·Basic Research·

Effect of titanium dioxide nanoparticles on zebrafish embryos and developing retina

Ya-Jie Wang [1], Zi-Zi He [1], Yang-Wu Fang [1], Yang Xu [2], Ya-Nan Chen [1], Guan-Qun Wang [1], Yong-Qiang Yang [1], Zhuo Yang [1], Yu-Hao Li [1]

[1]Key Laboratory of Tumor Microenviroment and Neurovascular Regulation, Nankai University School of Medicine, Tianjin 300071, China
[2]State Key Laboratory of Medicinal Chemical Biology, College of Chemistry, Nankai University, Tianjin 300071, China
Co-first authors: Ya-Jie Wang, Zi-Zi He, Yang-Wu Fang
Correspondence to: Yu-Hao Li; Zhuo Yang. Key Laboratory of Tumor Microenviroment and Neurovascular Regulation, Nankai University School of Medicine, Tianjin 300071, China. liyuhao@nankai.edu.cn; zhuoyang@nankai.edu.cn
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Abstract

• **AIM: To investigate the impact of titanium dioxide nanoparticles (TiO_2 NPs) on embryonic development and retinal neurogenesis.**

• **METHODS: The agglomeration and sedimentation of TiO_2 NPs solutions at different dilutions were observed, and the ultraviolet-visible spectra of their supernatants were measured. Zebrafish embryos were experimentally exposed to TiO_2 NPs until 72h postfertilization (hpf). The retinal neurogenesis and distribution of the microglia were analyzed by immunohistochemistry and whole mount *in situ* hybridization.**

• **RESULTS: The 1 mg/L was determined to be an appropriate exposure dose. Embryos exposed to TiO_2 NPs had a normal phenotype. The neurogenesis was initiated on time, and ganglion cells, cones and rods were well differentiated at 72 hpf. The expression of *fms* mRNA and the 4C4 antibody, which were specific to microglia in the central nervous system (CNS), closely resembled their endogenous profile.**

• **CONCLUSION: These data demonstrate that short-term exposure to TiO_2 NPs at a low dose does not lead to delayed embryonic development or retinal neurotoxicity.**

• **KEYWORDS:** titanium dioxide nanoparticles; retina; microglia; zebrafish
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INTRODUCTION

In recent years, titanium dioxide nanoparticles (TiO_2 NPs) have been widely used for a variety of purposes in a range of commercially available products, such as cosmetics, sunscreen, paint, and building materials, due to their high physical stability, anti-corrosive properties, and photocatalysis [1]. Some TiO_2 NPs enter the aquatic system, raising concern about their toxicity from aqueous exposures. Zebrafish (*danio rerio*) is a useful model organism not only for studies of vertebrate development and gene function but also for studying the bio-distribution and bio-toxicity of a variety of nano-materials because this organism has external fertilization, large numbers of spawns, transparent eggs and embryos, and rapid development [2,3]. Several studies with TiO_2 NPs have tested the toxicity on zebrafish models. Under ultraviolet light (UV) irradiation, there was greater mortality of the zebrafish larvae of the groups exposed to TiO_2 NPs[4]. In adult fish, TiO_2 NPs show limited toxicity. Histological examination of gill, liver, brain and gonad tissue showed little evidence of treatment-related morphological changes. Only reproduction was affected; exposure to TiO_2 NPs resulted in a lower cumulative number of viable embryos[5]. Moreover, the blood brain barrier (BBB) is a tight barrier that protects the brain, and most molecules cannot cross this barrier. But nanoparticles can cross the BBB or enter the brain through the nerve endings of the olfactory bulb, which makes the brain a target for these particles[6]. Zebrafish retina develops from a neuroepithelial sheet of undifferentiated cells, which then differentiates in a scheduled spatio-temporal pattern to produce the mature laminated retina[7]. Based on this, the zebrafish retina is rapidly becoming a major model for the study of neurogenesis. However, there is still a lack of knowledge about the effects of TiO_2 NPs on zebrafish embryonic development and the neurogenesis.

In this study, we observed the agglomeration and sedimentation of TiO_2 NPs solutions and measured the characteristic absorption peaks in the supernatant of each solution. It was determined that 1 mg/L was the concentration that should be used to represent aqueous exposure. Zebrafish embryos were exposed to TiO_2 NPs until 72h postfertilization (hpf). We then examined the phenotypes, determined the onset of retinal neurogenesis and

the distribution of microglia by *in situ* hybridization, and studied the cell differentiation of the major retinal neurons of the embryos using immunohistochemistry. Exposure to TiO_2 NPs at a low dose did not induce malformation and delayed embryonic development. The neurogenesis was initiated on time, and ganglion cells, cones and rods were well differentiated at 72 hpf. The migration of macrophages into the brain and distribution of microglia resembled their endogenous profile. These results indicate that the toxicity to embryos that were aqueously exposed to TiO_2 NPs is limited. Our study may help to further evaluate the short-term impact of TiO_2 NPs on embryonic development, as well as the neurotoxicity of these nanoparticles.

MATERIALS AND METHODS

Dispersion and Suspension Preparations of TiO_2 NPs Commercial TiO_2 NPs (P-25 type, 21 nm average size) were a gift from Dr. Ren GG and were provided by the Research Institute of Science and Technology (RSTI), University of Hertfordshire, England. These nanoparticles contained a photoactive, largely anatase form of TiO_2. The TiO_2 NPs varied in size from 20 to 50 nm as previously described [8]. The TiO_2 NPs were suspended in ultrapure water (Promega, Madison, WI, USA) at a concentration of 20 mg/mL as a stock solution. The stock solution was dispersed by an ultrasonic vibrator for 30min, after which the suspension was diluted in 1× Holt buffer (60 mmol/L NaCl, 0.67 mmol/L KCl, 0.3 mmol/L $NaHCO_3$, 0.9 mmol/L $CaCl_2$, pH 7.2) to a working concentration of 20, 10, 5, 1, 0.75, 0.5 and 0.2 mg/L.

Measurement of the Absorption Spectrum Five milliliters of the TiO_2 NPs-Holt buffer suspension at each concentration was placed in six-well plates and allowed to stand for 4h to achieve thorough sedimentation. Three milliliters of the supernatant from each well was used to measure the absorption spectra with a UV-2450-visible spectrophotometer (Shimadzu, Japan) using a 1 cm×1 cm quartz cuvette.

Experimental Animals Wild-type zebrafish (*Danio rerio*, AB strain) were used in this study and raised at 28.5℃ with a 10/14h dark/light cycle [9]. Embryos were collected after natural spawns, incubated with E3 embryo medium at 28.5℃, and developmentally staged by hpf. All procedures for using animals were approved by the Institutional Animal Care Committee at Nankai University.

Aqueous Exposure Embryos were collected immediately after fertilization, sorted to remove feces and infertile eggs, and placed into 6-well plates, with 50 embryos in each well. One group of embryos, the exposed group, was treated at one- to four-cell stage until 72 hpf with TiO_2 NPs-Holt buffer at a concentration of 1 mg/L. The same number of embryos formed the unexposed group; these embryos were incubated in Holt buffer as a control. The survival of the embryos was monitored by mortality at 24 hpf, and hatching was evaluated by the hatching rate at 48 hpf. The aqueous exposure described above was repeated 3 times.

Immunohistochemistry Immunohistochemistry was performed using standard procedures [10,11]. In brief, embryos were fixed overnight in 4% paraformaldehyde (PFA) at 72 hpf, cryoprotected with 20% sucrose in 0.1 mol/L phosphate buffer (pH 7.2), and frozen in Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Torrance, CA, USA). Serial cryosectioning at 10 μm were performed. After drying, sections were rinsed in 0.1 mol/L phosphate buffer containing 0.5% Triton X-100 (PBST), incubated with 20% normal sheep serum (NSS) in PBST, and incubated overnight at 4℃ in primary antibodies. Three primary antibodies were used in this study: Zn12, Zpr1 and Zpr3 [all diluted at 1:500; Zebrafish International Resource Center (ZIRC), Eugene, OR, USA]. In the next day, sections were washed extensively with PBST, incubated in a Cy3 conjugated secondary antibody (diluted at 1:500, Millipore, Billerica, MA, USA) for 1.5h at room temperature. DAPI (4',6-diamidino-2-phenylindole, Sigma, St. Louis, MO, USA) was used as a counterstain to label the nuclei. After washing in PBST, sections were sealed with mounting media and glass coverslips. Twenty animals were processed from each group.

For whole mount 4C4-antibody staining, 4% -PFA fixed embryos were incubated with antibody blocking buffer [10% NSS in PBST/bovine serum albumin (BSA) /dimethyl sulfoxide (DMSO)] for 4h to block non-specific binding sites, and incubated overnight at room temperature in 4C4 antibody (a gift from Dr. Peter Hitchcock), diluted in antibody blocking buffer at 1:200. On the following day, after washing with PBST, embryos were incubated again in antibody blocking buffer for 2h, then were incubated for 4h at room temperature in Cy3 conjugated secondary antibody (Millipore), and diluted in antibody blocking buffer at 1:200. Then embryos were washed thoroughly in PBST and cleared step by step through a glycerol series [30%, 50%, 70% in glycerol/phosphate-buffered saline (PBS), and twice in 100% glycerol]. Eleven animals were processed from each group.

Whole Mount *in situ* Hybridization Embryos were raised in to 0.003% 1-phenyl-2-thiourea (PTU, Sigma) to block pigmentation until 72 hpf. Whole mount *in situ* hybridization was performed followed a standard protocol [12,13]. Two mRNA probes were used in this study. *Atonal homolog 7* (*atoh7*) (GenBank NM_131632) probe was used to detect early differentiation of ganglion cells, and *lmx* (GenBank NM_131672) probe was used to label microglia. Digoxigenin (DIG)-labeled probes were synthesized with SP6 polymerase. Embryos were hybridized with probes concentration of 2 ng/μL overnight at 55℃. On the second day, the embryos were washed and incubated in an alkaline-phosphatase-conjugated antibody (Roche Diagnostics,

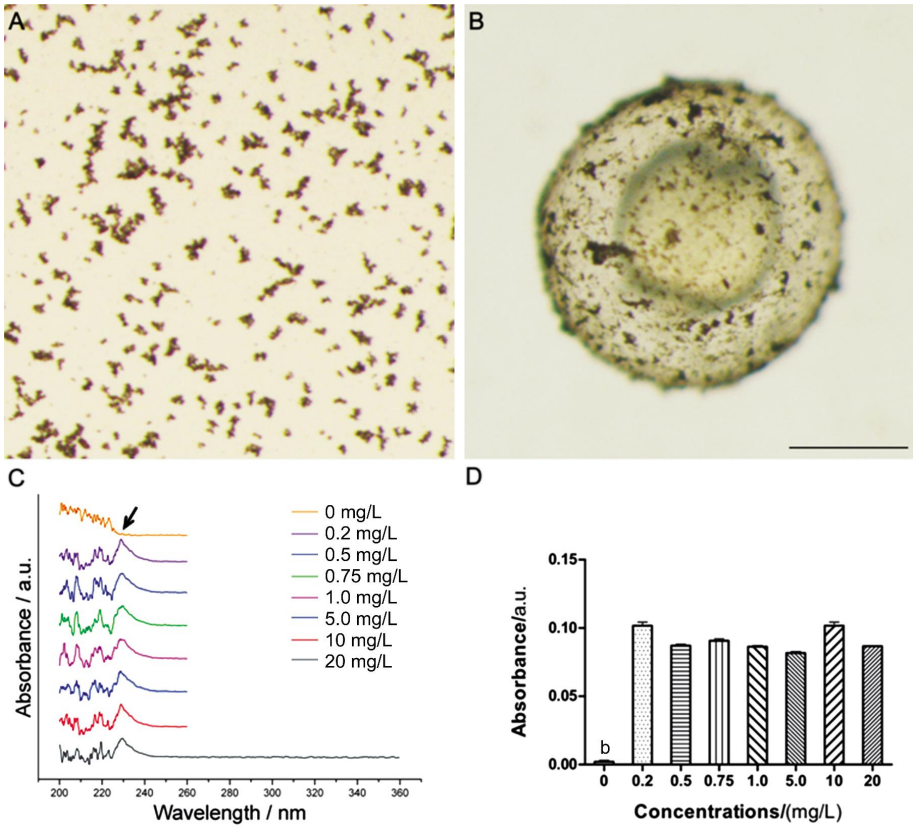

Figure 1 The sedimentation of TiO_2 NPs and measurement of absorption peaks of supernatants A, B: Agglomeration and sedimentation of TiO2 NPs on the bottom of six-well plate and 3-hpf embryos' chorion at a concentration of 20 mg/L; C: Absorption peak of supernatants from seven TiO_2 NPs-Holt buffer suspensions and Holt buffer; D: Statistical analysis of absorbance ($^bP<0.001$). Scale bar: A-B, 500 μm.

Indianapolis, IN, USA) at a dilution of 1:1500. Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Roche) was used as the enzymatic substrate on the third day.

Photography and Image Analysis Images of the immunohistochemistry were captured with an FV 1000 confocal microscope (Olympus, Japan). Images of whole mount *in situ* hybridization were photographed with an SZX10 dissecting microscope (Olympus).

Statistical Analysis Statistical analysis was performed using GraphPad software (version 5.0 c, GraphPad Software, La Jolla, USA). The mortality and hatching rate of the TiO2 NPs-exposed group or unexposed control group were represented as the average percentage of the mortality and hatching rate from 3 repeated tests, respectively. The comparison between groups was based on Chi-square test. After the fluorescent images of the 4C4 whole mount immunostaining were converted to 8-bit grayscale, the positive signals were determined on each image by Image J software (1.38X, NIH, http://rsb.info.nih.gov/ij/). Then the number of 4C4 positive cells, total area and average area on each image were calculated by Excel software. Intergroup comparisons between unexposed and TiO_2 NPs groups were based on student's *t*-test. The level for statistical significance was set at a P value of 0.05.

RESULTS

Exposed Concentration of TiO_2NPs Is Set at 1 mg/L At concentrations higher than 1 mg/L, TiO_2 NPs did not retain their 'nano-size' due to agglomeration, which led to sedimentation either on the bottom of the 6-well plates (Figure 1A) or on the chorions of embryos [14] (Figure 1B). At concentrations of 1 mg/L or lower, little sedimentation was found. Measuring the absorption spectrum revealed a characteristic absorption peak at 229 nm in the supernatants of the seven TiO_2 NPs-Holt buffer suspensions at different dilutions (Figure 1C). However, no such peak was found in Holt buffer (Figure 1C, black arrow). Statistical analysis of the absorbance showed that there was no significant difference among the seven supernatants, while the absorbance of Holt buffer was much lower than those of the supernatants (Figure 1D, $^bP<0.001$).These findings indicated that after agglomeration and sedimentation, the seven supernatants had almost the same working concentration, regardless of the dilution of the suspensions. Therefore, we chose 1 mg/L as the aqueous exposure dose in this study.

Embryos Exposed to TiO_2 NPs Has a Normal Phenotype During Embryonic Development Embryos were treated with TiO_2 NPs at the one- to four-cell stage until 72 hpf to ensure consistent TiO_2 NPs exposure under a standard 10/14h dark/light cycle. Phenotypes were assessed at 3 hpf, 24 hpf, 48 hpf and 72 hpf. In both the TiO_2

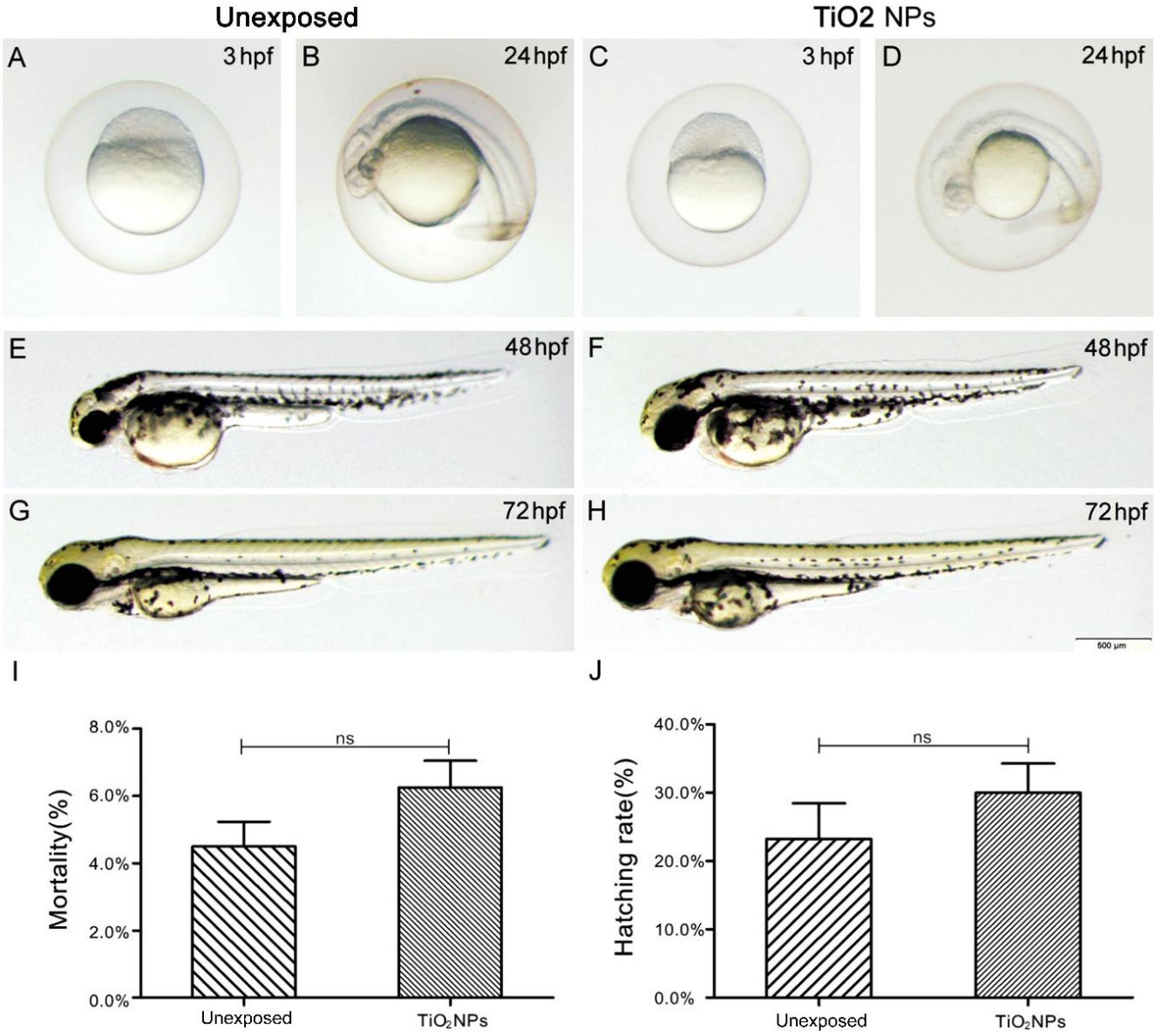

Figure 2 Phenotypes, mortality and hatching rate of embryos following aqueous exposure to TiO_2 NPs A-H: The phenotypes of embryos in the unexposed group and the TiO_2 NPs-exposed group at 3 hpf (A, C), 24 hpf (B, D), 48 hpf (E, F) and 72 hpf (G, H); I-J: The statistical analysis of the average mortality at 24 hpf and hatching rate at 48 hpf (n=50 embryos/group, 3 times repeated). Scale bar: A-H, 500 μm. ns: Not significant.

NPs-exposed group and the unexposed group, embryos were at the 1k-cell stage at 3 hpf [9] (Figure 2A, 2C). At 24 hpf, embryos were at the primula 5 stage; the tail bud increased in size, and the rudiments of the primary organs become apparent (Figure 2B, 2D). At 48 hpf, embryos were at the long pectoral fin bud stage, in which the remaining yolk was approximately the same size as the developing head (Figure 2E, 2F). At 72 hpf, embryos were at the protruding mouth stage; the head was nearly in line with the trunk and tail [15] (Figure 2G, 2H). No apparent malformation was observed at any time point. The embryo mortality at 24 hpf and hatching rate at 48 hpf in the TiO_2 NPs-exposed group were both higher than those in the unexposed group, but this difference was not statistically significant (Figure 2I, 2J). These data suggested that an exposure of TiO_2 NPs at 1 mg/L did not affect the survival or gross development of embryos.

Onset of Neurogenesis and Neuronal Differentiation Are Not Delayed Following TiO_2 NPs Exposure In zebrafish, retinal ganglion cells differentiate at 28 hpf, beginning at the ventro-nasal region of the eye [16,17]. *atoh7* is one of the basic helix-loop-helix (bHLH) transcription factors. It is used as a marker of retinal development because *atoh7* is required for the differentiation of retinal ganglion cells and expressed in cells immediately after their final mitosis [18,19]. *In situ* hybridization revealed that the expression of *atoh7* in TiO_2 NPs-exposed or unexposed retinas at 28 hpf matched that described previously [11] (Figure 3A, 3E). To evaluate the neuronal differentiation, expression of three cell-type specific antibodies were tested in this study. In ganglion cell layer, the Zn12 antibody can recognize the cell surface epitope on

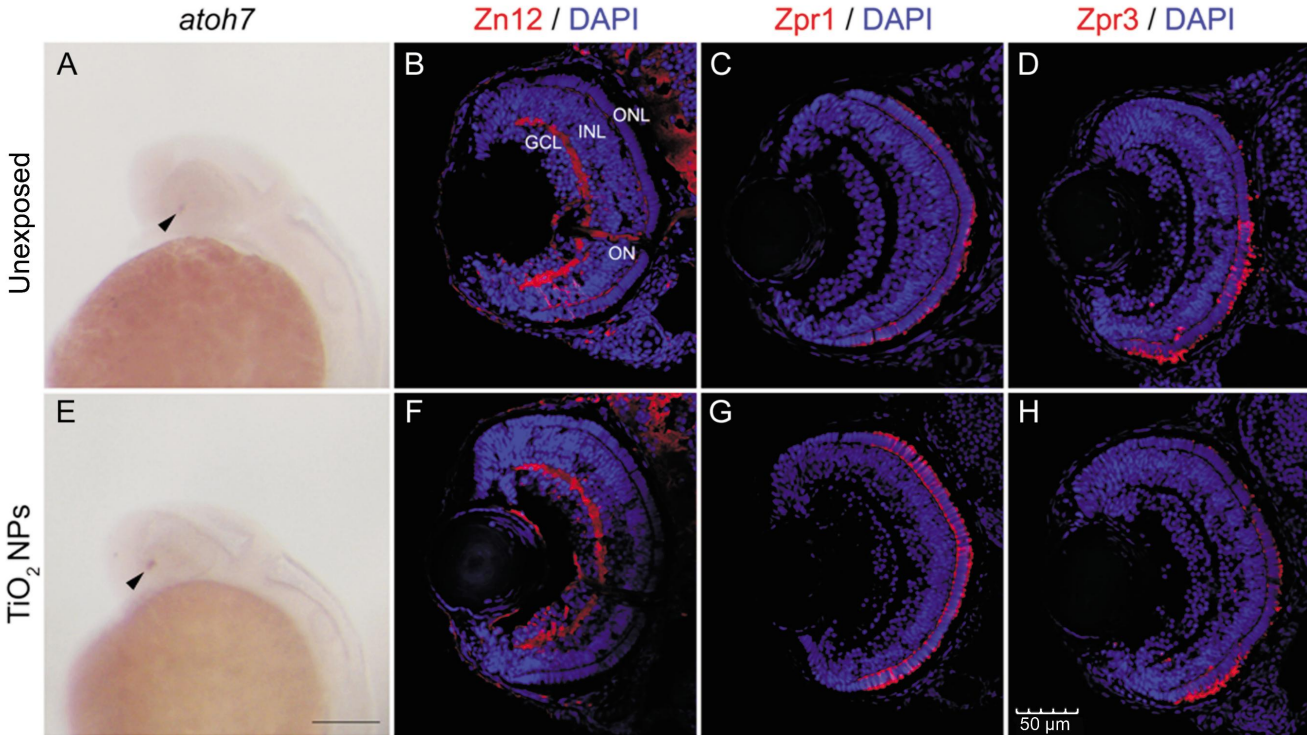

Figure 3 The onset of neurogenesis and neuronal differentiation in retinas following exposure to TiO_2 NPs A, E: The *in situ* analysis of *atoh7* expression during retina development at 28 hpf; B, F: Zn12 staining; C, G: Illustrate Zpr1 staining; D, H: Zpr3 staining. GCL: Ganglion cell layer; INL: Inner nuclear layer; ONL: Outer nuclear layer; ON: Optic nerve. Scale bar: A and E, 200 μm; B-D and G-H, 50 μm.

ganglion cells [16]. For photoreceptors in outer nuclear layer, the Zpr1 antibody was used to label a cell surface epitope on red/green-sensitive double cones, while the Zpr3 antibody labels an antigenic region on the rods [20-22]. At 72 hpf, ganglion cells, cones and rods were well-differentiated in retinas from both the exposed and unexposed groups. Meanwhile, ganglion cell layer, inner nuclear layer and outer nuclear layer were fully laminated (Figure 3B-D, F-H). These data showed that the onset of neurogenesis and neuronal differentiation were not delayed following exposure of TiO_2 NPs.

Exposure of TiO_2 NPs Does Not Alter the Distribution of Microglia To understand whether TiO_2 NPs exposure can stimulate the increased microglia migration into the brain and retina, we examined the distribution of microglia by whole mount *in situ* hybridization with an mRNA *fms* probe. *fms* is expressed in the early macrophage lineage at 22 somites, after which it remains expressed in all tissue macrophage populations, including early microglia [23-25]. In TiO_2 NPs-exposed and unexposed animals at 48 hpf, *fms*-expressing cells were detected on the surface of the yolk sac (Figure 4A, 4D, arrows), and labeled cells were also seen in the head, including the brain and retina (Figure 4A, 4D, arrowheads). *fms* expression progressively intensified, and by 72 hpf a large number of expressing cells were observed (Figure 4B, 4E). As these microglia can also be specifically labeled with the 4C4 antibody, we examined the microglia using whole mount immunohistochemistry form the exposed and unexposed groups [10]. At 72 hpf, 4C4-positive cells were scattered in irregular patterns on the brain or retinas (Figure 4C, 4F, white arrowheads). To quantify these findings, the number of 4C4-positive cells, total positive areas and average area on the brain and retinas through the maximum surface of bilateral retinas were scored. The results revealed that there was no statistically significant difference between the unexposed controls and TiO_2 NPs-exposed embryos (Figure 4G-I). These data suggested that exposure to TiO_2 NPs did not alter the migration of macrophages into the brain and retina during embryonic development.

DISCUSSION

Because the size of the pores on the chorion was approximately 0.5-0.7 μm which was larger than that of the TiO_2 NPs (20-50 nm in diameter) in this study, TiO_2 NPs entered the embryos across the chorion *via* pores [8,26]. The agglomeration and sedimentation of TiO_2 NPs block the chorion pores, attenuating the exchange of nutrients. This may be the main cause of the abnormal development or death of the embryos. In this study, we selected 1 mg/L as the TiO_2 NP exposure dose for two reasons. First, we observed the sedimentation in seven TiO_2 NPs-Holt buffer suspensions, from 0.1 to 20 mg/L. At higher concentrations, sedimentation was observed at the bottom of the six-well plate or on the chorion, observed via microscopy (Figure 1A, 1B), while little sedimentation was found in solutions at concentration of 1 mg/L or less. Second, we prepared seven

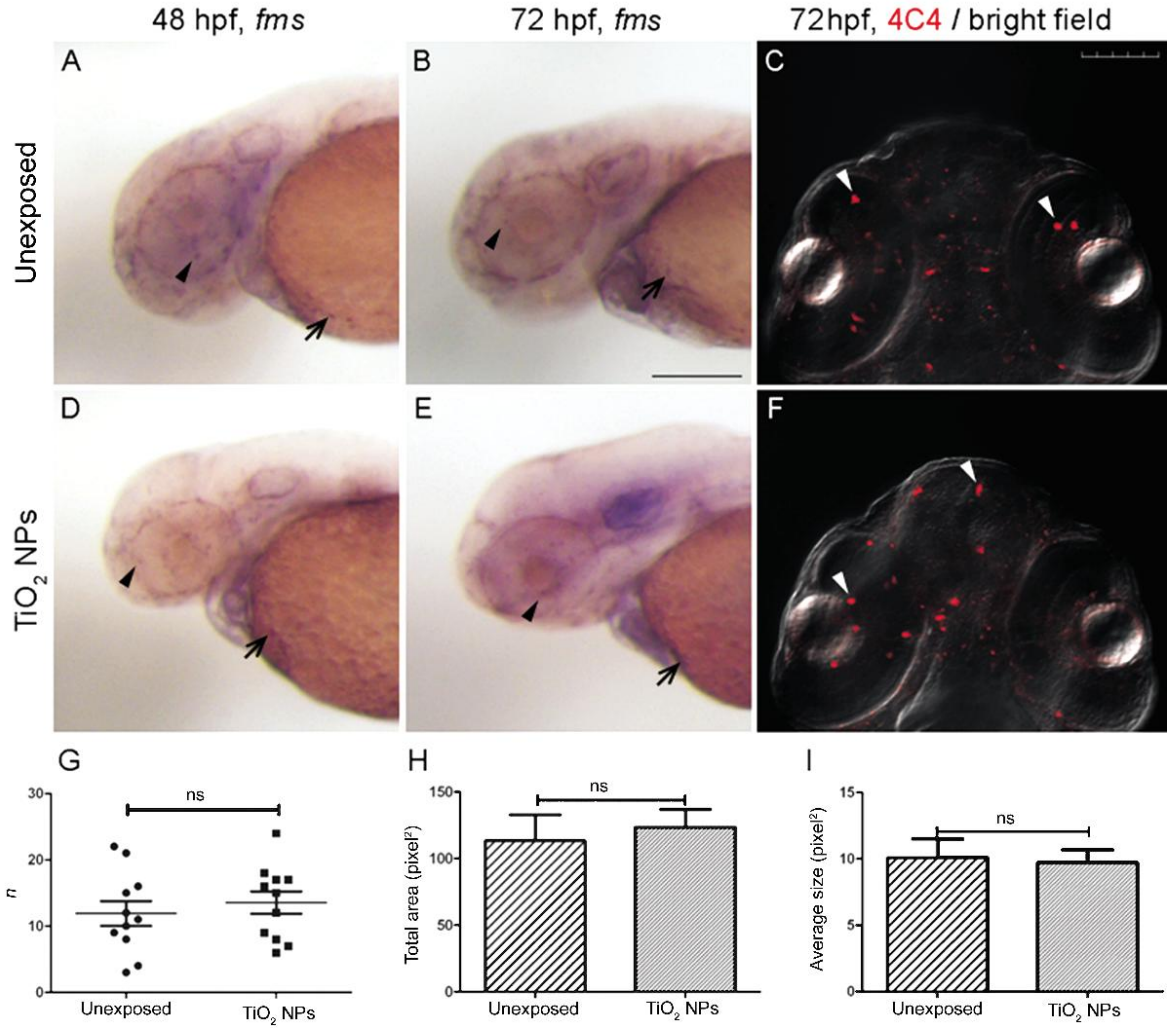

Figure 4 Expression of *fms* mRNA and 4C4 antibody in the brain and retina A, B, D and E: Whole mount *in situ* hybridization with the riboprobe *fms*; C, F: 4C4 immunostaining on embryos at 72 hpf; G-I: The quantitative comparison of the number, total area and average area occupied by 4C4-positive cells (n=11 embryos/group). Scale bar: A, B, D, E, 200 μm; C, F, 50 μm. ns: Not significant.

TiO_2 NPs-Holt buffer suspensions, from 0.1 to 20 mg/L. After allowing the suspensions to stand until thoroughly sedimented, the supernatants of all seven suspensions shared an absorption peak at 229 nm (Figure 1C). This result indicated that the concentration in the various supernatants was similar. Zebrafish embryos were consistently exposed in TiO_2 NPs-Holt buffer until 72 hpf. The phenotypes of the embryos remained similar to those of unexposed controls at 3, 24, 48 and 72 hpf time points (Figure 2A-H). Additionally, there was no significant difference in mortality or hatching rate between the TiO_2 NPs-exposed animals and unexposed controls (Figure 2I-J). Therefore, we believe that an aqueous TiO_2 NPs exposure at 1 mg/L will not result in a disturbance in gross development.

The zebrafish retina is an ideal model to investigate the development of neurons due to its scheduled spatio-temporal pattern of neurogenesis. Like vertebrates, the zabrafish retina differentiates from neuroepithelial cells. A small cluster of ganglion cells are the first progenitors which withdraw from the cell cycle at 28 hpf [17]. By 48 hpf, lamination has spread across the retina and most cells of the inner nuclear layer are post-mitotic. After 10-hour delay, the cells in the outer nuclear layer begin to differentiate, while the photoreceptors can be identified at 72 hpf[18,19]. In this study, *atoh7* was used as a marker to explore whether the differentiation of ganglion cells initiated on time. *atoh7* mRNA was detected at 28 hpf in TiO_2 NPs-exposed retinas as well as in unexposed controls (Figure 3A, 3E). At 72 hpf, three nuclear layers, including ganglion cell layer, inner nuclear layer and outer nuclear layer, were well-formed, while ganglion cells, cones and rods were differentiated in retinas from both the exposed and unexposed groups (Figure 3B-D, F-H). We demonstrated that neuroepithelial cells were present during the process of neurogenesis at an early time point and that the differentiation of three major cell types (ganglion cells, cones and rods) was not disrupted by exposure of TiO_2NPs.

Microglia are the resident macrophages and the principle responders which resides in the central nervous system (CNS). After activation by injury, microglia phagocytose cellular debris and modify the local immune response[27,28]. In zebrafish, early macrophages migrate from the yolk sac to the brain and retina at 26-30 hpf. At 55-60 hpf, the migrating

cells undergo a phenotypic transformation from macrophages to resting microglia, at which point they begin to form regular networks and to display a smaller cell body with more delicate ramifications[25,29]. In this study, the expression of *fms* mRNA and the 4C4 antibody, which were specific to microglia in the CNS, closely followed their endogenous profile (Figure 4). These results may partially explain why exposure to TiO_2 NPs does not disrupt retinal neurogenesis. Microglia are required by retinal progenitors to initiate the switch from proliferation to differentiation. The absence of microglia from the brain and retina results in a severe delay in neurogenesis [11]. However, although NPs can stimulate microglia to damage neurons *in vitro*, we believe that an exposure to TiO_2 NPs at 1 mg/L is insufficient to trigger the host defense *via* microglia[6,30].

In conclusion, overall, our results indicate that TiO_2 NPs aqueous exposure at a low dose did not induce embryonic developmental malformations, and little disturbance on neurogenesis and neuronal differentiation was found in zebrafish retina. Further testing of long-term exposure should help to develop a thorough understanding of the environmental concerns and safe use of TiO_2 NPs.

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