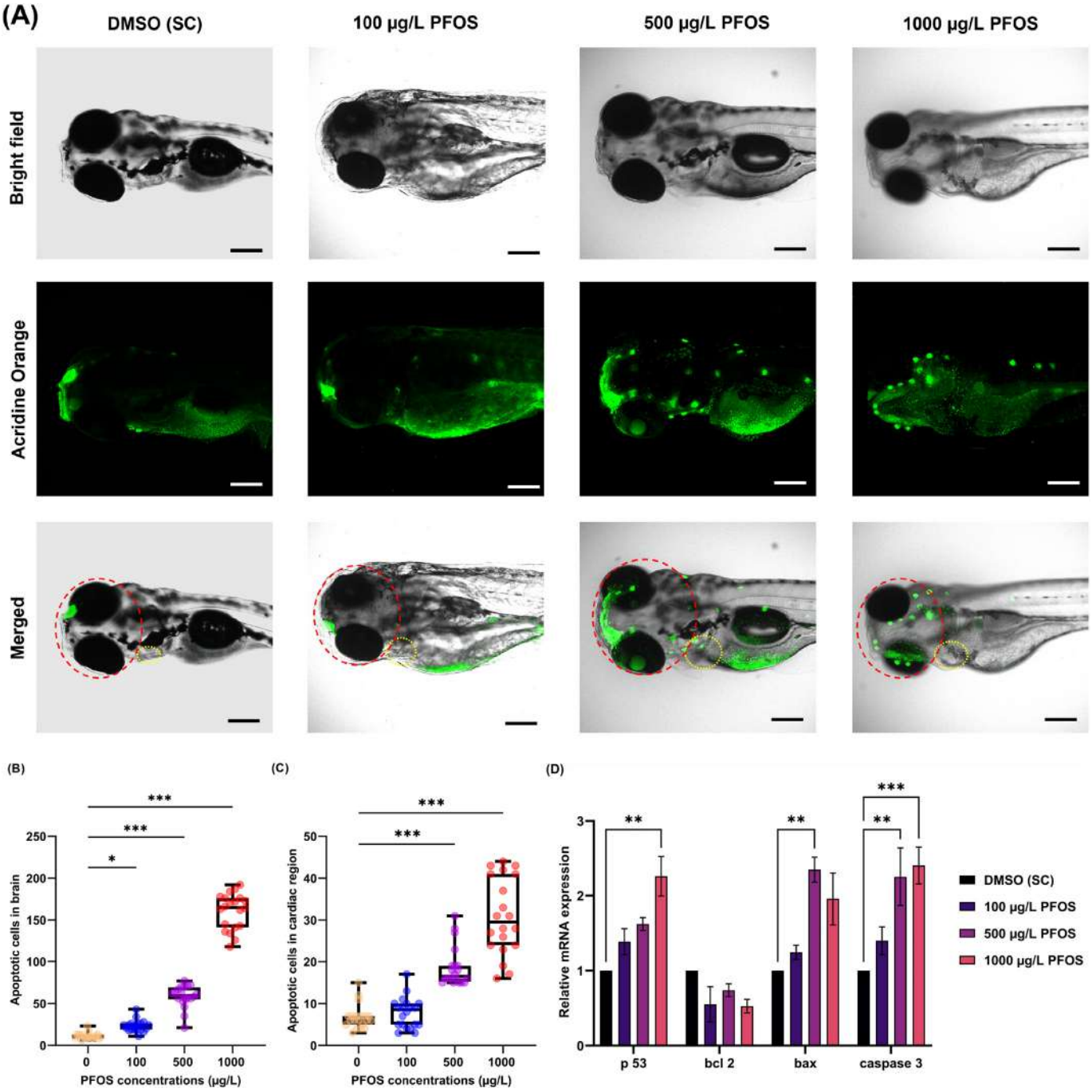

Fig. 7. (A) AO staining of PFOS treated 96 hpf zebrafish larvae showing apoptotic cells. The brain and cardiac region are marked by red and yellow dotted circles, respectively. Confocal images are *Z*-projections. Scale bar: 200 μm. Box plots showing the number of apoptotic bodies from the brain (B) and cardiac (C) region. The central line of the box reflects the mean value, while the upper/lower borders represent the maximum and minimum data points ($n = 20$). (D) The relative fold change in expression of genes involved in apoptotic pathway, as measured by qRT-PCR. β-actin was utilized throughout the process as an internal control. Asterisks (*) represent statistically significant differences between the PFOS-exposed and control groups. Significance level: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3.7. PFOS hindered neurotransmission and dopamine signaling pathway

AChE enzyme catalyzes the breakdown of acetylcholine and is essential for neuronal signal transmission. While examining AChE activity in the present study, a dose–response relationship for PFOS exposure was observed (Fig. 9A). These findings indicated that PFOS inhibits AChE in a way analogous to other conventional cholinesterase inhibitors. The dopamine content was also increased in PFOS-treated embryos (Fig. 9B). To determine if PFOS-induced neurotoxicity would alter mRNA transcript levels (*th1*, *dat*, *drd2a*, *drd3b*, and *drd4*) involved in dopamine signal transduction, we performed qRT-PCR. The results indicated the mRNA levels of *th1* were significantly higher, reaching to 1.51-fold change in 1000 μg/L PFOS-exposed embryos (Fig. 9C). However, the *dat* gene expression was decreased to 0.87-, 0.66-, and 0.72-fold in different concentration of PFOS (100, 500 and 1000 μg/L) compared with control. Significant upregulation of *drd2a*, *drd3b*, and *drd4* genes was detected in 500 and 1000 μg/L PFOS-treated embryos (Fig. 9C). Dopaminergic hyperactivity and locomotor hypoactivity were detected in PFOS-exposed larvae as evidenced by these data indicating that PFOS hinders neurotransmission and dopamine signaling.

4. Discussion

This study represents the developmental and neurobehavioral effects of PFOS in zebrafish embryos and the underlying mechanisms responsible for this toxicity. While examining the relationship between neuroinflammation and dopamine signaling in developing zebrafish, we addressed gaps in existing knowledge. While previous research by Shi et al. indicated that PFOS exposure led to decreased survival rates, increased malformation rates, and reduced body length in zebrafish embryos, our study is more comprehensive in scope, as it monitored embryonic development over a wider developmental window (24–96 hpf) (Shi et al., 2008; Kim et al., 2021). Our findings revealed that PFOS exposure caused delayed hatching, increased mortality, and malformations, including shortened body length, tail curvature, pericardial edema, yolk sac edema, and reduced eye size. Notably, the hatching rate decreased each time measured, suggesting that PFOS may adversely impact hatching enzyme activity and somite development (Peng et al., 2018; Zoupa and Machera, 2017). While we also assessed the total pigmented area in 96 hpf embryos, no significant changes were observed. Our results highlight the significant ecological risks posed by PFOS on aquatic organisms, as changes in hatchability, mortality, and deformity during embryonic development are potential indicators of developmental toxicity due to environmental contaminant stress, as previous studies have shown (Hill et al., 2005; Yang et al., 2022). Further research is needed to better understand the effects of PFOS exposure on embryonic development and identify potential interventions.

Behavior is seen as a highly organized, neural network-driven process that assures the fitness of organisms. As a result, behavioral alterations are frequently regarded as sensitive measures in ecotoxicological investigations for identifying neurological dysfunction caused by pollutant exposure (Legradi et al., 2018; Ford et al., 2021). The motor function of zebrafish embryos was analyzed through STC and TER (Drapeau et al., 2002). The STC is the first locomotor activity of zebrafish embryos (starts at 17 hpf), which occurs due to an active neuronal network and is solely dependent on electrical connection (Saint-Amant and Drapeau, 2001). Meanwhile, this STC assay in zebrafish embryos has been reported as an alternate screening tool for developmental neurotoxicity upon toxicants exposure (Thion et al., 2018). Vliet et al. studied the possibility of STC to distinguish modes of action and sensitive levels for pharmacologically active chemicals that interfere with neurotransmission but fail to discriminate the neurotoxic action (Vliet et al., 2017). Later, Zindler et al. connected the effects of recognized neurotoxicants on coiling activity with acute exposure consequences in embryos (Zindler et al., 2019). As de Oliveira et al. mentioned, the developmental window showing the most stable tail coiling behavior was between 22hpf to 30 hpf. Therefore, we have chosen this period to monitor the tail coiling behavior (de Oliveira et al., 2021). In our study, PFOS exposure decreased STC frequency, suggesting that primary motor neurons might be the target of PFOS associated with spinal deformities and delayed hatching (Chen et al., 2022). The second assay for analyzing motor function, i.e., TER assay, should be performed between 48 and 72 hpf window, as 26 hpf is too early to measure TER, while 96 hpf is an entirely active stage (Saint-Amant and Drapeau, 1998; Colwill and Creton, 2011). Moreover, TER involves local spinal motor and sensory neurons that activate in response to touch. Our data demonstrated that exposure to PFOS elicits a sluggish avoidance reaction in zebrafish larvae, consistent

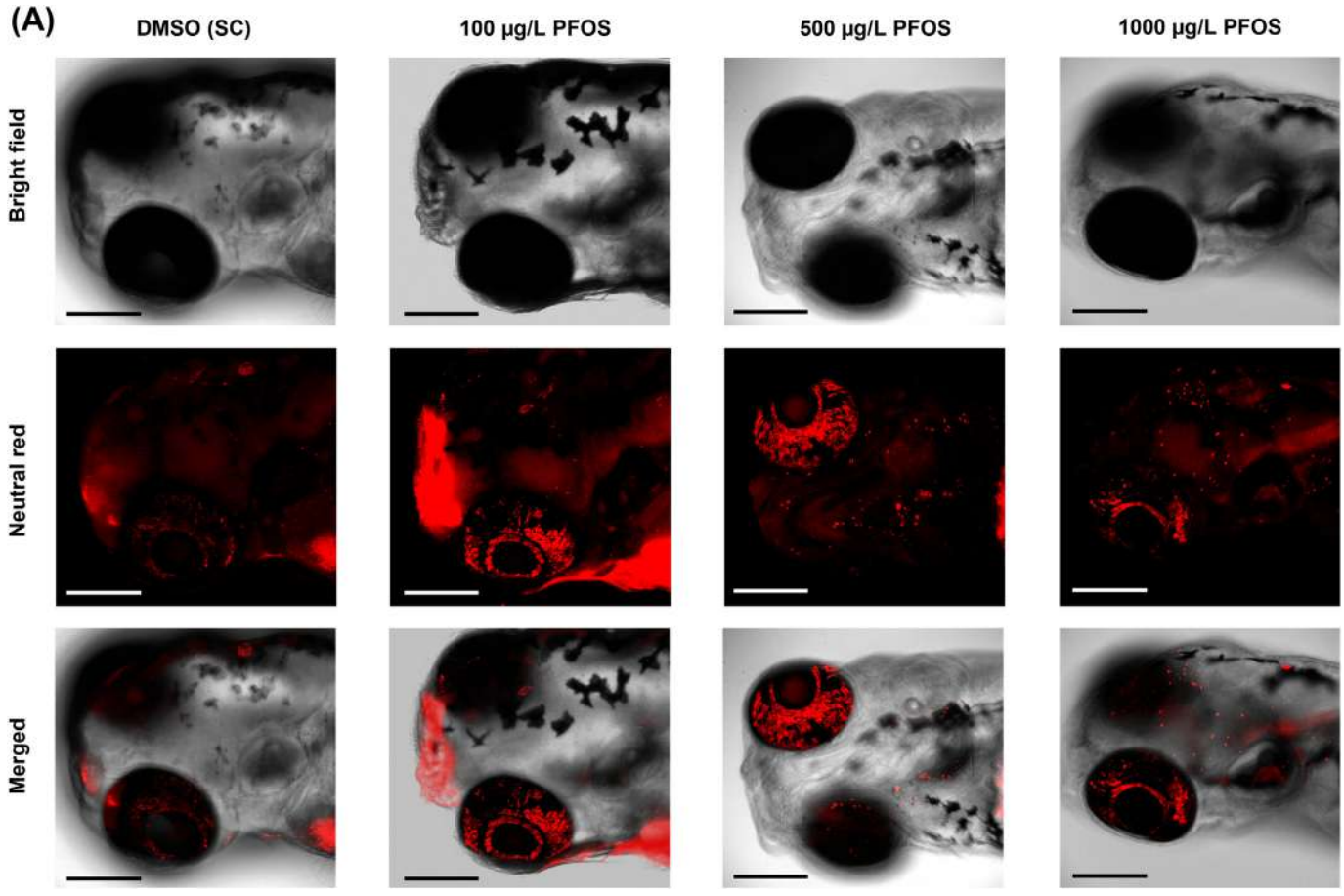

Fig. 8. (A) PFOS treated 96 hpf zebrafish larvae stained with NR showing brain microglial cells. Vehicle control larvae exhibited few microglia cells compared to treatment groups. Confocal images are *Z*-projections. Scale bar: 200 µm. B) Box plot showing the number of microglia cells stained with NR. The central line of the box reflects the mean value, while the upper/lower borders represent the maximum and minimum data points ($n = 20$). (C) The relative fold change in mRNA transcripts involved in neuroinflammation as measured by qRT-PCR. β-actin was utilized throughout the process as an internal control. Asterisks (*) represent statistically significant differences between the PFOS-exposed and control groups. The statistical significance was represented as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

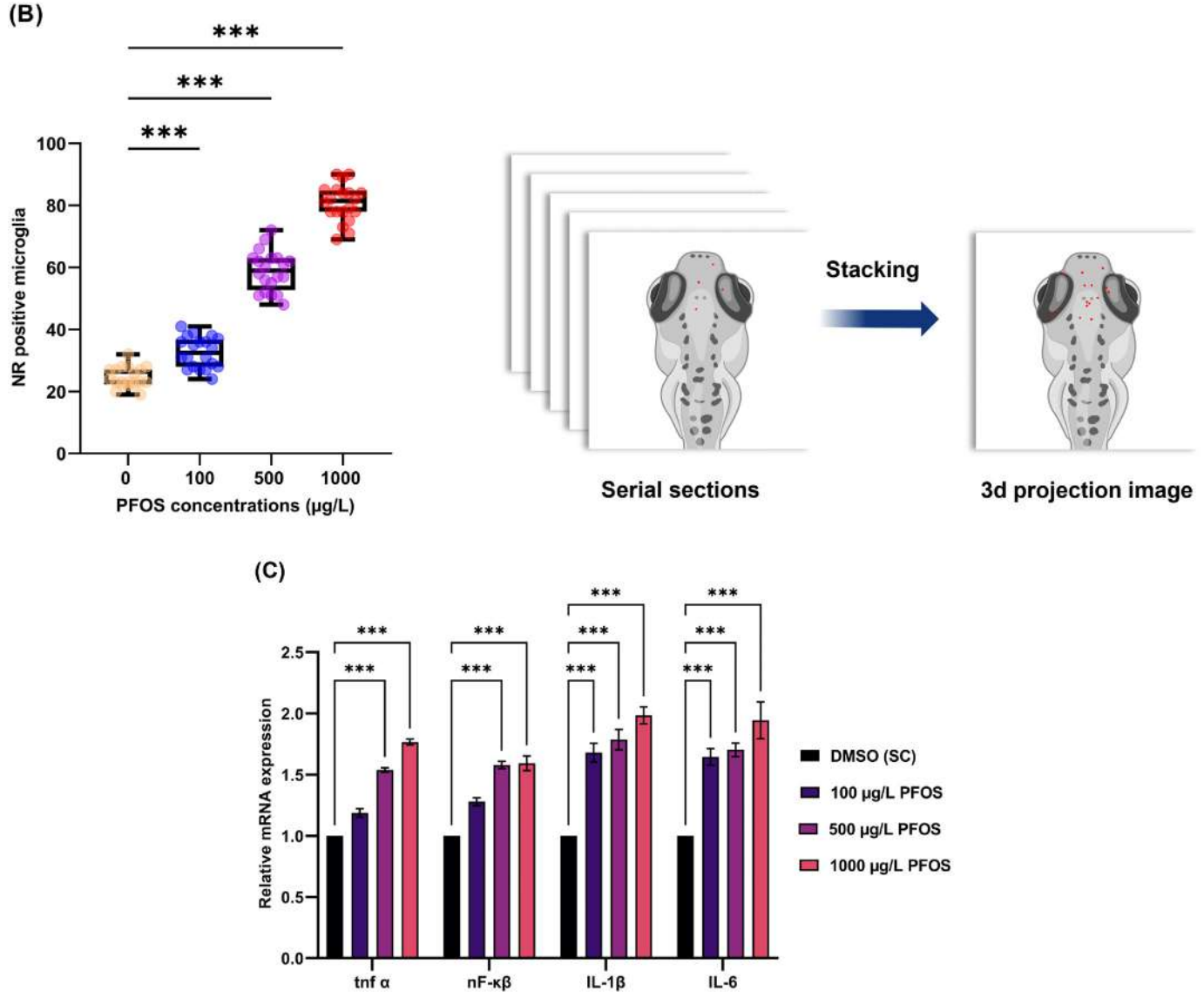

Fig. 8 (*continued*).

with the response stated by Koide et al. when they exposed larvae to CO_2 (Koide et al., 2018). Furthermore, we arbitrarily divided the arena into four traveling zones to quantify the response rate. Subsequently, almost half of the treated larvae from the 500 and 1000 μg/L PFOS treated embryos could not cross zone 3 and mainly remained in zone 1 or 2 upon touch stimuli. The lethargic voluntary movement of treated zebrafish larvae could result from motor neuron dysfunctions. Our research is the first to indicate that PFOS disrupts TER in zebrafish larvae, as far as we know.

To validate the results of TER, we further observed the response of voluntary movement without any touch stimuli. Our study demonstrated that the total distance traveled and the mean velocity of treated larvae markedly decreased after exposure. Even some larvae in the higher PFOS dose appeared adrift, swimming without a set path, suggesting that PFOS might disrupt the neurobehavioral pattern of zebrafish embryos. Our results are consistent with Feng et al. (Feng et al., 2022), who showed zebrafish embryos' impaired locomotory behavioral response in response to polystyrene nanoplastics. Previous investigations on organophosphorus chemicals in zebrafish have shown that exposure to paraoxon, chlorpyrifos, and diazinon decreases overall swimming activity (Koenig et al., 2016; Yen et al., 2011). According to the last report, spine abnormalities are associated with reduced frequency of STC and motor behavior (Moravec et al., 2017; Qian et al., 2020). Taken together, our results raise the possibility that PFOS exposure disrupts the nervous motor system and impairs neurotransmission in zebrafish larvae.

Cellular apoptosis is commonly induced as a homeostatic mechanism to maintain cell proliferation and differentiation in ideal physiological processes and pathogenesis throughout organisms (Ellis et al., 1991; Wong, 2011). Previous findings have reported that increased ROS production might result in apoptosis during embryogenic zebrafish (Zhu et al., 2015; Hung et al., 2021). Liu et al. said increased apoptotic bodies in the brain with increased apoptotic gene expression levels after INH exposure resulted in neurodevelopmental toxicity in embryonic zebrafish (Liu et al., 2021). Similarly, in our study, PFOS-exposed groups exhibited aberrant cellular response with increased apoptotic bodies in the brain and cardiac region during AO staining. For further investigation of apoptotic pathways, gene expression of apoptotic-related genes was analyzed. DNA-damaging agents or environmental pollutants activate the tumor suppressor gene, *p53*, to induce programmed cell death or apoptosis. Notably, in response to *p53* activation, anti-apoptotic genes (*bax*) expression downregulates and upregulates pro-apoptotic genes (*bcl-2* and *bcl-xl*) expression. Studies have shown that *caspase-3*, a key executioner, gets triggered during apoptotic induction and plays a pivotal role in activating downstream pathways (Deng et al., 2009; Zhao et al., 2016). So, we speculated this phenomenon in our study and found that PFOS exposure led to significant upregulation of *p53*

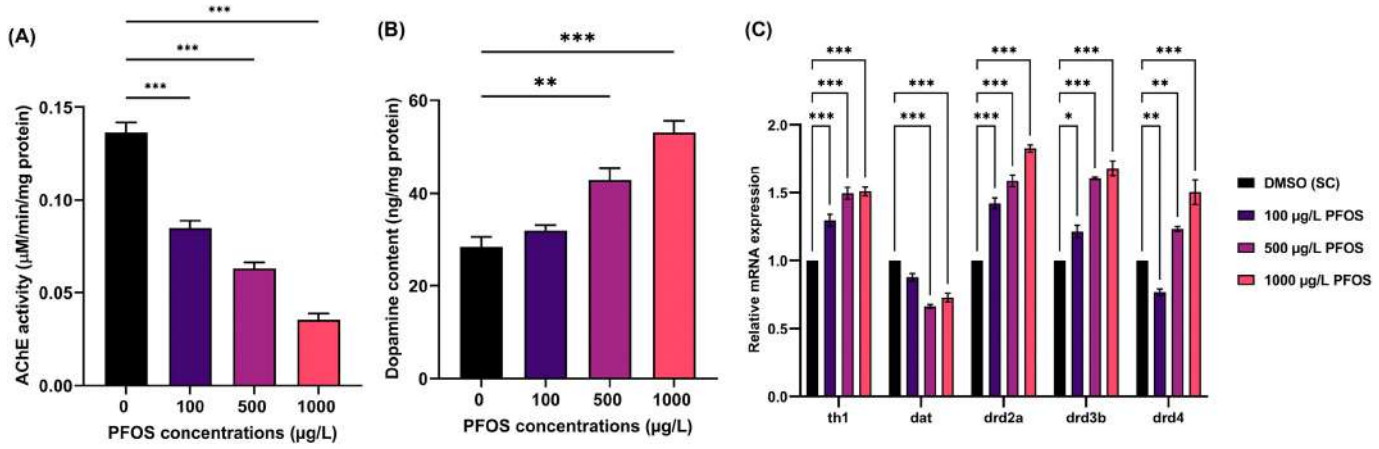

Fig. 9. (A) The activity of AChE and (B) Average dopamine concentration was measured in 96 h zebrafish embryos homogenate ($n = 6$). (C) The relative fold change in mRNA transcripts involved in dopamine signaling pathway as measured by qRT-PCR. β-actin was utilized throughout the process as an internal control. Asterisks (*) represent statistically significant differences between the PFOS-exposed and control groups. Data plotted as the mean ± SEM. The statistical significance was represented as * P < 0.05, ** $P < 0.01$, and *** $P < 0.001$.

gene expression followed by increased transcript level of bax and slight alterations in the *bcl-2* mRNA levels, resulting in the initiation of apoptosis. Furthermore, PFOS-induced *p53* activation upregulates *caspase-3* expression, suggesting that PFOS perturbs apoptosis during the early life stages of zebrafish. Therefore, PFOS initiates cellular apoptosis through a p53-mediated pathway by activating bax/bcl-2 and caspase-3 expressions. These results corroborated prior research showing significant perturbation in apoptotic-related gene expression that might lead to developmental damage and neurotoxicity in embryonic zebrafish (Deng et al., 2009; Wu et al., 2016).

Microglia are largely mononucleated phagocytic cells that serve as the brain's immune system and modulate neuronal connectivity (Lenz and Nelson, 2018). As adult brain microglia are derived from the early embryonic macrophage population, early disruption of microglia in developing embryos may disrupt the immune system of the maturing CNS, acting as the primary mediators of inflammation (Mehl et al., 2022; Thion et al., 2018). In the present study, the results showed that PFOS exposure during the early embryonic stage of zebrafish led to an altered number of microglia in the brain, suggesting that PFOS might be contributing to the inappropriate inflammation and ultimately leading to neurological disorders as reported earlier that when microglia get activated, they secrete a cocktail of pro-inflammatory factors such as cytokines (TNF-α and IL-1b) and chemokines, which contribute to neuronal damage. Similarly, Arsenic trioxide-induced neurotoxicity led to evident microglia activation and secreted inflammatory factor IL-1β (Mao et al., 2016). In the current study, we speculated that the transcript levels of inflammatory factors (TNF-α, NF-κB, IL-1β, and IL-6) were elevated, which is persistent with the previous report (Yuan et al., 2019). In summary, the present finding indicates that PFOS exposure can cause increased neuroinflammation in zebrafish larval brains by activating microglia.

To explore the mechanisms underlying PFOS-induced inhibitory actions, we further investigated the dopaminergic signaling at neurotransmitter and molecular levels, critical for neurodevelopmental toxicity and behavioral abnormalities in zebrafish (Irons et al., 2013). Evidence has reported that phthalates such as Di (2-ethylhexyl) phthalate (DEHP) disrupt the dopaminergic neurogenesis in larval zebrafish (Huang et al., 2022). In our study, DA levels showed a significant increase in zebrafish embryos at different concentrations of PFOS (100, 500, and 1000 μg/L). Notably, our result was consistent with the results of cuprizone, in which exposure begins in the embryonic stage and significantly increases DA content and perturbs normal neurodevelopment (Liu et al., 2022). Briefly, tyrosine hydroxylase (TH) catalyzes dopamine conversion into L-DOPA, therefore serving as the rate-limiting step in dopamine synthesis. Once the intracellular dopamine level exceeds dopamine active transporter (DAT), a membrane protein undergoes dopamine reuptake from presynaptic neurons. Hence, DAT is essential for regulating and maintaining dopamine release and its turnover in the body (Liu et al., 2021). Therefore, the altered mRNA expression of *th* and *dat* in treated larvae indicates that PFOS might lead to dysfunction of the developing brain by affecting the neurotransmitter homeostasis. Dopamine receptors (DRs) are transmembrane G protein-coupled receptors at which dopamine binds and hence plays a major role in regulating dopaminergic neurotransmission. The current study showed that *drd2a*, *drd3b*, and *drd4* expressions were induced in different concentrations of PFOS treatment groups relative to the control group. Recently, Liu et al. demonstrated that cuprizone-induced dopaminergic hyperactivity had been linked with locomotor-deficit zebrafish behavior (Liu et al., 2022). Thus, altered levels of dopamine levels and gene expression of dopamine signaling pathway after PFOS exposure suggests disruption in dopaminergic signal transduction resulting in neurodevelopment toxicity. In this regard, it is plausible that locomotor-deficit behavior in fish after PFOS exposure could be linked to aberrant expression of DRs. These findings indicate that hindrance in neurotransmitter transmission and signal transduction of the dopaminergic pathway can harm normal brain development and potentially lead to adverse neurological disorders.

5. Conclusion

The present study demonstrated that PFOS might cause developmental neurotoxicity by interfering with neurotransmitter release, motor neuron development, and gene expressions. PFOS exposure also disrupted the dopamine signaling system, altering neurotransmission and motor neuron responses in zebrafish, resulting in behavioral impairment. These outcomes may help understand the locomotor behavioral dysfunction of exposed zebrafish larvae, and the underlying process might be connected to the homeostasis of dopamine biosynthesis and metabolism. To my understanding, we present the first molecular report for the association between microglial activation and neurobehavior in PFOS-exposed zebrafish larvae considering neurotransmission and neuroinflammation. Thus, the pioneer findings of the present study will provide a better understanding and serve as a reference to determine the hazardous risk information of PFOS exposure and the underlying pathway of neuroprotection via microglial activation and hyperactive dopaminergic transmission during neurological insults. Moreover, the molecular crosstalk between microglial cells and dopaminergic neurons may shed new light on the therapeutic strategy for treating neurological disorders like Parkinson's and schizophrenia.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.164030.

Ethical aspects

All experimental procedures were performed in accordance with the ethical standards for animal experimentation, and meticulous efforts were made to ensure that the animals suffered as little as possible and to reduce external sources of stress, pain, and discomfort. The current study has not exceeded the number of animals needed to produce reliable scientific data. This article does not refer to any study with human participants performed by any authors.

CRediT authorship contribution statement

Archisman Mahapatra: Conceptualization, Investigation, Methodology, Writing- Original draft preparation, Software, Visualization, Reviewing and Editing. Priya Gupta: Methodology, Software, Data curation, Writing- Original draft preparation, Formal analysis, Reviewing and Editing. Anjali Suman: Writing – Review & Editing. Shubhendu Shekhar Ray: Writing - Review & Editing. Guilherme Malafaia: Project administration, Funding acquisition, Validation, Writing - Review & Editing. Rahul Kumar Singh: Supervision, Conceptualization, Project administration, Funding acquisition, Writing - Review & Editing.

Data availability

Data will be made available on request.

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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