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Neurotoxic impact of acute TiO_2 nanoparticle exposure on a benthic marine bivalve mollusk, *Tegillarca granosa*

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ABSTRACT

The release of nanoparticles (NPs) into the ocean inevitably poses a threat to marine organisms. However, to date, the neurotoxic effects of NPs remains poorly understood in marine bivalve species. Therefore, in order to gain a better understanding of the physiological effects of NPs, the impact of acute (96 h) TiO_2 NP exposure on the *in vivo* concentrations of three major neurotransmitters, the activity of AChE, and the expression of neurotransmitter-related genes was investigated in the blood clam, *Tegillarca granosa*. The obtained results showed that the *in vivo* concentrations of the three tested neurotransmitters (DA, GABA, and ACh) were significantly increased when exposed to relatively high doses of TiO_2 NPs (1 mg/L for DA and 10 mg/L for ACh and GABA). Additionally, clams exposed to seawater contaminated with TiO_2 NP had significantly lower AChE activity. In addition, the expression of genes encoding modulatory enzymes (AChE, GABAT, and MAO) and receptors (mAChR3, GABAD, and DRD3) for the neurotransmitters tested were all significantly down-regulated after TiO_2 NP exposure. Therefore, this study has demonstrated the evident neurotoxic impact of TiO_2 NPs in *T. granosa*, which may have significant consequences for a number of the organism's physiological processes.

1. Introduction

With recent advances in materials science, nanoparticles (NPs) are increasingly being used in a variety of products. It is predicted that the production of NPs will continue increasing and will exceed 500,000 tons per year by the end of 2020 (www.nanoproject.org), which will inevitably lead to an increase in the release of NPs into the environment. Like many other contaminants, it has been suggested that NP pollutants could reach the ocean *via* direct surface runoff, chemical deposition, atmospheric circulation, and dumping of effluents (Barmo et al., 2013; Baker et al., 2013).

Titanium dioxide nanoparticles (TiO_2 NPs) are among the most widely used nanoparticles and are found in paints, sunscreens, inks, foods, pharmaceuticals, surface coatings, and water treatments (Chen and Selloni, 2014). Therefore, compared with other NPs (Cu, Ag, Fe, ZnO NPs, *etc.*), TiO_2 NPs have the highest model-predicted concentrations in a variety of environmental media, such as seawater, intertidal zones, and oceanic sediments (Liu and Cohen, 2014). Upon entering seawater, TiO2 NPs are affected by its high ionic strength, high carbonate content, and other characteristics, to form aggregates that eventually deposit on the sea floor (Keller et al., 2010). It is predicted that the environmental concentration of TiO_2 NPs could eventually reach the mg/L levels and become even more concentrated in sediments (Gottschalk et al., 2013; Sun et al., 2016), which could pose a great threat to marine organisms, especially benthic sessile invertebrates such as the bivalve mollusks.

Therefore, increasing concerns have been raised over the physiological and ecological impacts of NPs on marine organisms. In recent years, NP exposure in various marine species has been revealed to cause a series of detrimental physiological impacts, including reducing fertilization success (Nielsen et al., 2009; Gallo et al., 2016; Kadar et al., 2013), constraining metabolism and growth (Hanna et al., 2013; Jarvis et al., 2013), retarding embryonic development (Ringwood et al., 2009, 2010; Libralato et al., 2013), and hampering immune responses (Moore et al., 2009; Rocha et al., 2014). For instance, it has been shown that the immune responses of many marine bivalve species, such as *Crassostrea virginica* (Abbott Chalew et al., 2012), *Tegillarca granosa* (Shi et al., 2017a), *Mytilus galloprovincialis* (Canesi et al., 2010), *M. coruscus* (Huang et al., 2016), *Ruditapes philippinarum* (Marisa et al., 2015), *Perna viridis* (Wang et al., 2014), and *Chlamys farreri* (Xia et al., 2017), can be hampered by exposure to NPs, which may render the organisms more susceptible to pathogenic infections.

It has also been suggested that NPs may be neurotoxic in teleosts and mammals (Sheng et al., 2016; Ze et al., 2016); however, it remains poorly understood in bivalve mollusks. For instance, in the zebrafish, *Danio rerio*, the concentration levels of norepinephrine (NE), dopamine

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(DA), and 5-hydroxytryptamine (5-HT) were found to be significantly reduced after 45 days exposure to low doses (5, 10, 20, or 44 μg/L) of TiO_2 NPs (Sheng et al., 2016). Similarly, in CD-1 (ICR) female mice, 9 months of exposure to 1.25, 2.5, or 5 mg/kg of body weight TiO_2 NPs led to a metabolic disorder of glutamate, the most abundant excitatory neurotransmitter in vertebrates (Ze et al., 2016). However, to the best of our knowledge, only one study has reported that NPs are neurotoxic in marine bivalve species (Xia et al., 2017). Through the screening of various biomarkers, it was unintentionally revealed that the acetylcholinesterase (AChE) activity in *C. farreri* was significantly increased after 14 days of exposure to TiO_2 NPs (Xia et al., 2017). Since AChE (E.C.3.1.1.7) is a specialized enzyme that not only hydrolyzes the neurotransmitter ACh but also participates in the maturation and regeneration of neurons (Matozzo et al., 2005; Xia et al., 2017; Fulton and Key, 2001), the activity of AChE is generally regarded as an indicator of neurotoxicity (Jebali et al., 2013; Lavado et al., 2006). Therefore, the detected alteration in AChE activity indicates that TiO2 NPs are significantly neurotoxic to the scallop *C. farreri* (Xia et al., 2017).

Neural signal transduction by neurotransmitters is one of the key biological processes of an organism. When nerve impulses reach the synapse, neurotransmitters are released and bind with the corresponding receptors on the membrane of the target cell, such as muscles and glands, which subsequently regulates physiological functions (Hurst et al., 2013; Ishii and Kurachi, 2006). It has been confirmed that both excitatory and inhibitory neurotransmitters, such as dopamine (DA), acetylcholine (ACh), and gamma-aminobutyric acid (GABA), play crucial roles in neural signal transduction in mollusks (Gainey and Greenberg, 2003; Karhunen et al., 1993). However, it remains unknown whether the *in vivo* concentrations of neurotransmitters are affected by exposure to NPs.

The blood clam, *Tegillarca granosa*, is an important commercial marine bivalve species that is widely distributed throughout the coastal regions of the Indo-Pacific (Shao et al., 2009; Zha et al., 2017). Inhabiting the intertidal mudflat, where the concentration of NPs is predicted to be higher than other parts of the ocean (Sun et al., 2016; Peng et al., 2015), the blood clam may be under serious threat of exposure to NPs. Therefore, in order to gain a better understanding of the neurotoxicity of NPs in marine bivalve mollusks, the impact of acute (96 h) TiO_2 NP exposure on the *in vivo* concentrations of three major neurotransmitters (DA, GABA, and ACh), the activity of AChE, and the expression of neurotransmitter-related genes in the blood clam was investigated in the present study.

2. Materials and methods

2.1. Animal collection and acclimation

Mature *T. granosa* with shell heights of 28.35 ± 2.45 mm were collected from Qingjiang, Wenzhou, Zhejiang province of China in June 2017. After being transported to the laboratory, the clams were kept in a 1000-L plastic tank and acclimated for 14 days in sand-filtered, flowing seawater (pH at 8.09 ± 0.02, temperature at 31.6 ± 0.54 °C, and salinity at 18.02 ± 0.24‰) with constant aeration before the beginning of the experiment. During the acclimation period, clams were fed with pre-prepared fresh microalgae *Tetraselmis chuii* (20000 cells per mL) at a rate of ~5% dry tissue weight twice a day (Shi et al., 2017b; Zhao et al., 2017).

2.2. Characterization of TiO_2 nanoparticles

The TiO_2 NPs used in this study were bought from the Shanghai Klamar Reagent Co. Ltd, China. According to our previous analysis (Shi et al., 2017a), the TiO_2 NPs used in the present study have a crystal structure of anatase with an average diameter of 35 ± 5 nm and BET surface area of 60.65 m^2/g. A stock solution (1 g/L) was made every day before use by dissolving the TiO_2 NPs in ultrapure water and sonicating for 15 min. The stock solution was diluted to the nominal exposure concentrations with 0.1 mm membrane-filtered seawater. The working concentrations were determined in triplicate by inductively coupled plasma mass spectrometry (ICP-MS, PE NexION 300X, USA) according to the methods described by Orians and Boyle (1993) and were presented in Table 1.

Table 1
Working concentrations measured for different TiO_2 NPs exposure treatments.

Nominal concentrations (mg/L)	Working concentrations (mg/L)
0	0.0028 ± 0.0001
0.1	0.098 ± 0.001
1	1.003 ± 0.002
10	9.996 ± 0.001

2.3. Exposure experiment and sampling

The exposure concentrations of 0.1, 1, and 10 mg/L were chosen for the TiO_2 NPs exposure experiments based on the predictions of previous studies (Gottschalk et al., 2013; Davis et al., 2010). Therefore, one control group was not exposed to any TiO_2 NPs, and three experimental treatment groups were exposed to 0.1, 1, or 10 mg/L of TiO_2 NPs, with three replicates per group. After two weeks of acclimation, 360 clams were randomly assigned to one of 12 individual plastic tanks (4 treatments × 3 replicates) with 30 L seawater containing the corresponding concentrations of TiO_2 NPs. During the experiment, the seawater was changed daily, and the exposure concentrations of TiO_2 NPs were maintained by re-adding TiO_2 NP stock solution. An exposure time of 96 h was used in the present study based on the previous work of Zhu et al. (2011) and no individual mortality was observed during the experiment.

2.4. Quantification of the concentrations of neurotransmitters

After exposure to TiO_2 NPs for 96 h, 10 clam individuals were randomly selected from each experimental group to quantify the *in vivo* concentrations of each neurotransmitter. In brief, after dissection on ice, the gill of each clam was removed and immediately frozen in liquid nitrogen. The frozen gill tissues were then homogenized in ice-cold PBS (0.01 M, pH 7.4, w/v (mg/ml) = 1/1) on ice by an electric homogenizer (ART, MICCRA D-1, Germany). Homogenates were subsequently centrifuged at 2000 rpm for 20 min at 4 °C. The precipitate was discarded and the supernatant was used to determine the concentrations of neurotransmitters. In the present study, three neurotransmitters including DA, ACh, and GABA, were measured using commercial ELISA kits (ML090244, ML095412, and ML086216, MLBIO biotechnology Co. Ltd., Shanghai, China) with a microplate reader (Thermo Multiskan Go, USA) at the absorption wavelength of 450 nm following the corresponding instructions provided by the manufacture.

2.5. Determination of the activity of AChE

A total number of 9 clams from each experimental group were used to determine the activity of AChE. Following the manufacturer's instructions, the activity of AChE was measured using commercial kits (A024, Nanjing Jiancheng Bioengineering Institute, China) with a microplate reader (Thermo Multiskan Go, USA) at the absorption wavelength of 412 nm. The total protein contents of the supernatant was determined with a commercial kit (A045, Nanjing Jiancheng Bioengineering Institute) using the bicinchoninic acid (BCA) method (Smith et al., 1985). The activity of AChE was subsequently calculated with one activity unit representing the hydrolysis of 1/6 μmol substrate per mg protein per minute at a temperature of 37 °C.