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Progressive impairment of learning and memory in adult zebrafish treated by Al_2O_3 nanoparticles when in embryos

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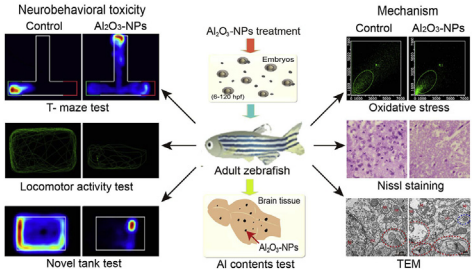
HIGHLIGHTS

- Al_2O_3-NPs causes progressive impairment of learning and memory during the development of zebrafish from embryo to adult.
- Impairment of zebrafish embryos induced by Al_2O_3-NPs can last and aggregate until they grow up to become adults.
- Neural cell death and autophagy may be involved in the mechanism of progressive learning and memory deficits.
- Al_2O_3-NPs can cause oxidative stress in the brain of adult zebrafish after exposure to Al_2O_3-NPs in its embryo stage.

GRAPHICAL ABSTRACT



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ABSTRACT

Al_2O_3 Nanoparticles (Al_2O_3-NPs) have been widely used because of their unique physical and chemical properties, and Al_2O_3-NPs can be released into the environment directly or indirectly. Our previous research found that 13 nm Al_2O_3-NPs can induce neural cell death and autophagy in primarily cultured neural cells in vitro. The aim of this study was to determine where Al_2O_3-NPs at 13 nm particle size can cause neural cells in vivo and assess related behavioural changes and involved potential mechanisms. Zebrafish from embryo to adult were selected as animal models. Learning and memory as functional indicators of neural cells in zebrafish were measured during the development from embryo to adult. Our results indicate that Al_2O_3-NPs treatment in zebrafish embryos stages can cause the accumulation of aluminium content in zebrafish brain tissue, leading to progressive impaired neurodevelopmental behaviours and latent learning and memory performance. Additionally, oxidative stress and disruption of dopaminergic transmission in zebrafish brain tissues are correlated with the dose-dependent and age-dependent accumulation of aluminium content. Moreover, the number of neural cells in the telencephalon tissue treated with Al_2O_3-NPs significantly declined, and the ultramicroscopic morphology indicated profound autophagy alternations. The results suggest that Al_2O_3-NPs has dose-dependent and time-dependent progressive damage on learning and memory performance in adult zebrafish when

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treated in embryos. This is the first study of the effects of Al_2O_3-NPs on learning and memory during the development of zebrafish from embryo to adult.

1. Introduction

Nanosized alumina (Al_2O_3-NPs) account for 20% of the global market for all commercially produced nanoparticles (NPs) (Oesterling et al., 2008). Al_2O_3-NPs have been widely used in pharmaceuticals and water treatment (Kagan et al., 2005; Willhite et al., 2014), polymers and tyres (Stadler et al., 2010), and aerospace platforms (Barako et al., 2018). Notably, the safety of these particles remains debatable. Some have claimed that compared with other metal oxides nanoparticles, the toxicity of Al_2O_3-NPs is relatively low (Wang et al., 2009; Ng et al., 2015). However, an increasing number of studies are highlighting the toxic effects of Al_2O_3-NPs on biological models. It has been reported that although the toxicity of Al_2O_3-NPs can drastically vary depending on morphology (Park et al., 2016) and size (Mirshafa et al., 2018), manufactured Al_2O_3-NPs of different particle sizes or forms can penetrate biological barriers (Chen et al., 2008; Zhou et al., 2018) and accumulate in multiple organs (Canli et al., 2019), leading to neurotoxicity (Dong et al., 2019), immunotoxicity (Braydich-Stolle et al., 2010), genotoxicity (Balasubramanyam et al., 2009; Zhang et al., 2017), and toxicity in the heart (El-Hussainy et al., 2016), lung (Li et al., 2017), liver (Yousef et al., 2019), and kidney (Anand et al., 2019). Despite the widely reported toxic effects of Al_2O_3 NPs, the specific mechanisms in the biological systems remain far from being elucidated.

In humans, the primary target of aluminium (Al) toxicity is the brain, where it has been associated with incidences of dialysis dementia, osteomalacia, Alzheimer's disease (AD), and Parkinson's disease (Krewski et al., 2007; Shah et al., 2015). By contrast, Al_2O_3-NPs contain Al toxicity, their nanoparticles can present in all organs, and the brain is the most affected organ (Canli et al., 2019). In particular, with their small size, Al_2O_3-NPs might penetrate the blood–brain barrier (Chen et al., 2008) and impair the cerebral neural cells associated with learning and memory. Thought-provoking results suggested that Al_2O_3-NPs, if aggregated in the brain, were very difficult to exclude from the brain in vivo (Zhou et al., 2018). As a result, they might cause serious long-term toxicity to the brain and its learning and memory functions. Our previous article indicated that the accumulation of Al_2O_3-NPs in the brain caused neurobehavioral changes and impaired learning and memory performance both in vivo and in vitro (Zhang et al., 2011, 2013, 2018). Therefore, we hypothesised that long-term accumulation of Al_2O_3-NPs in brain tissue can induce progressive impairment of learning and memory.

In terms of toxic mechanisms, exposure to Al_2O_3-NPs has been shown to cause mitochondria-dependent apoptosis and oxidative stress (Li et al., 2016). Other studies have shown cytotoxicity and apoptosis in cerebral cortex astrocytes of rats: In a dose-dependent manner exposed to Al_2O_3-NPs, these effects were associated with significantly greater reactive oxygen species (ROS) accumulation and inflammation induction (Dong et al., 2019). Promoted amyloid protein deposition and induced neurotoxicity were reported in the brain of ICR male mice exposed to Al_2O_3-NPs (Shah et al., 2015). In addition, mitochondrial damage and neurodegeneration were observed in mice exposed to Al_2O_3-NPs (Li et al., 2016). Together, these studies suggest a possible toxic mechanism of Al_2O_3-NPs that involves oxidative stress and inflammation. However, detailed molecular mechanisms by which Al_2O_3-NPs induce neurotoxicity and learning and memory impairment have not been defined.

The toxicity of Al_2O_3-NPs is closely related to their nanoparticle size (Mirshafa et al., 2018) and nanomorphology (Park et al., 2016) in a dose-dependent manner (Li et al., 2017). In our previous article, we reported a learning and memory impairment in 50 nm Al_2O_3-NPs treated mice (Zhang et al., 2011). In addition, we compared the neurodevelopmental toxic effects of a particle size of 50 nm with 13 nm Al_2O_3-NPs, and the results showed that the 13 nm particle size had stronger neurodevelopmental toxicity (Zhang et al., 2017). Therefore, in this study, we evaluated dose-dependent and time-dependent effects of 13 nm Al_2O_3-NPs at various concentrations and assessed the long-term toxicity in adult zebrafish aged 6 months, 9 months, and 12 months, after treatment with Al_2O_3-NPs during embryonic stages.

We designed a set of experiments to evaluate whether Al_2O_3-NPs could accumulate in the brain and would impair the performance of learning and memory in the adult zebrafish treated with Al_2O_3-NPs during their embryotic stage. We observed the characterisation of Al_2O_3-NPs by using an electron microscope and determined zeta potential with a nanoparticle size analyser. A locomotor activity test, exploratory activity test, and T-maze test determined the neurobehavioral and learning and memory alternations in adult zebrafish at age 6 months, 9 months, and 12 months. Moreover, oxidative stress and neurotransmitters in the brain were measured to provide additional evidence of learning and memory alternations and mechanistic exploration. Finally, the number of neuronal cells was counted by using Nissl staining, and the ultrastructure of neural cells at the zebrafish telencephalon was observed using an electron microscope.

2. Materials and methods

2.1. Nanoparticles size and zeta potentials characteristics

Al_2O_3-NPs used in this study were purchased from Sigma Aldrich (St. Louis, MO, USA). Al_2O_3-NPs stock suspensions were freshly prepared in fish water medium (Brand et al., 2002) (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4 \cdot 7H_2O$, pH = 7.2). The Al_2O_3-NPs suspension was ultrasonically (100 W, 30 kHz) mixed for 30 min, and then 10 μL of the solution was dropped onto a carbon-coated copper grid. After the carbon-coated copper grids were dried, the nanoparticle dispersion was characterised by using a transmission electron microscopy (TEM, JEM-100CX, Tokyo, Japan) operated at an accelerating voltage at 80 kV. The average particle size of the Al_2O_3-NPs suspension was analysed by image analysis software (Olympus, Tokyo, Japan). The Al_2O_3-NPs suspension was ultrasonically (100 W, 30 kHz) mixed for 30 min; next, the average hydrodynamic size and Zeta potentials were measured by Nano Zetasizer (Malvern Instrument Ltd., Malvern, UK) at 25 °C.

2.2. Zebrafish

Adult zebrafish specimens of the wild-type TU strain were purchased from National Zebrafish Resource Centre of Institute of Aquatic Biology, Chinese Academy of Sciences (Wuhan, China). Zebrafish were maintained under a 14/10-h light/dark cycle (light

onset: 8 a.m.; light offset: 10 p.m.), and their temperature (28 ± 0.5)°C and pH (7.0–7.4) were monitored and controlled. Fertilised eggs were provided by the same facility. Fertilised eggs were incubated in fish culture medium in an incubator (Shanghai Zhicheng, China) at 28.5 °C. During the experiments, before 120 h post-fertilisation (hpf), embryos were not fed, and the medium was changed daily to ensure optimal water quality. From 120 hpf to months of age, the food for zebrafish was gradually transferred from zebrafish special feed (Shanghai Haisheng Biotech, China) to brine shrimp. After 1 month of age, all fish were fed twice daily with brine shrimp. Fish were group-housed with <20 fish per 3 L tank. All the zebrafish experiments were performed per the "Policies on the Use of Animals and Humans in Neuroscience Research" approved by the Society for Neuroscience in 1995 and were supervised by the Animal Administration and Ethics Committee of Shanxi Medical University.

2.3. Al_2O_3 nanoparticles exposure

Embryos in 6 hpf were divided into 5 groups: 1 control and 4 treatment groups. The treatment groups were treated continuously with fish culture medium containing Al_2O_3-NPs at concentrations of 0, 6.25, 12.5, 25, 50, or 100 mg/L until 120 hpf. The medium was changed daily. At the age of 6 months, 9 months, and 12 months, we used the adult zebrafish to perform experiments at various concentrations and time points. All behavioural tests were conducted between 9:00 am and 5:00 pm, during the light phase (Liu et al., 2018). A detailed description of the experimental design is presented in Supplementary Fig. 1.

2.4. T-maze test

The main purpose of the T-maze experiment was to determine the learning and memory ability of zebrafish, and the zebrafish T-maze was conducted according to a method in the literature (Liu et al., 2018). The T-maze was divided into 2 parts: 1 long arm and 2 short arms, and all arms were comprised of transparent acrylic glass plates (Long arm length: width: height = 40 cm: 10 cm: 10 cm; short arm length: width: height = 20 cm: 10 cm: 10 cm). At the foremost end of the long arm, a starting point of length: width: height = 10 cm: 10 cm: 10 cm was designed; the left and right arms were covered with green- and red-coloured sleeves, respectively (Avdesh et al., 2012). Water in the maze was filled up to 6 cm height, and the water temperature was maintained at 28 °C throughout the experiment. One of the 2 chambers was used as the enriched chamber (EC zone) and contained a small amount of brine shrimp to offer a favourable habitat for the fish. Before the test, all zebrafish adapted to the T-maze environment. During the training period, each zebrafish was placed in the T-maze alone. Zebrafish behaviour was recorded by a camera (Panasonic System Networks, Fukuoka, Japan) and analysed by Ethovision XT10 (Noldus, Netherlands).

The testing paradigm included 3 phases: (1) A 4-day habituation phase, to acclimate the zebrafish to their novel environment. All zebrafish were introduced to the T-maze environment, but the number was gradually reduced each day (16, 8, 4, and 1) until a single fish was placed in the maze and allowed to freely swim. (2) A 4-day training trial. Accordingly, the sight of the stimulus group was established as a potential reward in reward-related associative learning task (Al-Imari and Gerlai, 2008). During training trials, a single fish was placed in the start zone of the T-maze and released after 30 s. The fish was allowed to explore the T-maze for 4 min. Each focal fish experienced 4 days. All fish received associative learning training. The latency was defined as the time necessary for the zebrafish to swim swam start zone to the EC zone. During the training time, if a zebrafish did not find the EC zone in the 4-min testing time, it would be guided into EC zone and allowed to remain in it for 1 min while it found the EC zone. The test started at 9:00 a.m. (Liu et al., 2018), once per day, for consecutive 4 days. (3) The probe trial was conducted the day after the last training trial, to evaluate memory performance. During this phase, all zebrafish were separately allowed to swim in the same T-maze environment for 4 min, but without food as rewards. The cumulative time (total time the fish stayed in EC zone) of zebrafish spent in the EC zone was analysed (Supplementary Fig. 2).

2.5. Locomotor activity test

The locomotor activity test was conducted to assess swimming behaviour of zebrafish in the darkness. In this test, a small test trapezoidal water tank was designed (length × width: 10 × 6 cm at the bottom, length × width: 11.5 × 7.5 cm at the top, 6 cm high). The tank was filled with ~150 mL of fish water: approximately 3 cm depth at 28 °C. Each zebrafish was placed in a test trapezoidal water tank for 1 min to adapt to the dark condition; next, a video recorder recorded the behaviours of the zebrafish for 3 min (Panasonic System Networks, Fukuoka, Japan). Average speed, freezing time movement ratio (total percentage of time when speed less than 1 cm/s) (Audira et al., 2018), and time spent in outer zone percentage (area within approximately 1.5 cm of the edge of the experimental tank) were analysed by Ethovision XT10 software (Noldus, Netherlands).

2.6. Novel tank test

The novel tank test measured zebrafish's reactivity to acclimate to the novel environment (Egan et al., 2009). In this test, each fish was placed in each test trapezoidal water tank (length × width: 24 × 19 cm at the bottom, length × width: 33 × 21 cm at the top, 18 cm high) and filled with ~1000 mL of fish water at approximately 3 cm depth at 28 °C. Each zebrafish was placed in the test tank for 1 min; next, the video recorder was started immediately and recorded for 5 min (Panasonic System Networks, Fukuoka, Japan). Average speed, freezing time movement ratio, and time spent in outer zone percentage were analysed by Ethovision XT10 software (Noldus, Netherlands).

2.7. Determination of aluminium content in brain tissues

After the completion of all the behavioural analyses, the 6-month-old and 12-month-old zebrafish were sacrificed for brain aluminium content determination. A complete brain was taken and absorbed into a 2 mL EP microtube. To each tube, 2 mL of high grade pure nitric acid was added; after being kept overnight at 20 °C, the contents were poured into the digestion microtube. After digestion by a microwave digestion instrument (CEM corporation, Matthews, USA), acid was driven out in the fume hood by an acid catcher until the remaining soybean granules were small and liquid droplets. The dilute nitric acid of 2 mL 0.4% was dissolved again and transferred to a microtube for determination. The sample was detected by inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer, USA). First, a series of standard curves were determined. Next, the sample was diluted 10 times in the tube; the aluminium content was determined by ICP-MS. Dilute nitric acid (3%) and standard solution (20, 40, 60, 80, 100 μg/L) were frequently used to adjust the accuracy during the sample testing process.

2.8. Tissue preparation and total protein determination

After the completion of all the behavioural analyses of the 6-

month-old and 12-month-old zebrafish, they were sacrificed by quickly freezing them on ice. The whole brain was extracted as soon as possible at 4 °C to ensure the freshness of the brain tissue. The whole brain was extracted for each independent assay. Three zebrafish whole brains were used to prepare a single homogenate for each sample; they were homogenised by an Ultrasonic Processor (SONICS, USA) on ice in volumes of 50 (v/w) of phosphate-buffered saline (PBS) at a pH of 7.2. Samples were centrifuged at 4000 rpm for 20 min at 4 °C, and the supernatant was kept in microtubes in the freezer at −80 °C for further analysis. Total protein analysis was performed using a BCA Protein Assay Kit (Beijing Kangwei Century Biotechnology Co., Ltd., China), and data were obtained at 590 nm by using a microplate reader (Biotek Instruments Co., Ltd., USA).

2.9. Determination of neurotransmitters in brain tissues

Acetylcholine (ACh) and dopamine (DA) levels were determined by using ELISA kits (purchased from Shanghai Jianglai Biological Technology Co., Ltd.), respectively. The whole-brain tissue lysates were subjected to measure 2 different neurotransmitters activity by using ELISA kits per the manufacturer's instructions. The tests were performed in triplicate to ensure consistency. The assay kits used in our experiment are based on the sandwich ELISA method, which involves a specific antibody for the detection of the chemicals of interest. First, the target-specific antibodies were immobilised onto 96-well microplates. Next, the tissue homogenates (10 μL)—Sample diluent (40 μL) and Horseradish peroxidase (HRP) (100 μL)—conjugated target-specific antibodies were applied onto a microplate and incubated at 37 °C for 1 h. After washing with washing buffer, chromogen A and B (50 μL) were applied onto the microplate and incubated at 37 °C for 15 min. Last, stop solution (50 μL) was applied to stop colour development, and the absorbance was analysed at 450 nm by using a microplate reader (Biotek Instruments Co., Ltd., USA).

2.10. Quantification of oxidative stress markers

Zebrafish were sacrificed on ice, and the whole brain was extracted. The brain tissue cell suspension was prepared by cutting it into a 2 mL microtubes, and 1 mL of 0.25% EDTA-pancreatin solution was added (Biological Industries, Israel). The whole brain was extracted for each independent assay. One zebrafish whole brain was used to prepare a single-cell suspension for each sample, which were operated on ice. Samples were centrifuged at 7000 rpm for 20 min at 4 °C. The excess EDTA-pancreatin was washed by PBS, and the production of intracellular ROS was measured by the oxidation sensitive probe 2, 7-dichlorofluorescein diacetate (DCFH-DA) (Applygen, Beijing, China). DCFH-DA working solution was 20 μM, and incubated at 37 °C for 60 min. Next, the cells were washed twice with cold PBS and resuspended in the PBS for analysis of intracellular ROS by a FACScan flow cytometry (guava easyCyte, Millipore Corporation, USA).

The whole-brain tissue lysates were subjected to measure the lactate dehydrogenase (LDH) activity, as described in the method in Subsection 2.9, and as per the manufacturer of the LDH assay kit (Nanjing Jiancheng Bioengineering Institute, China). According to the instructions provided by the reagent manufacturer, analyses were run on a 96-Well Cell Culture Plate (ExCell Biology, Inc., China), and data was obtained by a fully automated microplate reader at the wavelength of 450 nm. Protein quantification was performed using a BCA Protein Assay Kit (Beijing Kangwei Century Biotechnology Co., Ltd., China), and the data was analysed at 590 nm by using a microplate reader.

2.11. Total neural cells counting

The whole brain of the zebrafish that had completed behavioural analysis was taken out, and total nerve cells were counted through Nissl staining. First, 4% paraformaldehyde was used to fix the brain. Next, whole brains were collected, post-fixed, dehydrated, embedded in paraffin, and then cut into 5 μm longitudinal section telencephalon by sliding microtome (Sakura, Japan). Tissue sections with equal location, thickness, and depth were selected (Magnain et al., 2019) and then stained with Nissl staining (Solarbio, Beijing, China). After telencephalon sections had been deparaffinised and rehydrated, the slides were stained in methyl violet solution for 8 min at 20–25 °C. After clearing in distilled water, the slides were gradually dehydrated for 3 min in successive baths of ethanol at concentrations of 70%, 80%, 95%, and 100%. All slides were then given two 3-min passes in 100% dimethylbenzene and coverslips were applied with neutral balsam. Finally, under an optical microscope (Olympus, Tokyo, Japan) at 400 magnification, the numbers of nerve cells were counted by image analysis software (Image-Pro plus, Media Cybernetics, USA).

2.12. Histopathology and TEM

Zebrafish were sacrificed on ice. Brains were carefully removed, and the telencephalons were fixed in 2.5% glutaraldehyde for 4 h at room temperature followed by continuing fixation for 8 h at 4 °C. Post-fixation was performed in 1% osmium tetraoxide in sodium cacodylate buffer for 1–2 h at 20 °C, stained with 2% uranyl acetate in 50% ethanol. After dehydration through a graded series of ethanol, specimens were kept in epoxy resin for 16 h before embedding. Sections were cut longitudinally at 60 nm thickness on ultra-microtome. The ultrastructure of cells undergoing autophagy were observed and captured under a TEM (JEM-100CX, Japan) operated at an accelerating voltage at 80 kV.

2.13. Statistical analysis

For statistical analysis, the experimental values were compared between groups, and data were expressed as means ± SEM (standard error of the mean) or median ± interquartile range. Data were checked for normality and homogeneity of variance by using the Kolmogorov–Smirnov one-sample test and Levene's test, respectively. Statistical analysis was performed on parametric by data using one-way or two-way analysis of variance (ANOVA) with Dunnett's or Tukey's multiple comparisons. When nonparametric tests were required, statistical analysis was performed by using the Kruskal-Wallis test with Dunn's post hoc analysis. Graph-Pad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA) was used to determine significant differences between the exposed and control groups. Statistical significance of difference was set at $P < 0.05$.

3. Results

3.1. Characterisation of aluminium oxide nanoparticles

The Al_2O_3-NPs suspension was ultrasonically mixed for 30 min and then detected by TEM. The Al_2O_3-NPs in the suspension showed a polygonal shape without obvious aggregation. The TEM results of the Al_2O_3-NPs suspension were analysed and the mean particle size was (20.90 ± 9.51) nm (Supplementary Fig. 3). The Al_2O_3-NPs suspension was analysed by Nano Zetasizer, dynamic light scattering measurement: Mean hydrodynamic size of Al_2O_3-NPs was (86.89 ± 10.87) nm, and Zeta average potential was (49.43 ± 2.21) mv.

3.2. Aluminium content in the brains of adult zebrafish increased with age after Al_2O_3-NPs treatment in embryos

At different age time points, aluminium content in the brains of zebrafish treated with various concentrations of Al_2O_3-NPs was detected. A schematic showed the process for Al_2O_3-NPs exposure and different time points (Fig. 1A). In Fig. 1B, compared with 6-months old, at 12-months old, the brain aluminium content was significantly increased (Time factor $F_{(1,60)} = 55.61$, $P < 0.001$; Treatment factor $F_{(5,60)} = 14.89$, $P < 0.001$). At 6 months old (Fig. 1C), the brain aluminium content in Al_2O_3-NPs treated groups at the concentrations of 50 and 100 mg/L were significantly increased compared with the control group ($P < 0.05$). At 12 months old (Fig. 1D), the brain aluminium content increased significantly even at the concentrations of 12.5, 25, 50, and 100 mg/L Al_2O_3-NPs in the treated groups ($P < 0.05$).

3.3. Al_2O_3-NPs exposure reduced learning and memory performance in adult zebrafish

The T-maze test for learning and memory performance in zebrafish revealed that Al_2O_3-NPs treatment at concentrations of 25 mg/L and above had longer latency compared with the control group at 6 months old, and so did that compared with the 6.25 mg/L group (Time factor $F_{(3,360)} = 8.299$, $P < 0.001$; concentration factor $F_{(3,360)} = 8.402$, $P < 0.001$; Fig. 2A). At 12 months old, even at the lower concentrations (12.5 mg/L and above) of Al_2O_3-NPs, the treatment group had longer latency (Time factor $F_{(3,360)} = 5.397$, $P < 0.001$; concentration factor $F_{(3,360)} = 4.635$, $P < 0.001$; Fig. 2A). During the experimental memory period, at 6 months old ($F_{(5,90)} = 3.793$, $P = 0.004$; Fig. B), the cumulative time of zebrafish staying in EC zone was significantly decreased in Al_2O_3-NPs treated groups of concentrations of 25 mg/L and above, compared with the control group ($P < 0.05$). At 12 months old ($F_{(5,90)} = 3.088$, $P = 0.013$; Fig. 2B), the cumulative time significantly decreased even at lower concentrations of 12.5 mg/L and above, compared with the control group ($P < 0.05$). A representative heat map of adult zebrafish memory behaviour in the T-maze at 6 months, 9 months, and 12 months is presented in Fig. 2E. The heat map of the zebrafish indicates that the control group was more likely to appear in EC zone and that the exposed group contained some zebrafish unable to identify direction of the EC zone.

3.4. Al_2O_3-NPs exposure changed locomotor activity of adult zebrafish under dark conditions

The zebrafish were exposed to Al_2O_3-NPs for 6 days as embryos. The locomotor activity changed in adult zebrafish, and this increase with age. Fig. 3 (A, C, E) shows that compared with the adult zebrafish in the control group, for the average speed and time spent in the outer zone, the percentage of zebrafish that were 6 months, 9 months and 12 months old significantly reduced ($P < 0.05$), and the freezing time movement ratios significantly increased ($P < 0.05$). Fig. 3 (B, D, F) shows that compared with those that were 6 months old, for average speed and time spent in the outer zone, the percentage of zebrafish that were 12 months old significantly reduced ($P < 0.05$), and the freezing time movement ratios significantly increased ($P < 0.05$). Fig. 3G shows the locomotion trace of the zebrafish treated with various concentrations of Al_2O_3-NPs in embryos and recorded at 6 months, 9 months, and 12 months of adult zebrafish. The results demonstrated smooth circling swimming activity in the control group; nonetheless, the locomotion of zebrafish with increasing exposure to concentrations of Al_2O_3-NPs was gradually adhered to the wall, and the trace was likely to be toward a specific side of the container rather than swimming freely and evenly in the container.

3.5. Al_2O_3-NPs exposure reduced novel exploratory ability of adult fish under light conditions

The zebrafish were treated with Al_2O_3-NPs for 6 days as embryos, and the exploration behaviour in a novel environment was determined in adult zebrafish aged 6, 9, and 12-months (Fig. 4). The results showed that compared with the control group, the average speed (A), and time spent in outer zone (E) in zebrafish at 6, 9, and 12 months of age were significantly reduced ($P < 0.05$), whereas the freeze time ratio (C) was significantly increased ($P < 0.05$). Fig. 4 (B, D, F) indicates that compared with the 6-month-old zebrafish, for

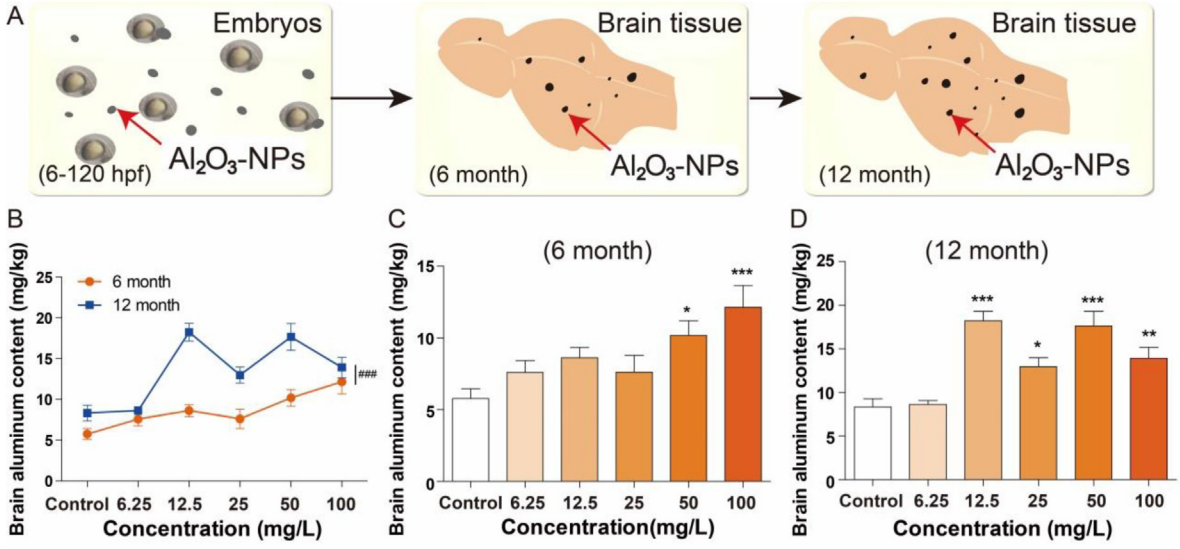

Fig. 1. Effects of aluminium content in brain tissues of adult zebrafish at 6 months and 12 months treated by various concentrations of Al_2O_3-NPs. (A) Schematic of the process for Al_2O_3-NPs exposure and detection time points. Brain aluminium content in brains of zebrafish treated with various concentrations of Al_2O_3-NPs were compared (B) at 6 months (C) and 12 months (D). Data are shown as means ± SEM and were analysed. (n = 6; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with control; ### $P < 0.001$, compared with 6 months old).

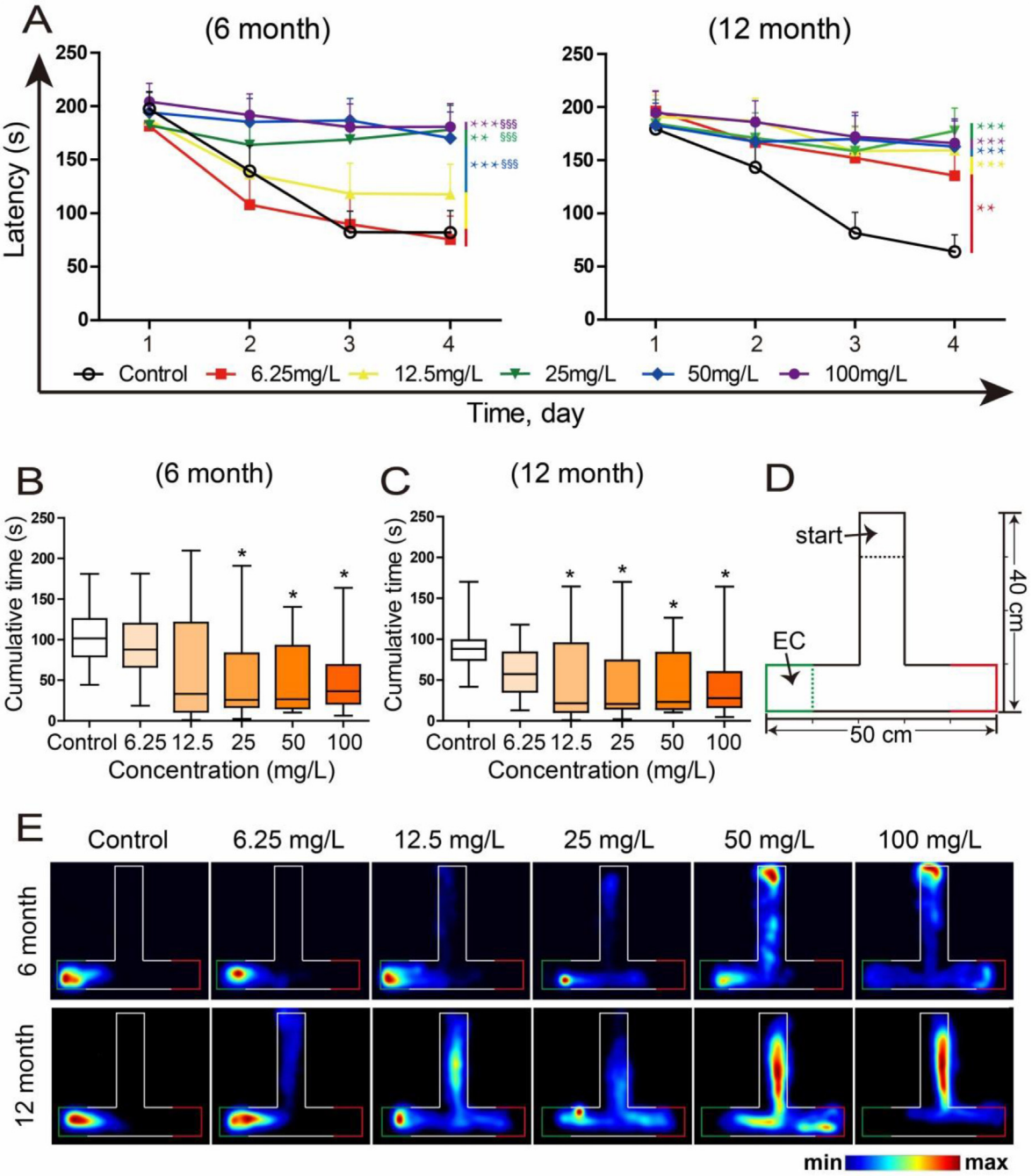

Fig. 2. Comparison of learning and memory performance at different time points after exposure of Al_2O_3-NPs to zebrafish. Latency at 6 months old and 12 months old (A) and Cumulative time in EC zone at 6 (B) months old and 12 months old were compared (C). Diagram of the T-maze apparatus (D). Representative heat map of adult zebrafish memory behaviour in the T-maze at 6 months old and 12 months old (E). Data were analysed and are expressed as means ± SEM (n = 16; compared with the control group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control; §§§ $P < 0.001$, compared with 6.25 mg/L group).

the 9-month-old and 12-month-old zebrafish, the percentage that had average speed and time spent in the outer zone percentage significantly reduced ($P < 0.05$). Fig. 4G shows that the zebrafish displayed concentration-dependent reductions and that the behaviour of zebrafish adhering to the wall gradually decreases with increased dosages, and the trace was likely to stay in some area of the container other than swimming freely in the container.

3.6. Al_2O_3-NPs exposure reduced the expression of neurotransmitters in the brain of adult zebrafish

Neurotransmitters affect a variety of physical and psychological functions, for example, heart rate, sleep, appetite, and emotional and fear behaviours. To investigate the effect of Al_2O_3-NPs exposure on neurotransmitter expression, the relative amount of neurotransmitter in the brain was measured biochemically using ELISA.

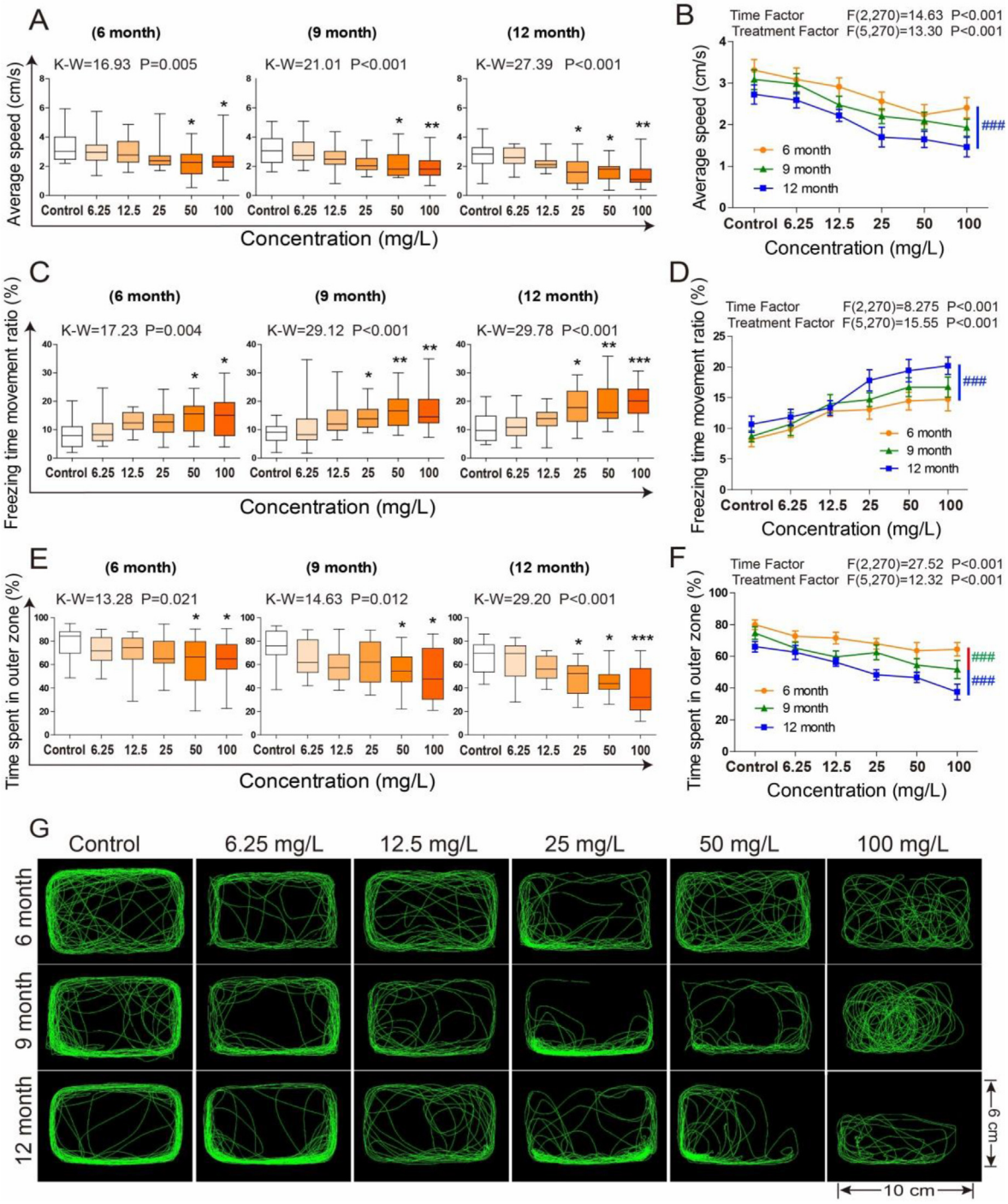

Fig. 3. Comparison of locomotor activity in various ages of zebrafish after exoposure to Al_2O_3-NPs in embryos. The average speed (A, B), freezing time movement ratio (C, D) and time spent in outer zone percentage (E, F) were analysed. Locomotion tracking patterns of 6 months, 9 months, and 12 months of adult zebrafish of 6 months, 9 months, and 12 months (G). The differences among various ages were analysed by Kruskal-Wallis (K-W) tests, followed by Dunn's multiple comparisons. Box plots in B, D, and F compare the data of anxiety as means ± SEM, and the differences among which were analysed by two-way ANOVA tests, and followed by Dunnett's multiple comparison test (n = 16; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with control; ### $P < 0.001$, compared with 6 months old).

The fixed amount of the total soluble protein in the brain was subjected to determine the expression level of acetylcholine (ACh) and dopamine (DA), and this was also determined by ELISA.

For ACh content in zebrafish brain tissue, Supplementary Fig. 4A shows the following: In the comparison of brain tissue of zebrafish between the 6-month-old and 12-month-old groups, that in the 12-month-old group significantly decreased (Time factor $F_{(1,60)} = 87.59$, $P < 0.001$; concentration factor $F_{(5,60)} = 9.438$, $P < 0.001$). At 6 months old, compared with the control group, the amount of ACh in the brain tissue of the medium and high concentration (25 mg/L and above) Al_2O_3-NPs group was significantly reduced ($P < 0.05$); at 12 months old, even in the lower (12.5 mg/L and above), were significantly reduced ($P < 0.05$). Supplementary Fig. 4B shows the comparison of the DA contents in the brain

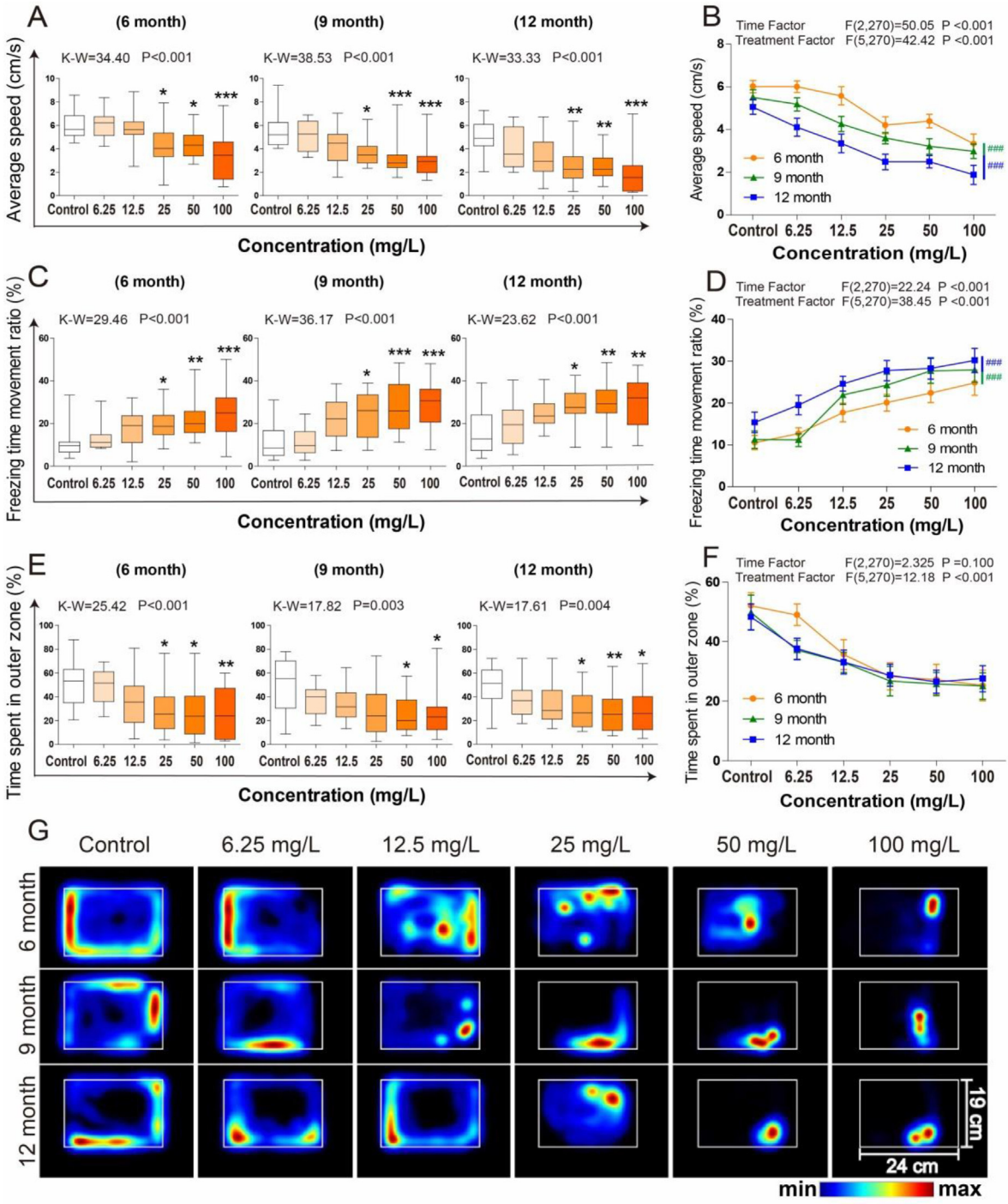

Fig. 4. Comparison of exploration behaviour at different time points after exposure of Al_2O_3-NPs in zebrafish embryos. The behaviour endpoints of average speed (A, B), freezing time movement ratio (C, D) and time spent in outer zone percentage (E, F) were analysed. Representative heat map of zebrafish exploratory activity at 6 months, 9 months, and 12 months (G). Box plots (A, C, E) represent the data of average speed, freezing time ratio, and time spent in the outer zone in 6 months, 9 months, and 12 months of adult zebrafish, the data (B, D, F) were compared by a Kruskal-Wallis test with Dunn's multiple comparisons test as follow-up tests. Data are expressed as the means ± SEM. (n = 16; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with control; ### $P < 0.001$, compared with 6 months old). K-W = Kruskal-Wallis statistic.

tissue between the 6-month-old and 12- month-old groups (Time factor $F_{(1,60)} = 15.90$, $P < 0.001$; concentration factor $F_{(5,60)} = 17.80$, $P < 0.001$); at 6-months old or 12-months old, compared with the control group, the amount of DA in the brain tissue of the concentrations of the 12.5 mg/L and above Al_2O_3-NPs groups were significantly reduced ($P < 0.05$).

3.7. Al_2O_3-NPs exposure on oxidative stress response in zebrafish brain

To investigate the induction of oxidative stress in brain tissue of zebrafish treated with various concentrations of Al_2O_3-NPs, ROS generation was monitored through increases in fluorescence intensity of dichlorofluorescein. In Fig. 5A, levels of ROS increase with

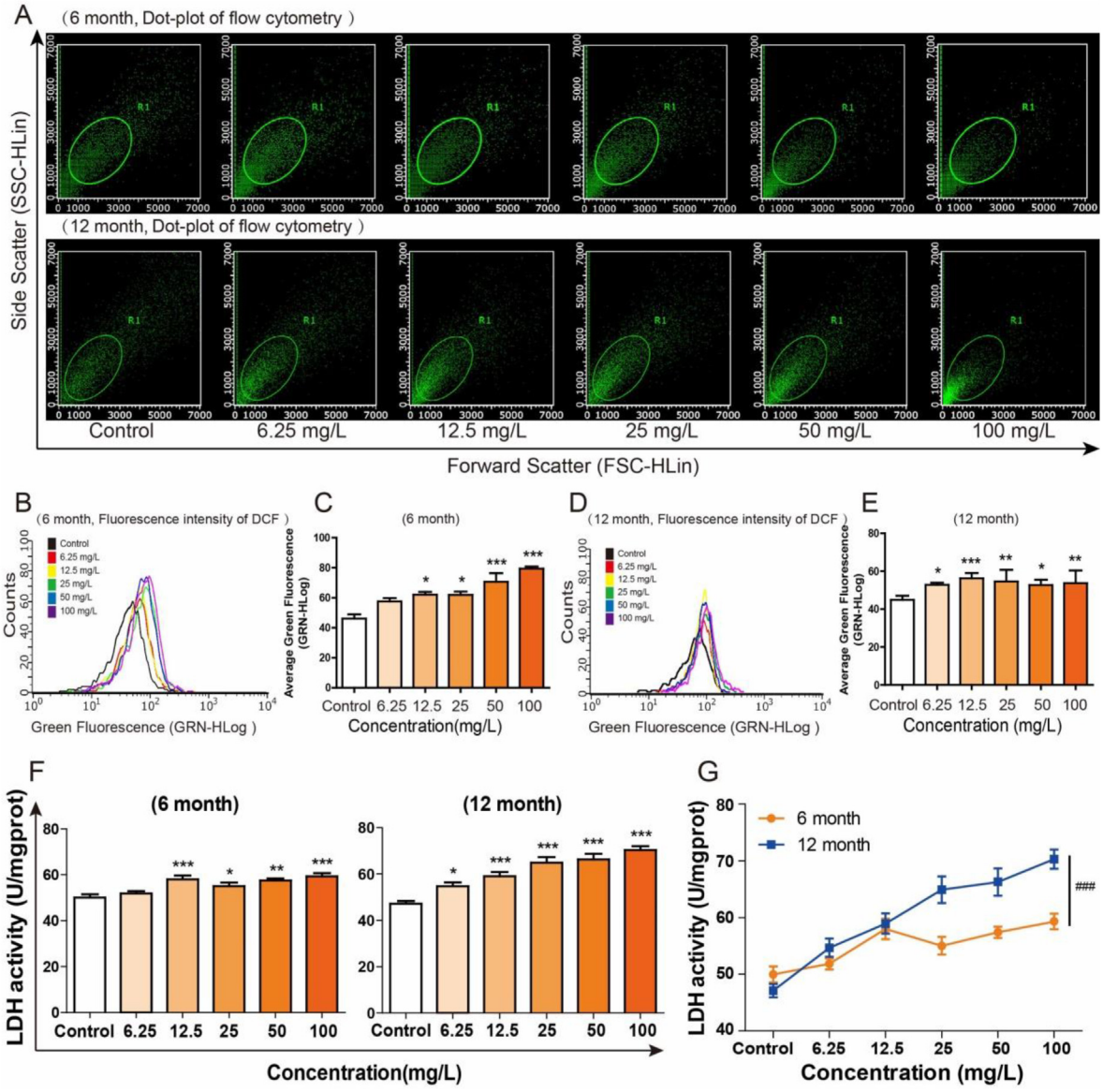

Fig. 5. Effects of oxidative stress in brain tissue of adult zebrafish exposed to Al_2O_3-NPs in embryos. The dot-plot of flow cytometry analysis (A) shows the expressions of ROS in brain tissue exposed to various concentrations of Al_2O_3-NPs in embryos and measured at 6 months and 12 months. The expression of ROS was depicted by a histogram of flow cytometry analysis at 6 months and 12 months (B, D). It shows the mean fluorescence intensity level of ROS positive cells in the brain of zebrafish at 6 months and 12 months (C, E). The expressions of LDH activity on brain tissue exposed to various concentrations of Al_2O_3-NPs at 6 months and 12 months were compared (F, G). Data are expressed as the means ± SEM. (n = 6; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with control; ### $P < 0.001$, compared with 6 months old.)

the increase of treatment dosage of Al_2O_3-NPs, and the cells are more likely to accumulate at the origin of the coordinates, suggesting that the nerve cells may have been more severely damaged with the increase of treatment dosage of Al_2O_3-NPs. At 6 months old ($F_{(5,30)} = 13.51$, $P < 0.001$; Fig. 5B, C), the ROS levels in the brain of zebrafish treated with Al_2O_3-NPs at concentrations of 12.5 mg/L and above were significantly increased ($P < 0.05$) and compared. At 12 months old ($F_{(5,30)} = 5.418$, $P = 0.001$; Fig. 5D, E), the ROS levels even in the lower concentrations of treatment dosage of Al_2O_3-NPs (6.25 and 12.5 mg/L and above) were significantly increased ($P < 0.05$).

At various time points, the LDH activity of zebrafish was measured, and different concentration groups of Al_2O_3-NPs treatment were detected. At 6 months old ($F_{(5,30)} = 7.378$, $P < 0.001$; Fig. 5F), the LDH levels in zebrafish treated with Al_2O_3-NPs at concentrations of 12.5 mg/L and above were significantly increased compared with the control group ($P < 0.05$). So did the LDH levels at 12-months old ($F(5,30) = 20.43$, $P < 0.001$; Fig. 5F). Compared with 6 months old, the levels of LDH activity in the brain tissues were significantly increased ($P < 0.01$) at 12 months old (Time factor $F_{(1,60)} = 28.63$, $P < 0.001$; concentration factor $F_{(5,60)} = 25.79$, $P < 0.001$; Fig. 5G).

3.8. Al_2O_3-NPs exposure reduced the numbers of neurons and accompanying autophagy

Nissl staining was used to count the numbers of total neurons in the telencephalon. The neurons in control group were clear and intact. However, in the Al_2O_3-NPs exposure groups, the neurons in the Al_2O_3-NPs treated groups at the concentrations of 12.5 mg/L and above were significantly decreased ($F_{(5,30)} = 19.89$, $P < 0.001$; Fig. 6A). Schematics of a brain section (Fig. 6B, C, D were presented.

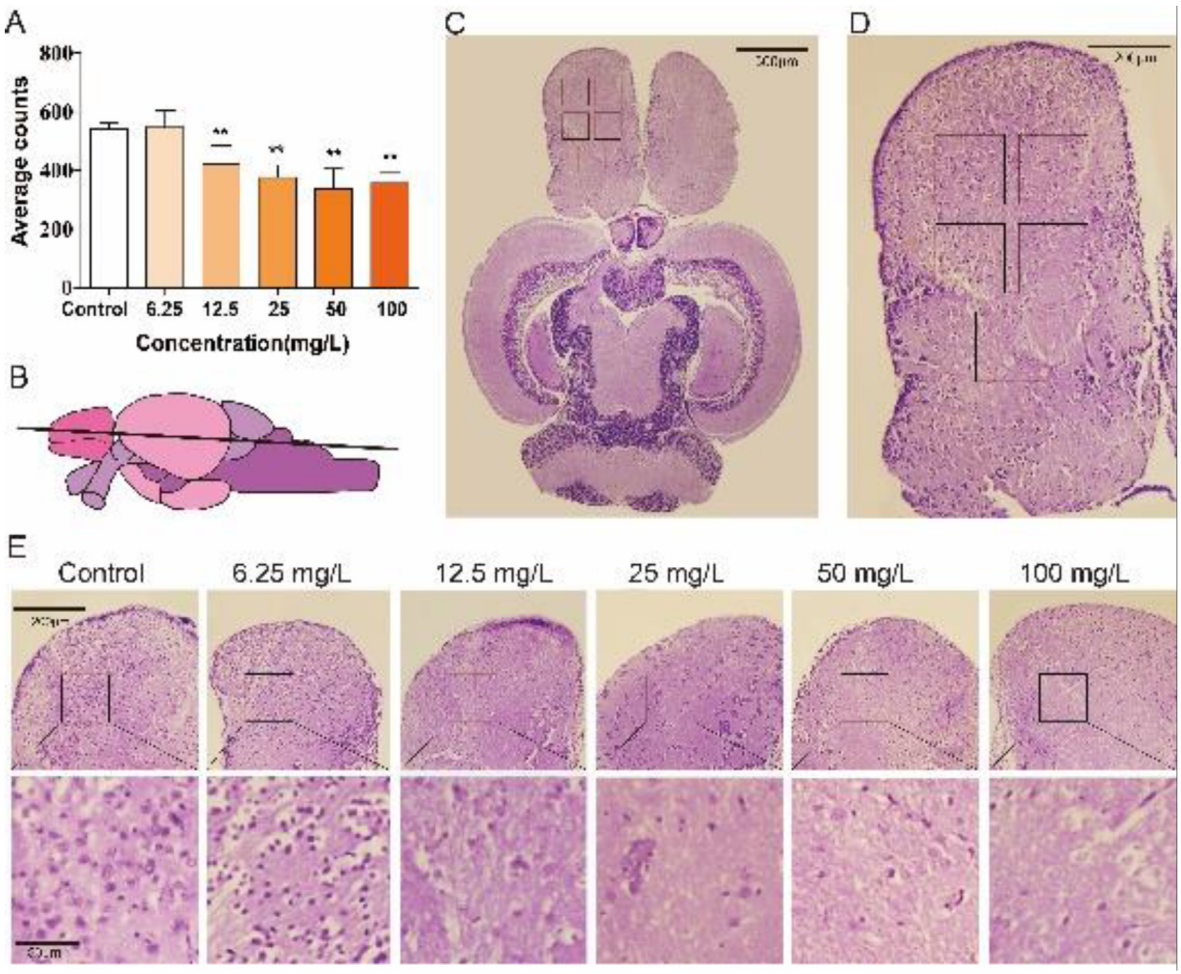

Fig. 6. Nissl staining of brain tissue induced by Al_2O_3-NPs. Data show the counts of survival neurons in six Al_2O_3-NPs treated groups (A). Schematics of a brain section are shown as the level of brain slice (B), the whole-brain overview (C), and the counting boxes in the brain (D). Representative Nissl staining in the Al_2O_3-NPs treated groups (E). Data are expressed as the means ± SEM and were analysed by one-way ANOVA tests followed by Dunnett's multiple comparison tests (n = 6; ** P < 0.01).

Representative Nissl staining imagines (Fig. 6E) showed that the number of nerve cells in the telencephalon decreased gradually with the increase of the concentrations of Al_2O_3-NPs in the exposed group.

For accurate observation of ultramicroscopic structures, the telencephalons were collected for TEM at 6 months of age. In the control group, the nuclear membrane was clear and intact, the mitochondria were intact, and the endoplasmic reticulum organelles were clearly visible (Fig. 7A; "MI" indicates a mitochondrion, "ER" indicates endoplasmic reticulum, "N" indicates a microglial nucleus). In the exposure group, Al_2O_3-NPs agglomerates appeared in the nucleus and were observed (Fig. 7B; yellow square). The microglia proliferation (Fig. 7C; "N" indicates a microglial nucleus.), myelination (Fig. 7D; purple arrowheads), and Golgi bodies swelling (Fig. 7E; yellow square) were also observed. Additionally, the mitochondria had severe damage, including swelling, breaking, vacuole, and rupture of membrane structure (Fig. 7G; "MI" indicates a mitochondrion), accompanied by autophagy in the same period (Fig. 7F; a large phagosome). A large number of autophagosomes (the blue-dotted circles indicate autophagosome, and g1 represents one of them) and autolysosomes (the red-dotted circles indicate autolysosome, and g2 represents one of them) were observed in the exposure group (Fig. 7G).

4. Discussion

Al_2O_3-NPs are widely used in medicine, cosmetics, ceramics, textiles, and electronics (Meziani et al., 2009). Some have claimed that the toxic effect of Al_2O_3-NPs is relatively low, but the Al2O3-NPs in these studies were either at a low dose or short-term exposure was used (Wang et al., 2009; Canli et al., 2017). Therefore, the toxicity of Al_2O_3-NPs may be underestimated. In this study, we demonstrated the long-term effect of Al_2O_3-NPs on learning and memory performance of adult zebrafish when treated during the embryonic period. The progressed effect of Al_2O_3-NPs on neurobehaviors of adult zebrafish at different ages was studied. In addition, the possible mechanisms of learning and memory alternations and related mechanisms of oxidative stress, neurotransmitters, neural cell count, and autophagy were studied.

Size effect plays a critical role in the study of the toxicity of Al_2O_3-NPs. Alumina of different particle sizes induces toxicity in rat brains and isolated mitochondria, and compared with micron alumina, nanosised alumina can induce more severe oxidative stress and induce depression-like behaviour in rats (Mirshafa et al., 2018). In our previous article, we also explored the size effect of Al_2O_3-NPs. Compared with the 50-nm-sized Al_2O_3-NPs, the 13-nm-sized Al_2O_3-NPs had stronger genetic toxicity (Zhang et al., 2017). Therefore, more attention should be paid to the toxicity of Al_2O_3-NPs with a smaller particle size, which is one of the reasons why we

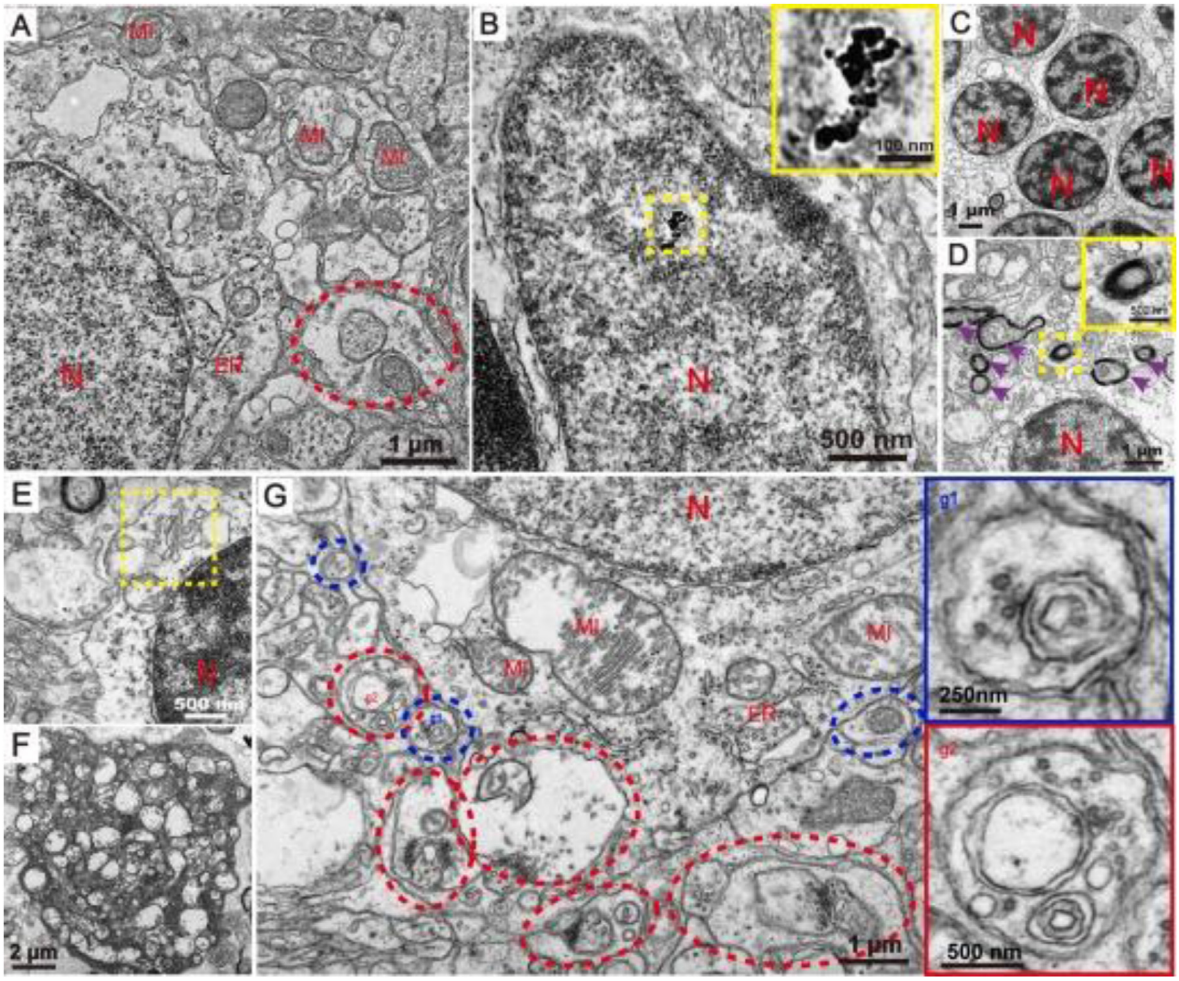

Fig. 7. Ultramicroscopic structural changes in telencephalon under a TEM. A. Normal organelles in the brain of control zebrafish; B. Al_2O_3-NPs agglomerates appeared in the nucleus; C. Microglia proliferation; D. Myelination; E. Golgi bodies swelling; F. Accumulation of many large autophagy-related structures in a large phagosome. G. Mitochondria had severe damage, accompanied by autophagy. The blue-dotted circles indicate autophagosomes, and g1 represents one of them. The red-dotted circles indicate autolysosomes, and g2 represents one of them. The purple arrow indicates a myelination. "MI" indicates a mitochondrion. "ER" indicates endoplasmic reticulum. "N" indicates a neuronal nucleus. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

focus on 13-nm-sized Al_2O_3-NPs in this study.

Al_2O_3-NPs has an accumulation effect (Park et al., 2017), and Al_2O_3-NPs are more likely to accumulate in the brain compared with other organs (Canli et al., 2019); thus, the brain is more vulnerable to the damage. NPs had the ability to cross the blood–brain barrier and reach the central nervous system (D'Agata et al., 2017) and might impair the central nervous system. In our previous article, we found that aluminium content increased in the hippocampus of mice treated with Al_2O_3-NPs, which indicated the NPs had penetrated the blood–brain barrier and accumulated in the brain. The subsequent dose-dependent impairment of learning and memory indicated the toxicity of Al_2O_3-NPs (Zhang et al., 2018). In this study, we had consistent results, that is, aluminium content in the brain tissue of adult zebrafish treated with Al_2O_3-NPs during the embryo age increased (Fig. 1). We observed a dose-dependent and time-dependent trend, which indicates that the Al_2O_3-NPs can accumulate in the brain. Based on those results, we further determined the effects of Al_2O_3-NPs on learning and memory performance, and other related mechanisms.

A series of behavioural experiments were used to evaluate the zebrafish neurotoxicity caused by Al_2O_3-NPs. A T-maze experiment was used for zebrafish learning and memory tests (Wenk, 2001). A locomotor activity test was mainly used to evaluate the anxiety response of zebrafish in the dark (Fontana et al., 2018). A novel tank test was mainly used for the exploration activity test when the zebrafish were placed in a new environment (Chakravarty et al., 2013). The analysis of zebrafish learning and memory, anxiety, exploring behaviour and swimming trajectory can better evaluate the effect of Al_2O_3-NPs on zebrafish neural behaviour.

A T-maze was used as an assessment tool and provides a brief overview of the cognitive status in a zebrafish model. This tool has been one of the most widely used behavioural paradigms to study detailed features of spatial working memory (Wenk, 2001). In the present repetitive learning task, an individual zebrafish was trained to explore and travel to the deeper end of the T-maze to find shrimp as reward food (Roy and Bhat, 2017). If the zebrafish could chose the correct arm, which is the location of the reward food, it received the food to eat. If the zebrafish could not choose the correct arm within the designed 4 min period, it was guided to the location of the enriched area and knew that there was a place that contained the reward food. During the 4 days of training, the control group was more likely to reach the EC area in a shorter period with the prolonged number of training days.

By contrast, the Al_2O_3-NPs treated groups need a longer time to reach the EC area (Fig. 2). The higher the dose, the longer the time necessary. We found that latency increased in the middle to high

concentration groups (25 mg/L and above) in 6-month-old zebrafish. In the 12-month-old zebrafish, the latency significantly increased even at lower concentrations of Al_2O_3 (6.25 mg/L) and above the treatment groups (Fig. 2A). After the training, the memory capability of zebrafish was tested. We found that the cumulative time of Al_2O_3-NPs groups in the EC area was significantly shorter. Our results showed that the learning and memory performance decreased in the middle-high concentrations of the Al_2O_3 treatment groups (25 mg/L and above) in 6-month-old zebrafish. In the 12-months old zebrafish, the cumulative time significantly decreased even at low concentrations (12.5 mg/L) and above of Al_2O_3 treatment groups (Fig. 2B). This, the results suggest that Al_2O_3-NPs can lead to a gradual decline in learning and memory in dose-dependent and time-dependent manners.

In recent years, the zebrafish has become an attractive model organism in neuroscience to study the neural bases involved in anxiety-like responses (Fontana et al., 2018). The locomotor activity test and novel tank test are suitable protocols to evaluate defensive responses in zebrafish (Aponte and Petrunich-Rutherford, 2019). In the locomotor activity test, the motivational aspect is the anxiety-like behaviour of the zebrafish in the sudden entry into the dark zone, and the main stimulus was light−dark changes (Maximino et al., 2010). Anxiety-like behaviour can be assessed by changes movements, freezing, and wall-hugging. In the novel tank test, the adaptation and exploration behaviour of the zebrafish in the new environment were used to evaluate the novelty stress, such as erratic movements and freezing (Quadros et al., 2016).

By nature, to avoid danger when zebrafish enter a dark area, they usually swim while avoiding the centre of an arena and move toward the wall or periphery of a novel environment (Levin et al., 2006). The tendency of wall-hugging is called thigmotaxis, which is a valid index of an anxiety indicator (Steenbergen et al., 2011). Zebrafish larvae show thigmotaxis as early as 5 days post-fertilisation (Stewart et al., 2015; Quadros et al., 2019). In the locomotor activity test, the less time zebrafish spent at the periphery of the arena, the more an anxiety-like behaviour was validated (Baiamonte et al., 2016). Our results showed that the average velocity in the high and medium dose of Al_2O_3-NPs treated groups decreased, the freezing time ratio increased, and they spent less time at the periphery of the arena (Fig. 3). The results suggest that Al_2O_3-NPs can induce anxiety-like behaviour in zebrafish, and a trend of progressive damage was observed.

In the novel tank test, we examined the zebrafish's ability to explore new environments, by observing changes in the behaviour of zebrafish in the new environment. Chakravarty et al. used a novel tank test to test the ability of zebrafish to explore the diverse unpredictable stress factors in the new environment (Chakravarty et al., 2013). Our results showed that the average speed and outer zone exploration decreased, and freezing time movement ratio increased. This change was dose-dependent and time-dependent, and this damage gradually worsened with time and dose (Fig. 4). These results demonstrate that the ability of zebrafish to explore in the external environment reduces exposure to Al_2O_3-NPs, and a trend of progressive damage was observed.

Neurotransmitter levels are associated with learning and memory. Acetylcholine (ACh) is of importance in regulating the motor and cognitive functions in the cholinergic system (Behra et al., 2002), and ACh is a well-known enzyme that plays a pivotal role in learning and memory (Hasselmo, 2006). Dopamine (DA) is a catecholaminergic neurotransmitter in the vertebrate central nervous system, which plays a critical role in motor movement, cognition, reward, and feeding behaviour (Schultz, 2010). DA is also associated with neurodegenerative diseases (Kim et al., 2002) and is the major neurotransmitter in the brain for coping with stress (Thornqvist et al., 2019). Therefore, in this paper, we used biochemical assays to detect levels of ACh and DA neurotransmitters as further evidence for learning and memory and neurobehavioral changes (Supplementary Fig. 4). We found a significant reduction in the levels of ACh and DA in zebrafish brain tissues at the medium and high concentrations for Al_2O_3-NPs treated groups at 6 months old and 12 months old. Moreover, the alternations were time-dependent. Compared with 6 months old, the levels of ACh and DA decreased significantly at 12 months old. This reault suggests decreased nerve conduction ability, neurotoxicity, and nervous system damage (Rai et al., 2010). The declined neurotransmitters in the Al_2O_3-NPs treated group corresponded to the impaired learning and memory performance.

Cytotoxicity was induced by NPs, and an increase in ROS and oxidative stress was usually observed (Choi et al., 2010; de Lima et al., 2012). Some studies have demonstrated that excessive levels of ROS in brain tissue cells can trigger oxidative stress and are associated with inflammation (Petersen et al., 2004). Nanomaterials commonly cause oxidative damage to cells, and induce in vivo cytotoxicity in embryonic zebrafish (Verma et al., 2017). The degree of cytomembrane injury was assessed by detecting the LDH activity in tissue (Wolterbeek and van der Meer, 2005). LDH is an indicator of cell membrane integrity, and is a parameter that estimates cell survival (Wang et al., 2012). Our results showed that exposure to Al_2O_3-NPs increased the levels of ROS and LDH activity (Fig. 5). At 6 months of age, the levels of ROS and LDH activities were elevated in the brain tissue treated with Al_2O_3 at concentrations of 12.5 mg/L and above. By 12 months of age, ROS and LDH were elevated in all Al_2O_3 exposed groups. The level of ROS and LDH activity were dose-dependent and time-dependent and increased with age. These results suggest that Al_2O_3-NPs can increase the levels of ROS in zebrafish brain tissue, which likely contributed to a stress-elicited reduction in locomotor activity. Similar results have been found (Mocelin et al., 2015; Ahmad et al., 2016). In addition, the increase of LDH released from cells tantamount to cell disruption can indicate tissue damage. These observations clearly indicate that Al_2O_3-NPs cause progressive damage to oxidative stress in zebrafish brain tissue.

Learning and memory abilities are relevant to the hippocampus, and in zebrafish telencephalon show high homology with the hippocampus (Casini et al., 1997). Our previous research found that 13 nm Al_2O_3-NPs can induce neural cell death and autophagy in primarily cultured neural cells in vitro (Data unpublished). We used Nissl staining to further determine the effects of Al_2O_3-NPs on neural cells in the telencephalon region of the zebrafish brain. The control group had a normal number of neural cells. However, the number of surviving neural cells in the Al_2O_3-NPs treated groups significantly declined with the increase of Al_2O_3-NPs treatment dosage (Fig. 6). The mechanism of which might be related to oxidative stress and teratogenicity in zebrafish embryos (Ganesan et al., 2016) or to a location of active synaptic vesicle recycling through constant endocytosis and exocytosis (Fedorovich et al., 2010). TEM analysis demonstrated autophagy alternations, accompanied by microglia proliferation, myelination, Golgi bodies swelling, and mitochondria damage in the same period (Fig. 7). These observations clearly indicate that damage of Al_2O_3-NPs to neural cells of telencephalon can be one of the causal factors in the progressive damage of zebrafish learning and memory.

In summary, our results indicate a progressive impairment of learning and memory performance induced by Al_2O_3-NPs and its mechanisms related to the alternations of neurotransmitters, increased oxidative stress, decreased number of neural cells, and ultrastructural features changes in the neural cells, including mitochondrial damage, myelination, and autophagy. Some studies have also emphasised that exposure to Al_2O_3-NPs had mitochondrial dysfunction, oxidative stress, and neurodegeneration in mice

(Li et al., 2016). Notably, AD is one of the most prevalent forms of neurodegenerative disorders. The behaviour change in patients with AD mainly includes having progressive memory impairment, declined cognitive ability, and increased anxiety, and being easily irritated (Rao, 2017).

We observed that Al_2O_3-NPs can cause symptoms similar to AD in adult zebrafish, including progressive learning and memory disorders and anxiety-like symptoms. We found that Al_2O_3-NPs increased ROS levels in zebrafish brain tissue cells. Many studies have shown that the imbalance between the production of ROS, on the one hand, and antioxidant defences, on the other, contribute considerably to the pathogenesis and progression of AD (Garcia-Mesa et al., 2016; Wojsiat et al., 2018). Together, our results show that multiple factors involved in maintaining the stress, anxiety, learning, and memory ability in fish can be affected by exposure to Al_2O_3-NPs. In addition, our research based on zebrafish has made some useful explorations of the pathogenesis of AD. Further research can be done to performed to deepen the understanding of the molecular mechanism for Al_2O_3-NPs induced toxicity.

5. Conclusions

We successfully employed a panel of behavioural endpoint tests for the first time to observe the progressive impairment of learning and memory induced by Al_2O_3-NPs exposed in embryo and observed in adult zebrafish. The test panel comprised a brain aluminium content test, anxiety activity test, exploration activity test, learning and memory test, oxidative stress test, determination of neurotransmitters test, and neural cell counting test. We then assessed the toxic effects on adult zebrafish when exposed to a range of concentrations from 6.25 mg/L to 100 mg/L for 6 days in 6 hpf embryos. We found that the low concentration (6.25 mg/L) Al_2O_3-NPs dosage is relatively safer for the zebrafish before 6 months of age. With the increase of exposure dose and duration, all dosages of Al_2O_3-NPs, including the low concentration (6.25 mg/L), can induce toxic effects. We speculate that the accumulation of nanoalumina in a zebrafish brain may aggravate the progressive damage of a zebrafish nervous system induced by Al_2O_3-NPs.

Declaration of competing interest

The authors did not report any conflict of interest.

CRediT authorship contribution statement

Jin Chen: Data curation, Formal analysis, Writing - original draft. **Rong Fan:** Formal analysis. **Yanhong Wang:** Formal analysis. **Tao Huang:** Validation. **Nan Shang:** Validation. **Kaihong He:** Validation. **Ping Zhang:** Validation. **Ling Zhang:** Validation. **Qiao Niu:** Validation. **Qinli Zhang:** Conceptualization, Writing - review & editing, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.126608.

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