

Low-Dose Exposure of Silica Nanoparticles Induces Neurotoxicity via Neuroactive Ligand–Receptor Interaction Signaling Pathway in Zebrafish Embryos

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Objective: Silica nanoparticles (SiO₂ NPs) have been extensively employed in biomedical field. SiO₂ NPs are primarily designed to enter the circulatory system; however, little information is available on potential adverse effects of SiO₂ NPs on the nervous system.

Methods: The neurotoxicity of SiO₂ NPs at different concentrations (3, 6, 12 ng/nL) on zebrafish embryos was determined using immunofluorescence and microarray techniques, and subsequently confirmed by qRT-PCR.

Results: SiO₂ NPs disrupt the axonal integrity and decrease the length of axons in Tg (NBT: EGFP) transgenic lines. The number of apoptotic cells in the brain and central nervous system of zebrafish embryos was increased in the presence of 12 ng/nL of SiO₂ NPs, but the difference did not reach statistical significance. Screening for changes in the expression of genes involved in the neuroactive ligand–receptor interaction pathway was performed by microarray and confirmed by qRT-PCR. These analyses demonstrated that SiO₂ NPs markedly downregulated genes associated with neural function (*grm6a*, *drd1b*, *chrnb3b*, *adrb2a*, *grin2ab*, *npffr2.1*, *npy8br*, *gabrd*, *chrna3*, *gabrg3*, *gria3a*, *grmla*, *adra2b*, and *glra3*).

Conclusion: The obtained results documented that SiO₂ NPs can induce developmental neurotoxicity by affecting the neuroactive ligand–receptor interaction signaling pathway. This new evidence may help to clarify the mechanism of SiO₂ NPs-mediated neurotoxicity.

Keywords: silica nanoparticles, neurotoxicity, neuroactive ligand–receptor interaction signaling pathway, zebrafish

Introduction

Silica nanoparticles (SiO₂ NPs), one of the most effective nano-powders, are widely applied in various fields.^{1–3} Natural SiO₂ NPs have been identified as the main inorganic constituent of particulate matter (PM).^{4,5} Given the widespread use of SiO₂ NPs, their potentially harmful impact on human health has gained significant attention. It has been shown that SiO₂ NPs exert significant toxic effects on the respiratory,⁶ cardiovascular⁷ and reproductive systems.⁸ In zebrafish embryos, SiO₂ NPs can induce blood hypercoagulability by reducing the rate of blood flow.⁹ Importantly, the neurotoxic effects of SiO₂ NPs have also been documented. For instance, in adult zebrafish, SiO₂ NPs can disturb light/dark preference, inhibit exploratory behavior, and impair memory capability.¹⁰ In vitro, SiO₂ NPs exhibit size-dependent toxicity in N9 cells, which represent the residential macrophages of the central nervous system. Additionally, SiO₂ NPs induce the formation of mitochondrial ROS, secretion of IL-1 β , phosphorylation of GSDMD, and development

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of pyroptosis in N9 cells,¹¹ and upregulate apoptosis-promoting genes in Neuro-2a cells.¹² However, the impact of SiO₂ NPs on neurodevelopment in vivo has not yet been fully elucidated.

Given the prominent properties and genetic homology with most human genes, zebrafish has been employed as an efficient system for the assessment of SiO₂ NPs neurotoxicity.¹⁰ Additionally, zebrafish is frequently used in neurotoxicity studies due to the conserved nervous systems in comparison with mammals.^{13–15} Due to its high fecundity, cost-effectiveness, well-characterized and developmental stages, zebrafish has huge potential in high-throughput nanotoxicity screening.¹⁶ Moreover, because zebrafish embryos are transparent, the use of fluorescently tagged transgenes allows the visualization of the development of the neurological systems and the monitoring of neurological disorders in a living organism.¹⁷

The current study aimed to document the potential developmental neurotoxicity of SiO₂ NPs in vivo by taking advantage of the unique features of the zebrafish model. Specifically, the hypothesis was raised that SiO₂ NPs would lead to the underdevelopment of neurological systems in zebrafish by perturbing a specific stage in neurodevelopment. To test this possibility, experiments were performed to determine SiO₂ NPs-induced changes in 1) axonal integrity and pattern, 2) apoptosis of brain and central cells, and 3) the neuroactive ligand–receptor interaction signaling pathway. It was expected that the study would identify the mechanism of developmental neurotoxicity of SiO₂ NPs in zebrafish embryos.

Materials and Methods

SiO₂ NPs Preparation

SiO₂ NPs were produced by using the Stöber method from Jilin University, China. Briefly, 2.5 mL of tetraethylorthosilicate (TEOS, Sigma, St. Louis, MO) was transferred into a flask with 50 mL premixed ethanol, 2 mL ammonia and 1 mL water. The mixed solution was sustained at 40°C with sustained stirring (150 r/min) for 12 h. After that, the resulting suspension was centrifugated at a speed of 12,000 r/min for 15 min, aiming at isolating the particles. The detached particles were then rinsed with deionized water and dispersed in 50 mL deionized water as a stock medium. In addition, the suspensions were sonicated (160 W, 20 kHz, 5 min) (Bioruptor UDC-200; Diagenode, Liège, Belgium) prior to the experiments for avoiding possible aggregation of particles.

Zebrafish Husbandry

The zebrafish lines include Albino, Tg (fli-1: EGFP) and Tg (NBT: EGFP), which have been declared in our previous study.¹⁸ The certification number (International Association for Assessment and Accreditation of Laboratory Animal Care, 001458) was obtained from Hunter Biotechnology, Inc. The zebrafish were raised in a specific system (28°C, 80% humidity) accompanied with a round of 14 h light and 10 h dark. The collection of fertilized eggs was assisted by a stereomicroscope (Nikon, SMZ645, Japan) within 4 h post fertilization (hpf) for the following research. No less than 30 embryos were distributed for each group. Newly fertilized embryos were administered with SiO₂ NPs and those dead were excluded every day. All experiments were strictly implemented according to relevant laws and institutional guidelines.

Intravenous Microinjection

The doses of SiO₂ NPs were selected according to morphologic measurements with the no observed adverse effect level in zebrafish embryos as previous descriptions.¹⁸ The no observed adverse effect level of SiO₂ NPs is about 3 ng/nL under morphologic assessments by 15 parameters. Various doses of SiO₂ NPs (0, 3, 6 and 12 ng/nL) were injected with 10 nL at Duct of Cuvier (DC) by microinjector (PCO1500, Zgenebio, Taipei, Taiwan) with 30 zebrafish embryos for each well for 24 h. By contrast, the zebrafish embryos of control group were received ultrapure water by injection.

Neurotoxicity Measurement

After the Tg (NBT:GFP) transgenic zebrafish was treated with 3, 6 and 12 ng/nL of SiO₂ NPs for 24 h, neuron development and injury levels were measured. Images were captured under a fluorescence microscope (Nikon, AZ100, Japan).

Immunofluorescence

The immunofluorescence protocol has been stated in a previous study.¹⁹ Briefly, after being exposed to SiO₂ NPs for various doses, zebrafish was fixed with paraformaldehyde for 24 h. Zebrafish was then permeated with trypsin and fixed with paraformaldehyde, and immersed in the mouse antibody against sea urchin acetylated-tubulin (α -AT) (Sigma, USA) for 24 h. After washing for 3 times, the zebrafish was incubated with Rhodamine Red-X-AffiniPure goat anti-mouse IgG β IgM (H β L) (1:1000,

Jackson ImmunoResearch, USA) for 4 h, which is considered as a secondary antibody. Finally, zebrafish was observed under a fluorescence microscope. NIS-Elements D 3.10 software was employed for analysis of fluorescence intensity.

Cellular Apoptosis Assay

Cell apoptosis was measured in the brain and central nervous system using acridine orange (AO) staining, respectively. AO stains cells with disturbed plasma membrane permeability so it primarily stains necrotic or late apoptotic cells, however, normal cells are nonpermeable to AO. Brains of zebrafish were exposed to SiO₂ NPs (3, 6, and 12 ng/nL) for 24 h. Then, brains were rinsed three times with PBS and incubated in 5 mg/mL AO for 30 min in the dark at 28°C, followed by being rinsed at PBS. Finally, brain tissues were observed under a fluorescence microscope (Olympus BX61, Tokyo, Japan). The captured fluorescence was assessed and quantified by Volocity Demo 6.1.1 software (PerkinElmer, USA).

Microarray and Bioinformatic Analysis

The analysis of microarray and bioinformation was executed as previous descriptions.²⁰ The mRNA expression patterns of zebrafish embryos treated with SiO₂ NPs were measured by Zebrafish Gene 1.0 ST Array (AFFY-METRIX GeneChipVR, Santa Clara, CA). The data of microarray in the current study have been stored in NCBI Gene Expression Omnibus (GEO) and the series accession number GSE73427.

Statistical Analysis

All data were analyzed using SPSS 23.0 software. Data were described as means ± S.D. The least significant difference (LSD) test was implemented to evaluate the difference between two groups. Statistical significance was at the level of * $p < 0.05$.

Results

Characterization of SiO₂ NPs

The characterization of SiO₂ NPs has been reported in our previous studies.^{9,18} Briefly, the transmission electron microscope images showed that the SiO₂ NPs exhibited a near-spherical shape and were well dispersed with an average diameter of approximately 62 nm. The hydrodynamic sizes and Zeta potentials of SiO₂ NPs in ultrapure water were about 108 ± 1 nm and -38 ± 2 mV,

respectively. Additionally, no endotoxin was detected in SiO₂ NPs suspensions using limulus amebocyte lysate assay.

Neurotoxicity Induced by SiO₂ NPs in Zebrafish Embryos

The neuron damage induced by SiO₂ NPs was captured by images, which were observed under a multi-purpose zoom fluorescence microscopy (Figure 1). SiO₂ NPs had a significantly depressor effect on the length of axons Tg (NBT: GFP) transgenic zebrafish lines. Axons structure also showed significant damage after exposure to SiO₂ NPs, especially on the 6 and 12 ng/nL doses groups (Figure 2). The above results explain that SiO₂ NPs could damage the nervous system and result in neuron injury in zebrafish model.

Cellular Apoptosis Caused by SiO₂ NPs in Zebrafish Embryos

The number of apoptotic cells in the brain of zebrafish embryos was increased in the 12 ng/nL groups, but the difference did not reach statistical significance (Figure 3). Similarly, there were no significant differences between the control group and the SiO₂ NPs-exposed group regarding apoptosis of central cells in zebrafish embryos (Figure 4).

The Effect of SiO₂ NPs on Neuroactive Ligand–Receptor Interaction Pathway in Zebrafish Embryos

In line with gene chip high-throughput screening, the expression levels of genes in neuroactive ligand–receptor interaction pathway were detected in zebrafish embryos (Figure 5A). The related genes were verified by qRT-PCR analysis. In addition, as illustrated in Figure 5B, the genes regulating neuroactive ligand–receptor interaction (grm6a, drd1b, chrnb3b, adra2b, chrm5b, grin2ab, npffr2.1, npy8br, gabrd, chrna3, gabrg3, gria3a, adrb2a, grm1a, and glra3) were significantly decreased following SiO₂ NPs treatment.

Discussion

The widespread use of SiO₂ NPs in consumer products has caused controversy and concern due to its known neuro-, teratogenic, and developmental toxicity.^{21–23} Our previous study showed that nano-scale silica particles can induce more severe cytotoxicity and autophagy dysfunction than micro-scale silica particles in a size-dependent manner.²⁴

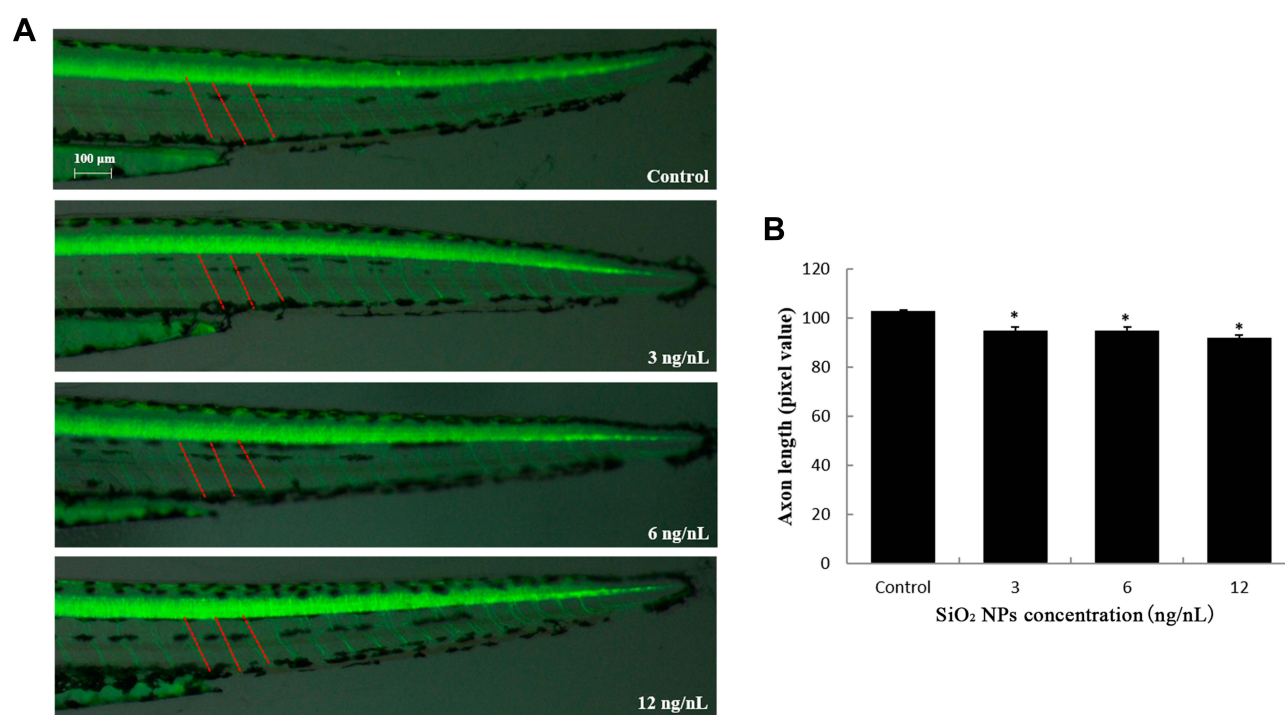


Figure 1 SiO₂ NPs induced neurotoxicity in zebrafish. **(A)** SiO₂ NPs disrupted the axonal integrity via the alteration in axon length in Tg (NBT:EGFP) lines. **(B)** Changes induced by SiO₂ NPs in the axon length of zebrafish embryos were detected by NIS-Elements D 3.10 software. Data are expressed as means S.D. from three independent experiments (* $p < 0.05$).

Kim et al observed that approximately 60 nm SiO₂ NPs affect cells differently than nanoparticles of other sizes due to enhanced uptake. Therefore, uptake of approximately 60 nm particles may be optimal to affect cellular physiology whereas larger particles exhibit more toxicity.²⁵ SiO₂ NPs are washed several times using deionised water during the preparation process, similar to the separation of water-extractable ions in PM_{2.5} particles.^{26,27} Also, SiO₂ NPs are stored at low temperature until use to maintain ion release. While they are very stable at room temperature and neutral pH, SiO₂ NPs may release some ions when exposed to strong acids, strong bases, or high temperatures. In this study, the internal environment of zebrafish was a neutral pH with low temperature. Therefore, SiO₂ NPs were very stable and no ions were released under experimental conditions, ensuring the accuracy of our findings.

Previous experimental studies have found that SiO₂ NPs can result in behavioural and morphological changes in zebrafish by modification of Parkinson-related genes.¹⁰ In addition, SiO₂ NPs can interfere with the structure of the blood-brain barrier (BBB) and induce BBB inflammation in Sprague-Dawley rats.²² The purpose of the current study was to detect the developmental toxicity of SiO₂

NPs and to illustrate its effect and underlying mechanism in a zebrafish model. We measured the neurotoxicity of SiO₂ NPs in zebrafish by transgenesis and immunofluorescence assays. We assessed the development of axons, which is a time-efficient method to screen for neurotoxicity.^{28,29} Our results demonstrated that SiO₂ NPs reduced the axon length and perturbed the axon pattern in zebrafish. There has been limited research on the effects of SiO₂ NPs on the structure of the nervous system. Our previous study discovered that PM_{2.5} particles are capable of disrupting axon integrity and reducing axon length in Tg (NBT:EGFP) transgenic zebrafish lines.³⁰ Taken together, our data demonstrated that exposure to SiO₂ NPs incurred neurotoxicity in zebrafish embryos, especially on the development of axons.

Our previous studies found alterations of some signalling pathways after exposure to SiO₂ NPs in zebrafish through microarray analyses. We used the KEGG database to analyse the 11 downregulated genes and found significant involvement of specific pathways. Neuroactive ligand–receptor interaction signalling pathway was directly related to neuro function.¹⁸ Neuroactive ligands influence neuronal function by binding to intracellular receptors, which has the capability of binding transcription factors

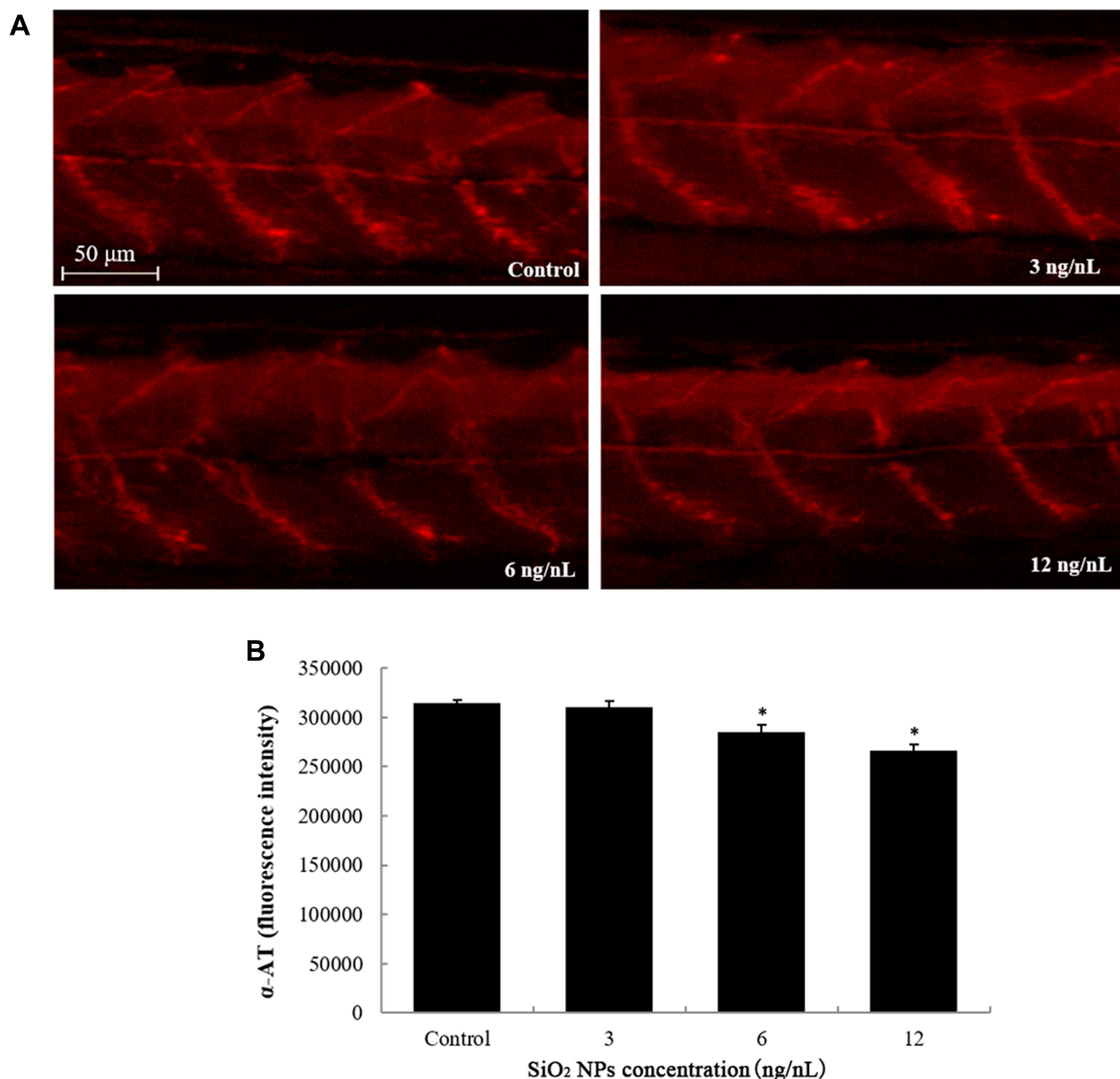


Figure 2 SiO₂ NPs induced neurotoxicity in zebrafish. **(A)** SiO₂ NPs caused disruption of axon pattern. **(B)** Changes induced by SiO₂ NPs in the axon structure of zebrafish embryos were detected by NIS-Elements D 3.10 software. Data are expressed as means S.D. from three independent experiments (* $p < 0.05$).

and regulating gene expressions.³¹ Disrupting the genes involved in neuroactive ligand–receptor interaction results in decreased memory function.³² qRT-PCR based on high-throughput genetic screening in our study showed that the expression of neuroactive ligand–receptor interaction signalling pathways were significantly decreased in zebrafish treated with SiO₂ NPs, which were in alignment with results from microarray analyses. Drd1b is a member of the dopaminergic receptor family, which belongs to the superfamily of G protein-coupled receptors. A recent study showed that dopaminergic receptors can modulate

extracellular matrix remodelling, resulting in physiological and pathological plasticity accompanied with disturbances or malformation of the neural extracellular matrix.³³ Expression levels of Gabrd, Chrna3, and Npy8br encoding for gamma-aminobutyric acid (GABA) receptor, cholinergic receptor, and neuropeptide receptor, respectively, are vital indicators for neurotoxicity.^{34–36} Our study previously demonstrated that the decreased expression of Chrna3 and Chrb3b were correlated with the expression of GABA receptor genes, indicating that SiO₂ NPs may inhibit the release of neurotransmitters in zebrafish

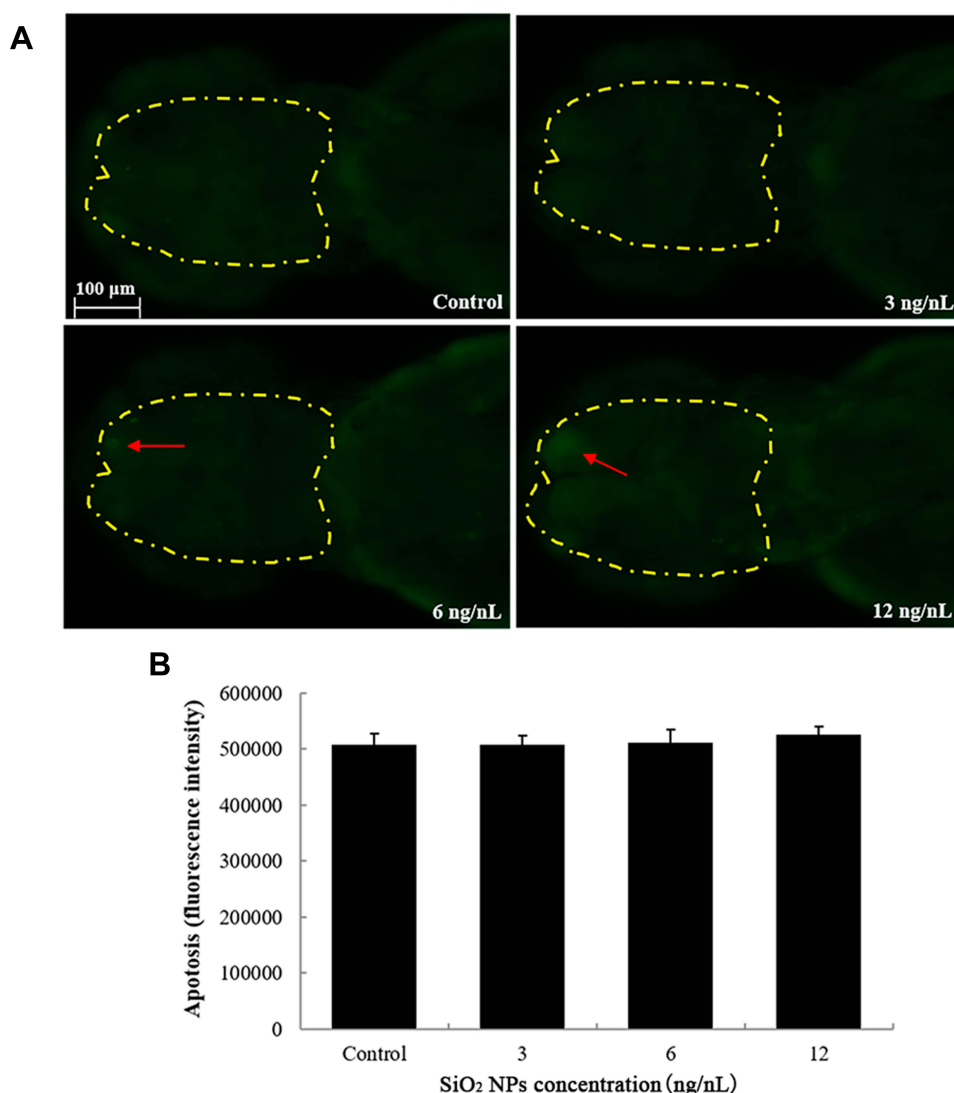


Figure 3 The apoptosis of brain cells induced by SiO₂ NPs exposure was determined using acridine orange staining in embryos of zebrafish after a 24 h exposure. **(A)** The images of apoptosis of brain cells were detected by fluorescence microscope. **(B)** The relative fluorescence of cellular apoptosis of brain was detected. Data are expressed as means S.D. from three independent experiments.

brains.³⁷ Neuropeptide FF is a member of the RF-amide peptide family, which plays an important role in controlling the feeding behaviour of vertebrates.³⁸ It binds to neuropeptide FF receptor 2 (NPFFR2), which is mainly expressed in the hypothalamus, superficial layers of the spinal cord, and thalamic nuclei and is involved in the regulation of extracellular signal-regulated protein kinase pathway as well as pain and sensory input.³⁹ The *Adra2b* gene has been shown to be associated with enhanced emotional memory⁴⁰ and Li et al found that SiO₂ NPs inhibits memory capability in adult zebrafish.¹⁰ In addition, *Gla3* seems to play a vital role in inflammatory pain sensitisation. A previous study found that SiO₂ NPs caused inflammation–coagulation response mediated by

the JAK1/TF pathway.⁹ These results suggested that SiO₂ NPs may cause neurotoxicity by initiating inflammation. In summary, these data indicate that the signaling pathways associated with neuroactive ligand–receptor interaction are indeed activated by SiO₂ NPs in zebrafish.

It is known that apoptosis plays a crucial role in SiO₂ NPs-induced neurotoxicity. It has been documented that SiO₂ NPs at low concentrations of 10 mg/mL change cellular morphologies and induce oxidative stress and apoptosis in both mouse neuro2a and human neuroblastoma cells.⁴¹ Lee et al also reported that SiO₂ NPs lead to caspase-dependent apoptosis through endoplasmic reticulum stress in neuronal cells.¹² In our study, we evaluated the apoptosis of SiO₂ NPs on zebrafish by acridine

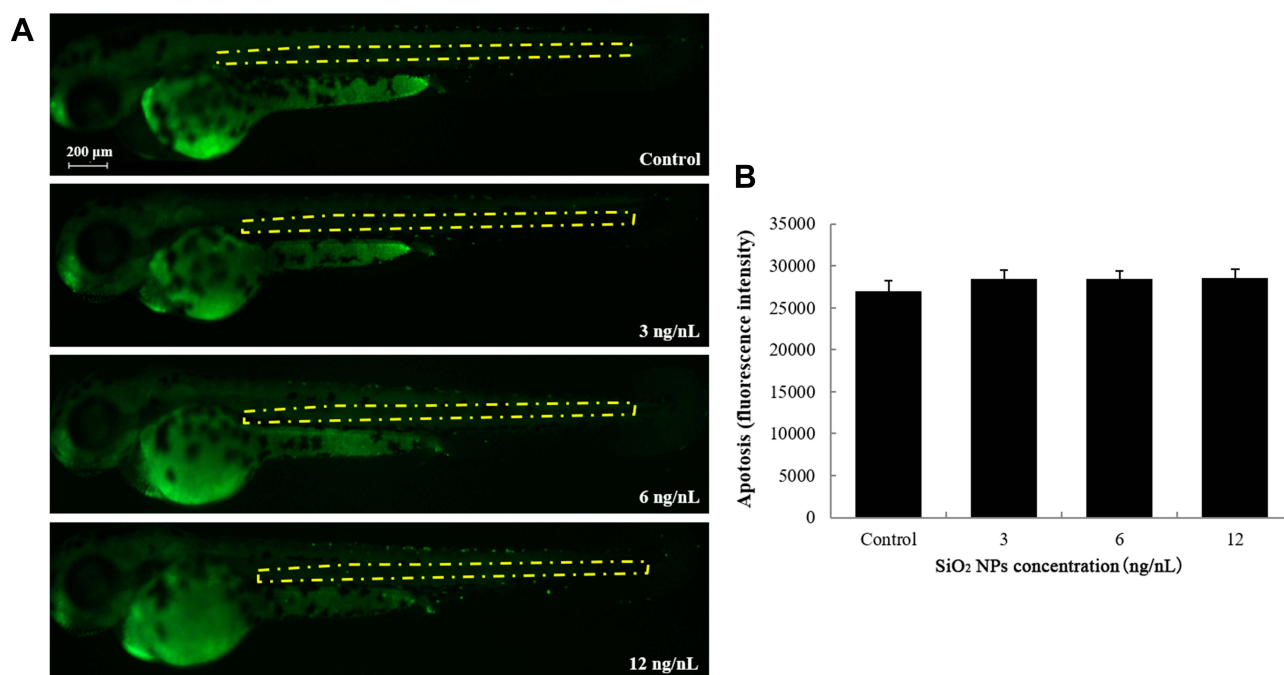


Figure 4 The apoptosis of central cells induced by SiO₂ NPs exposure was determined using acridine orange staining in embryos of zebrafish after a 24 h exposure. **(A)** Central cell apoptosis images were detected by fluorescence microscope. **(B)** The relative fluorescence of apoptosis of central cells was detected. Data are expressed as means S.D. from three independent experiments.

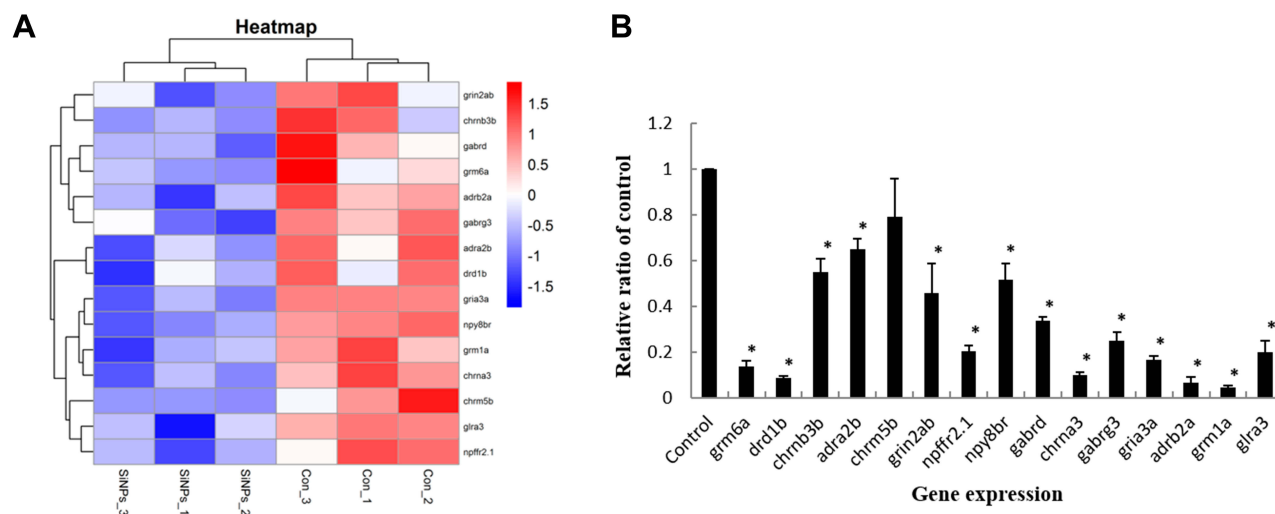


Figure 5 The effect of SiO₂ NPs on the neuroactive ligand–receptor interaction signaling pathway. **(A)** Heat map from microarray analysis of neuroactive ligand–receptor interaction-related genes. **(B)** qRT-PCR analysis showed that the genes involved in neuroactive ligand–receptor interaction. Data are expressed as means S.D. from three independent experiments (* $p < 0.05$).

orange staining. Our results showed that apoptosis rates in cells of the brain and central nervous system were increased after SiO₂ NPs treatment with highest dosage, although this difference was not statistically significant. The results suggest that in vivo low-dose SiO₂ NPs are unable to cause apoptosis of brain and central nervous system cells.

Conclusion

In summary, our results showed that exposure to SiO₂ NPs caused neurotoxicity with detrimental effects on neuron maturation processes such as axon formation in zebrafish. However, apoptosis was not significantly affected. In addition, gene expression suggested experiments suggest that the neuroactive ligand–receptor pathway played a role in

SiO₂ NPs-induced neurotoxicity. Our finding provides evidence to better elucidate the mechanism of neurotoxicity caused by exposure to SiO₂ NPs.

Disclosure

The authors report no conflicts of interest in this work.

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