Effects of nano-scale TiO₂, ZnO and their bulk counterparts on zebrafish: Acute toxicity, oxidative stress and oxidative damage

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Abstract

The acute toxicity and oxidative effects of nano-scale titanium dioxide, zinc oxide and their bulk counterparts in zebrafish were studied. It was found that although the size distribution of nanoparticles (NPs) was similar to that of the bulk particles in suspension, the acute toxicity of the TiO₂ NPs (96-h LC₅₀ of 124.5 mg/L) to zebrafish was greater than that of the bulk TiO₂, which was essentially non-toxic. The acute toxicities observed for ZnO NPs, a bulk ZnO suspension, and a Zn²⁺ solution were quite similar to each other (96-h LC₅₀ of 4.92, 3.31 and 8.06 mg/L, respectively). In order to explore the underlying toxicity mechanisms of NPs, ·OH radicals generated by NPs in suspensions and five biomarkers of oxidative effects, i.e. superoxide dismutase, catalase activities, malondialdehyde, reduced glutathione and protein carbonyl were investigated. Results showed that after the illumination for 96 h, the quantities of ·OH in the NP suspensions were much higher than ones in the bulk particles suspensions. The malondialdehyde content of zebrafish exposed to 50 mg/L TiO₂ NPs, was 178.1% and 139.7% of controls, respectively. The malondialdehyde levels in zebrafish exposed to 5 mg/L TiO₂ NPs and bulk TiO₂ were elevated (217.2% and 174.3% of controls, respectively). This discrepancy indicates the occurrence of lipid peroxidation which is partly due to the generation of ·OH. In contrast, exposure to 5 mg/L ZnO NPs and bulk ZnO suspension induced oxidative stress in the gills without oxidative damage. Oxidative effects were more severe in the livers, where the protein carbonyl content, in the light and dark groups exposed to 50 mg/L TiO₂ NPs, was 178.1% and 139.7% of controls, respectively. The malondialdehyde levels in the liver of fish exposed to 5 mg/L ZnO NPs and bulk ZnO were elevated (204.2% and 286.9% of controls, respectively). Additionally, gut tissues exhibited oxidative effects after exposure to NP suspensions. These results highlight the importance of a systematic assessment of metal oxide NP toxicity mechanisms.

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TiO₂
ZnO
ROS
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1. Introduction

Nano-scale (diameters between 100 and 1 nm) titanium dioxide and zinc oxide, which have broad UV spectrum attenuation properties, are used in a variety of applications, including sunscreen (Maier and Korting, 2005), wastewater treatment (Chen et al., 2004), and environmental remediation (Aitken et al., 2006). Direct and indirect release of these nanoparticles (NPs) into aquatic environments via bathing, sewage effluent (Daughton and Ternes, 1999; Handy and Shaw, 2007; Ternes et al., 2004) and other engineering applications (Chen et al., 2004; Nagaveni et al., 2004) have increased the exposure chances of humans and ecosystems to NPs (Nowack and Bucheli, 2007). Consequently, the potential impacts of TiO₂ and ZnO NPs on aquatic ecosystems have attracted special attention due to the unique properties of NPs (Handy and Shaw, 2007; Handy et al., 2008; Oberdörster et al., 2005; Wiencz et al., 2009).

Generally, the toxicity of NPs is associated with their small size and high specific surface area (Lovern and Klaper, 2006). Nanomaterials are theoretically expected to be more toxic than their bulk counterparts due to their greater surface reactivity and the ability to penetrate into and accumulate within cells and organisms (Carlson et al., 2008; Ipas et al., 2009; Mironava et al., 2010). However, investigations of aggregations of NPs with size distributions similar to those of their bulk particles in suspension have revealed that the toxicity mechanisms of NPs are more complex (Warheit et al., 2006; Zhang et al., 2008). In suspensions, NPs tend to form large particles and most of the aggregates will settle out of the suspensions in a few hours, which may reduce their toxicities (Adams et al., 2006). On the other hand, reactive oxidative species (ROS) generated through various mechanisms, such as illumination of NPs (Jiang et al., 2008) and disruption of intra-cellular metabolic activities (Long et al., 2006) may disturb the anti-oxidant system (Brown et al., 2004), leading to damage to lipids, carbohydrates, proteins and DNA (Kelly et al., 1998). Previous studies have shown that TiO₂ NPs induced oxidative stress in the brains of rainbow trout (Oncorhynchus mykiss) (Federici et al., 2007). ZnO NPs participated in damaging the membrane of Escherichia coli through ROS mechanisms, thus exhibiting an antibacterial action (L. Zhang et al., 2007). Another crucial factor that...
may affect the toxicity of metal oxide nanoparticles is the release of metal ions. It has been shown previously that the toxicity of nZnO was mainly attributed to the dissolved Zn ions (Aruoja et al., 2009; Franklin et al., 2007). However, contradictory results have been noted in recent studies, suggesting the possibility for a size dependence of toxicity, distinct from adverse effects associated with the presence of dissolved ions (Nair et al., 2009; Wong et al., 2010).

Although there have been a number of studies on the potential hazards of TiO₂ and ZnO NPs to aquatic ecosystems, their environmental impacts on aquatic organisms and toxicity mechanisms still have not been fully elucidated. Particularly, the toxicity tests on adult fish have mainly focused on carbon-based NPs (Oberdörster, 2004; Smith et al., 2007; Zhu et al., 2006). Moreover, those investigations of the toxicity of metal oxide NPs (TiO₂, ZnO NPs) to fish have concentrated on early developmental stages (Zhu et al., 2008, 2009). Few researchers have touched the toxicity of TiO₂ and ZnO NPs to adult fish. Based on the fact that zebrafish represents a bridge between in vitro cell culture models and in vivo mammalian models (Fako and Furgesom, 2009), the objective of this study was to evaluate the acute toxicity and oxidative effects of ZnO and TiO₂ NPs on adult zebrafish, especially the effects of particle formulations (nano or bulk). ·OH radicals generated by NPs and metal ions released from metal oxide particles.

2. Materials and methods

2.1. Preparation and characterization of particle suspensions

TiO₂ and ZnO NPs were purchased from the Nano Applied Research Center of Nanjing University of Technology. The surfaces of TiO₂ and ZnO NPs were not modified. Bulk TiO₂, ZnO and ZnSO₄·7H₂O were purchased from Tianjin Guangcheng Chemical Reagent Co., Ltd. The purities, diameters, crystal structures and zeta potentials of the NPs purchased from Tianjin Guangcheng Chemical Reagent Co., Ltd. and bulk particles were listed in Table 1.

Suspensions of nano-scale ZnO and TiO₂ and their counterparts were prepared with aerated single-distilled water and dispersed with a bath sonicator (JL-360, Shanghai Jieli Co., Ltd, at 100 W and 40 kHz) for 20 min instead of using stabilizing agents. The shape of the NPs and bulk ZnO and TiO₂ particles were determined using a transmission electron microscope (TEM) (JEM-100CXII, JEOL, Ltd., Japan) operated at 100 kV. The particle size distribution was determined by a Nano-Zetasizer (1000, Malvern Instrument Ltd., UK), which uses a dynamic light scattering (DLS) technique. During the measurement process, the effects of ionic strength, electrolyte type and electrolyte concentration on particle size (DLS) technique. During the measurement process, the effects of ionic strength, electrolyte type and electrolyte concentration on particle size were not considered since no electrolyte was added to the suspensions. The Zn²⁺ solution was prepared by dissolving ZnSO₄·7H₂O in aerated single-distilled water. The quantity of Ti⁴⁺ ion released from TiO₂ was extremely low, so Ti⁴⁺ ion toxicity in zebrafish was not investigated.

2.2. Animals

Adult zebrafish with a mean age of 120 d, mean length of 3.02±0.33 cm, and mean weight of 0.22±0.05 g were obtained from the Research Group for Molecular Pathology of Fish (Institute of Hydrobiology, Chinese Academy of Sciences). Fish were acclimatized in dechlorinated tap water with a natural light-dark cycle (12 h light/12 h dark) for at least 7 d in the laboratory before experimentation and fed twice daily with newly hatched brine shrimp (Artemia). During this period, the water temperature was maintained at 23±2 °C, and no fish died.

2.3. Toxicity tests

Two types of endpoints were assessed in the zebrafish: 1) lethality, and 2) oxidative stress and oxidative damage. Test concentrations for lethality were 0, 2, 5, 10, 30 and 50 mg/L for ZnO NPs, bulk ZnO and Zn²⁺ and 0, 10, 50, 100, 150, 200 and 300 mg/L for TiO₂ NPs and bulk TiO₂. Based on the lethality results, the exposure concentrations selected for the oxidative endpoints were 5 mg/L for ZnO NPs and bulk ZnO, and 50 mg/L for TiO₂ NPs and bulk TiO₂. These concentrations ensured representative results and adequate survival of fish for biochemistry tests.

Test suspensions were prepared and dispersed using a sonicator for 20 min immediately prior to use without the addition of salts or stabilizing agents. Seven fish were randomly exposed to each concentration for 96 h in a 2-L glass beaker containing 1.5 L of the test solution. To ensure a constant concentration, all the test solutions were changed every 24 h. The control group was provided with fresh water without NPs or bulk particles. Each treatment was run in triplicate and placed under the same conditions with a natural light/dark cycle.

In order to maintain water quality, fish were not fed on the day before or during the experimental period to minimize the absorption of the NPs and bulk particles in food and the production of feces. During the exposure period, the water temperature was 23±2 °C. The pH and dissolved oxygen content (DO) of the water monitored using a pH meter (YSI 63, Yellow Springs Inc., USA) and DO meter (YSI 85, Yellow Springs Inc., USA) were 6.7–7.2 and oxygen no less than 5.10 mg/L, respectively. The number of dead fish was recorded every 12 h, and they were removed immediately to avoid contamination of the exposure solutions.

2.4. Biochemistry

At the conclusion of the toxicity tests, superoxide dismutase (SOD) and catalase (CAT) activities, as well as the reduced glutathione (GSH), malondialdehyde (MDA) and protein carbonyl content of the zebrafish tissues were determined to assess oxidative stress and damage. Six fish were dissected for each particle type. Samples of gill, liver and gut tissues were collected, rinsed with cold saline, dried with filter paper, weighed, and then immediately snap frozen in liquid nitrogen before storage at −80 °C until they were analyzed. Tissue samples were homogenized in 100 volumes of ice-cold 0.1 mol/L phosphate buffer solution (containing 300 mmol/L sucrose and 0.1 mmol/L ethylenediamine tetra acetic acid and adjusted to pH 7.8) to yield a 1% (w/v) homogenate. This was then centrifuged at 4 °C at 10,000×g for 10 min. Samples of the supernatant were used to determine SOD, CAT, GSH, MDA and protein carbonyl content.

Table 1

<table>
<thead>
<tr>
<th>Particles</th>
<th>Purity (%)</th>
<th>Crystal structure</th>
<th>Advertised diameter (nm)</th>
<th>Diameter (nm)ᵃ</th>
<th>Hydrodynamic diameterᵇ (nm)</th>
<th>Zeta (mv)ᵈ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nano-TiO₂</td>
<td>99</td>
<td>Anatase</td>
<td>30</td>
<td>20–70</td>
<td>251–630 (403)</td>
<td>−13.1</td>
</tr>
<tr>
<td>Bulk TiO₂</td>
<td>99</td>
<td>Anatase</td>
<td>1000</td>
<td>128–949</td>
<td>272–597 (439)</td>
<td>−27.8</td>
</tr>
<tr>
<td>Nano-ZnO</td>
<td>99</td>
<td>Six-wurtzite</td>
<td>30</td>
<td>16–157</td>
<td>423–1722 (658)</td>
<td>−0.3</td>
</tr>
<tr>
<td>Bulk ZnO</td>
<td>99</td>
<td>Six-wurtzite</td>
<td>500</td>
<td>72–411</td>
<td>428–1076 (744)</td>
<td>−2.6</td>
</tr>
</tbody>
</table>

ᵃ Data developed according to TEM images in our lab.
ᵇ Particle size distribution in solutions, measured by the nano-sizer in our lab.
ᶜ The data are expressed as range (mean).
ᵈ Data generated by nano-sizer in our lab and the pH was 7.02 and 6.95 for nanoscale and bulk TiO₂, and 7.10,7.05 for nanoscale and bulk ZnO, respectively.
SOD and CAT activities and the GSH and MDA content of the gill, gut and liver tissues were assayed using commercially available kits according to the manufacturer’s protocol (Nanjing Jiancheng Bioengineering Institute, China). Protein concentration was determined by the dye binding assay of Bradford (1976) using bovine serum albumin as a standard. The protein carbonyl concentration was determined by a colorimetric method using 2,4-dinitrophenylhydrazine (Levine et al., 1990). One unit (U) of SOD activity was defined as the quantity of enzyme yielding 50% inhibition of nitrite formation in 1 mL of reaction solution. CAT activity was calculated in terms of H$_2$O$_2$ consumed in nmol/s · g of protein. The MDA, GSH and protein carbonyl concentrations were expressed as nmol MDA/mg protein, mg GSH/g protein and nmol protein carbonyl/mg protein, respectively. The analysis of SOD and CAT activities were completed within 1 d after toxicity testing was finished, and GSH, MDA and protein carbonyl content determination were completed within 7 d.

One additional fish was collected from each glass exposure beaker at the end of toxicity testing for observation of the oxidative effects on the cellular morphology of the gills. Gill tissue (the second gill arch) was rinsed three times with cold saline water and fixed in 2.5% (v/v) glutaraldehyde buffered with phosphate buffer saline (PBS, pH = 7.8) at 4 °C for 4 h. The tissue was washed with PBS and post-fixed in 1% (v/v) osmium tetroxide at room temperature for another 2 h. After dehydration in a graded ethanol series, the tissue was embedded in Epon 812 resin and sectioned. Ultrathin sections were stained with uranyl acetate and lead citrate for the examination by transmission electron microscopy (JEM-100CXII, JEOL, Ltd., Japan).

2.5. Determination of ·OH

The procedures for determining the ability of generating ·OH by NPs and bulk particles were adopted from the method described by Hislop and Bolton (1999) using a gas chromatograph equipped with a flame ionization detector (GC/FID) (SP3400, Beifen Ruili Analytical Instruments, Ltd., China) and 2-propanol as a scavenger. In the presence of ·OH generated by NPs, 2-propanol is oxidatively modified into acetone, which can then be detected by GC/FID. Accordingly, the concentration of ·OH was calculated from the concentration of acetone. Suspensions of NPs and bulk ZnO and TiO$_2$ were prepared as described in Section 2.1. To simulate the conditions of the toxicity tests, particle suspensions containing 0.9% (v/v) 2-propanol were placed in 50-mL colorimetric tubes and incubated at 28 °C under constant illumination with a parallel array of six standard fluorescent lamps (220 V, 40 W) for 120 h, and suspensions of TiO$_2$, ZnO NPs, ZnO NPs and their counterparts were placed in the dark as negative controls. Samples were removed from the solutions at 0, 24, 48, 72, 96 and 120 h and centrifuged at 10,000 × g for 10 min, and the supernatant filtered through a 0.22-μm filter (the first milliliter of the filtrate was discarded). Finally, the concentration of acetone in the filtrate was analyzed by GC/FID.

A quartz capillary chromatographic column was used for the GC/FID (FFAP, 30 m × 530 μm, Agilent Company). The gas chromatography conditions were as follows: injection volume of 2 μL and injector and detector temperatures at 150 °C and 300 °C, respectively. The oven temperature program was as follows: 70 °C initially, held for 4 min, then increased to 250 °C at a rate of 30 °C/min and held for 5 min. High purity nitrogen was used as the carrier gas at a flow rate of 40 mL/min. For the quantitative analysis, empirically determined standard curves for acetone and 2-propanol were employed: $c_{\text{acetone}} = 0.0144A + 0.153$ ($R^2 = 0.9995$) and $c_{\text{2-propanol}} = 0.0136A + 0.0946$ ($R^2 = 0.9995$), where $c_{\text{acetone}}$ and $c_{\text{2-propanol}}$ represent the concentrations of acetone and 2-propanol, respectively, and A is the area of each peak. As the capture efficiency of 2-propanol to ·OH was 86.7% (Hislop and Bolton, 1999), the concentration of ·OH was calculated as $c_{\text{OH}} = c_{\text{acetone}}/0.867$. The detection limit was 0.20 mmol/L, the recovery rate and the relative standard deviations were 96.8% and 3.6%, respectively.

2.6. Statistical analysis

Data were analyzed using the statistical package SPSS (Ver. 16.0, SPSS Inc., Chicago, IL, USA). The 96-h LC$_{50}$ values were calculated using a probit analysis method. Data for biochemical tests are expressed as the means ± SD from three independent experiments, and a one-way analysis of variance with Tukey’s multiple comparisons was run to compare the differences between treatment and control groups. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Characteristics of NPs and bulk particles in suspension

The TEM images and size distributions of NPs and bulk particles are shown in Fig. 1 and Table 2. In suspensions of TiO$_2$ and ZnO NPs and of bulk TiO$_2$ and ZnO, particles were collected into aggregates of irregular shape (Fig. 1). The mean size of the NP aggregates increased with increasing concentrations (Table 2). As shown in Fig. 1, the initial diameter of individual NPs (about 30 nm as provided by the suppliers) was much smaller than the corresponding bulk particles. However, the measured diameter ranges for NPs in suspensions of different concentrations were 400–1400 nm for ZnO and 200–500 nm for TiO$_2$, which were similar to the size ranges of bulk particles in suspension. In fact, the Nano-Zetasizer has underestimated the actual particle size, as its measuring principle is based on hydrodynamic properties that are calculated by the Stokes–Einstein equation. This method yields a hydrodynamic diameter that is a calculated particle diameter of a sphere that has the same measured motion in the solute as the actual particle (Wang et al., 2009; Jiang et al., 2009).

3.2. ·OH generated in NP suspensions

Fig. 2 shows the concentrations of ·OH produced by NPs in suspension. Although the size range of the NPs was similar to the bulk particles, their ability to generate ·OH was very different. Under the fluorescent light, both TiO$_2$ and ZnO NPs suspensions were able to generate ·OH. No ·OH was detected in the bulk TiO$_2$ and ZnO suspensions or in TiO$_2$ and ZnO NPs suspensions that were placed in the dark. The concentrations of ·OH in 5 mg/L ZnO NPs suspensions after 24 h, 48 h and 96 h of illumination were also all below the detection limit. Using regression analysis, a linear relationship between the concentration of ·OH and illumination time was found as shown in Fig. 2. The rate of ·OH generation by NPs increased with increasing NP concentration, but once the NP concentration reached 200 mg/L, no further increase was observed with increasing concentration. This may be because the NP suspensions in 50 mL colorimetric tubes were illuminated over a specific area, and the number of NPs exposed to the light remained constant when the concentration exceeded a certain value. The ·OH concentration in 50 mg/L TiO$_2$ NP suspensions (1.77 mmol/L) was much higher than that in 50 mg/L ZnO NP suspensions (0.46 mmol/L) after illumination for 96 h, indicating that the ·OH-generating-activity of TiO$_2$ NPs was much higher than that of ZnO NPs. Moreover, the 2-propanol concentration remained constant in the control and bulk particle groups during 120 h of illumination (data not shown). This indicates that 2-propanol was stable under the experimental conditions and that no ·OH was produced by the bulk particles.

3.3. Acute toxicity of NPs and bulk particle suspensions to zebrafish

The acute toxicity of TiO$_2$, NPs, ZnO NPs, bulk ZnO and Zn$^{2+}$ to zebrafish increased with particle concentration (Fig. 3), demonstrating a dose dependency. As shown in Fig. 3a, although TiO$_2$ NPs at a concentration of 50 mg/L produced no mortality in zebrafish, as in the
control group, the 300-mg/L TiO\textsubscript{2} NP suspension caused 100% mortality with a calculated 96-h LC\textsubscript{50} of 124.5 mg/L. In contrast, bulk TiO\textsubscript{2} suspensions of all concentrations showed no acute toxicity to zebrafish, and even the bulk TiO\textsubscript{2} suspension up to 300-mg/L resulted in 0% mortality after 96 h of exposure (data not shown).

As with TiO\textsubscript{2} NPs, a dose-dependent toxicity was also found for ZnO NPs and for bulk ZnO. When their concentrations exceeded 2 mg/L, death of the zebrafish occurred with mortality increasing as particle concentration increased; 100% mortality was observed at 30 mg/L of ZnO NPs and bulk ZnO suspensions with calculated 96-h LC\textsubscript{50} values of 4.92 mg/L and 3.31 mg/L, respectively. Zn\textsuperscript{2+} solutions were also toxic to zebrafish (96-h LC\textsubscript{50} of 8.062 mg/L). Using the statistical analysis method introduced in Section 2.6, it was found that the 96-h LC\textsubscript{50} for ZnO NPs and bulk ZnO were not significantly different ($p > 0.05$), but they were significantly different between ZnO NPs and Zn\textsuperscript{2+} ($p < 0.05$), as well as between bulk ZnO and Zn\textsuperscript{2+} ($p < 0.05$). This means that Zn\textsuperscript{2+} released by NP and bulk ZnO may not be the main mechanism of toxicity for the NPs and bulk ZnO suspensions observed in this study.

### 3.4. Oxidative stress and tissue damage caused by TiO\textsubscript{2} and ZnO

Exposure to 50 mg/L of TiO\textsubscript{2} NPs under either light or dark conditions did not cause significant changes in the SOD activities in gill tissues, but both groups displayed significant decreases in SOD activity in liver tissues and increased SOD activity in gut tissues (Fig. 4a). Compared to controls, the activities of SOD in liver and gut from the light exposure group were 70.2% ($p < 0.05$) and 149.6% ($p < 0.05$), respectively, and 76.6% ($p < 0.05$) and 132.7% ($p < 0.05$) for the dark group, respectively, suggesting that fish in the light exposure group had suffered higher oxidative stress than fish in the dark group.

### Table 2

<table>
<thead>
<tr>
<th>Particle (mg/L)</th>
<th>Concentration (mg/L)</th>
<th>Average diameter (nm)</th>
<th>Range of diameters (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO\textsubscript{2} NPs (Anatase)</td>
<td>300</td>
<td>394.6</td>
<td>245–617</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>514.4</td>
<td>402–633</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>403</td>
<td>251–630</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>413</td>
<td>270–539</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>371</td>
<td>249–488</td>
</tr>
<tr>
<td>Bulk TiO\textsubscript{2}</td>
<td>50</td>
<td>438.7</td>
<td>272–597</td>
</tr>
<tr>
<td>ZnO NPs</td>
<td>300</td>
<td>1381</td>
<td>489–2456</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1273</td>
<td>358–1798</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>658.3</td>
<td>423–1722</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>449.1</td>
<td>201–1269</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>616.5</td>
<td>504–753</td>
</tr>
<tr>
<td>Bulk ZnO</td>
<td>50</td>
<td>744.4</td>
<td>428–1076</td>
</tr>
</tbody>
</table>

**Fig. 1.** TEM images of suspensions of: (a) TiO\textsubscript{2} NPs, (b) bulk TiO\textsubscript{2}, (c) ZnO NPs, and (d) bulk ZnO. Scale bars are calibrated in nm.
exposure group. This was in accordance with the content of ·OH in the two groups. However, no change in SOD activity was observed in zebrafish exposed to the 50 mg/L bulk TiO2 suspension. The 5 mg/L ZnO NP suspension reduced SOD activity in liver tissue and augmented it in gut tissue (62.9%, p < 0.05 and 269.2%, p < 0.01 of controls, respectively). As to 5 mg/L bulk ZnO, inhibitory effects on SOD activity were observed in both liver and gut tissue (51.7%, p < 0.05 and 32.0%, p < 0.01 of controls, respectively).

The impact of NPs and bulk ZnO suspensions on CAT activity were the highest in liver tissue, followed by gut tissue, and then gill tissue (Fig. 4b). In all treatment groups, except for the group exposed to a 50-mg/L bulk TiO2 suspension, which had no observable effect, CAT activity in liver tissue was observed to be reduced in all other groups. Inhibition rates were 34.6% relative to controls for 50 mg/L TiO2 NPs in the light and 40.1% for TiO2 NPs in the dark. For ZnO NPs, inhibition was 46.9% compared to controls, and it was 48.2% for bulk ZnO suspensions (Fig. 4b). In gut tissue, 5 mg/L ZnO NPs and bulk ZnO suspensions stimulated and inhibited CAT activity, respectively, while both light and dark groups exposed to 50 mg/L of TiO2 NPs and bulk TiO2 suspensions experienced no effect on CAT activity in gut tissue. CAT activity in gill tissue was relatively insensitive to most of the treatments, although a 5 mg/L ZnO NPs suspension caused a small increase.

In liver tissue, the trends in alteration of GSH concentrations were similar to those of CAT activity (Fig. 4c). Exposure to 50 mg/L of TiO2 NPs, 50 mg/L of TiO2 NPs in the dark, 5 mg/L of ZnO NPs and a bulk ZnO suspension for 96 h generally caused a decrease in GSH content in liver tissue compared to controls (by 53%, p < 0.05; 48.3%, p < 0.05; 45.2%, p < 0.05; and 34.6%, p < 0.05, respectively). It was consistent with the generation of excessive ROS that reacted with and neutralized GSH. In contrast, significant increases of GSH concentrations were observed in the gut tissue of fish exposed to all groups of TiO2 suspensions and to the 5 mg/L ZnO NPs suspension, but not in the gut tissues of controls or the bulk ZnO group. In the groups exposed to suspensions of 50 mg/L of TiO2 NPs, 50 mg/L of TiO2 NPs in the dark, 50 mg/L of bulk TiO2 and 5 mg/L of ZnO NPs, compared to controls, the GSH contents in gut tissue were 212.9% (p < 0.05), 225.1% (p < 0.05), 230.2% (p < 0.05) and 350.8% (p < 0.01), respectively. GSH in gill tissue was relatively insensitive to these treatments, and only the 5 mg/L ZnO NP suspension induced a small increase in GSH content.

The protein carbonyl concentrations in all tissue samples studied were not significantly different from the controls except in the case of liver tissue from fish exposed to 50 mg/L TiO2 NPs (the contents in gill and gut are not shown). In the liver tissues of fish exposed to both light and dark, the treatment with 50 mg/L TiO2 NP suspensions resulted in significantly elevated protein carbonyl levels (178.1% and 139.7% of the control, respectively), consistent with oxidative damage to the liver (Fig. 4e). Lipid peroxides were also found in the gills and guts from the light and dark groups of fish exposed to 50 mg/L TiO2 NP suspensions (Fig. 4d). MDA concentrations in gills from the light and dark groups were 217.2% (p < 0.05) and 174.3% (p < 0.05) of controls, respectively, with 160.9% (p < 0.05) and 289.6% (p < 0.05) of controls, respectively, but they remained the same as the controls in gill and gut tissues.

3.5. Morphology of gill cells

Cytological changes in the gill tissues sampled from the exposure and control groups were observed under TEM. As shown in Fig. 5a, gill cells of fish in the control group possessed normal cell structure with intact cell membranes, nuclei and organelles (e.g. mitochondria), and normal appearance of the cytoplasm. Normal cell morphology of gill tissue was also observed in fish exposed to 50 mg/L bulk TiO2 suspensions (Fig. 5b). However, gill cells exposed to 50 mg/L of TiO2 NPs showed a number of morphological changes (Fig. 5c), including cell membrane damage (perhaps caused by ROS such as ·OH),
irregular cell outlines, pyknotic nuclei and a trend of complete disruption of gill cells.

Both 5 mg/L ZnO NPs and bulk ZnO induced marked damage to gill cells as indicated in Fig. 5d–e. Although cell membranes were intact, shrinkage or loss of cell cytoplasm was observed when compared to the control, and the shapes of nuclei were abnormal in fish exposed to 5 mg/L of ZnO NPs (Fig. 5d).

4. Discussion

4.1. Acute toxicity of TiO2 NPs, ZnO NPs and bulk particles to zebrafish

Although the particle size analysis indicated that the average diameters of TiO2 were similar to their bulk particles in suspension, as noted in other studies (Adams et al., 2006; Long et al., 2006), the acute
The toxicity of TiO$_2$ NPs (96-h LC$_{50}$ of 124.5 mg/L) was far higher than that of bulk TiO$_2$, which produced 0% mortality in zebra fish even at a concentration of 300 mg/L. Similarly, the toxicities of TiO$_2$ NPs to the nematode Caenorhabditis elegans (Wang et al., 2009) and to D. magna (Zhu et al., 2009) were also significantly higher than the toxicity of bulk TiO$_2$ suspensions. In the present study, TiO$_2$ NPs had no measurable effects on zebra fish at low doses (10–50 mg/L), whereas there were significant effects at higher levels (100–300 mg/L). These findings are consistent with the results from in vitro toxicity tests on a rat liver-derived cell line (Hussain et al., 2005). Although ·OH generated by TiO$_2$ NPs may result in oxidative stress and damage as measured in this study (Fig. 4), the toxicity of ·OH is not too strong to cause lethality of zebra fish because the ·OH generated by 50 mg/L TiO$_2$ NPs was only slightly lower than those by 100, 200 and 300 mg/L TiO$_2$ NPs (see Fig. 2). Therefore, it can be concluded that, at high concentrations, TiO$_2$ NPs had a dose–response toxicity.

Differed from those of TiO$_2$, the toxicities of ZnO NPs and the corresponding bulk particles were similar (96-h LC$_{50}$ of 4.92 and 3.31 mg/L, respectively). Previous investigations also indicated that the toxicity of ZnO NPs and bulk ZnO were identical (Hussain et al., 2005; Wang et al., 2009; Zhu et al., 2008, 2009). In addition to the toxicity caused by ZnO NPs and bulk ZnO themselves, the dissolved Zn$^{2+}$ ions were also toxic to zebra fish (Fig. 3b). This is in accordance with previous researches on the effects of ZnO and Zn$^{2+}$ on D. magna (Wiench et al., 2009), the bacterium Vibrio fischeri, the branchiopod Thamnocephalus platyurus (Heinlaan et al., 2008) and the microalga Pseudokirchneriella subcapitata (Aruoja et al., 2009). However, according to the literature, the aqueous solubility of ZnO is only 1.6–5 mg/L (EPA, 1995), and the concentration of Zn$^{2+}$ in a 2000 mg/L ZnO NPs suspension ranges from 0.3–3.6 mg/L (Lin and Xing, 2007). This suggests that the concentration of Zn$^{2+}$ in ZnO suspensions in this study would not have exceeded 3.6 mg/L, which was below the
oxidative damage in gill tissues. This was different from the 50 mg/L SOD, CAT activities and GSH contents despite the lack of observed suspensions in which concentrations of ·OH were below the detection status in the UK and global trends. Occup Med 2006;56:300–306.


References

4.2. Oxidative stress and tissue damage caused by TiO2, ZnO NPs and bulk particles

In addition to lethal toxicity test, the molecular biomarker responses were essential to determine the sublethal toxicities of the test chemicals to zebrafish and provide insights into toxic mechanisms of NPs. By monitoring several biomarkers, including SOD, CAT, GSH, MDA and protein carbonyl contents, the disturbances of the oxidative defense system caused by exposure to TiO2 NPs, ZnO NPs and their bulk particle suspensions were observed in the gill, gut and liver tissue of zebrafish (Fig. 4).

After 96 h of exposure to a 50-mg/L TiO2 NPs suspension and a 50-mg/L TiO2 NPs suspension in the dark, lipid peroxidation was observed in gill tissues, but 50-mg/L bulk TiO2 had no effect, which is consistent with the observed acute toxicity of TiO2 to zebrafish. Moreover, a 50-mg/L TiO2 NPs suspension induced a higher production of lipid peroxidation than a 50-mg/L TiO2 NPs in the dark. Conversely, gills of fish exposed to 5-mg/L ZnO NPs and bulk ZnO suspensions in which concentrations of ·OH were below the detection limit experienced equal amounts of oxidative stress, as indicated by SOD, CAT activities and GSH contents despite the lack of observed oxidative damage in gill tissues. This was different from the 50 mg/L TiO2 NPs suspension, which may perhaps be explained by the fact that the exposure concentration of ZnO NPs (5 mg/L) was too low to generate enough ROS to induce oxidative damage, and ZnO NPs acted on gill cells through a different mechanism.

The ·OH is generally considered to be one of the most toxic ROS species (Knight, 1995) and is able to oxidize almost all cellular components (Yamakoshi et al., 2003). Although the life-span of ·OH is extremely short, the gills of fish may directly contact NPs and ·OH in the water. Therefore, the lipid peroxidation in gill tissue caused by 50 mg/L TiO2 NPs observed in this study must be partly due to the ·OH, which is generated by 50 mg/L TiO2 NPs but not by 50 mg/L TiO2 NPs in the dark or bulk TiO2 suspension. Likewise, when Federici et al. (2007) exposed rainbow trout (O. mykiss) to TiO2 NPs suspensions, oxidative damage to lipids was found in the gill tissue.

Additionally, 50 mg/L TiO2 NPs suspension either in the light and dark induced lipid peroxidation in gills without oxidative stress because among all of the biomarkers, only MDA content changed significantly in gill tissue compared to controls. This resembles the oxidative effects of H2O2, which was used as a positive control in a study on the effects of C60 NPs on juvenile largemouth bass (Micropterus salmoides) (Oberdörster, 2004). It was demonstrated that ·OH probably acted on gill cell membranes directly, which is consistent with TEM images of gill tissue (Fig. 5c). As to the 5 mg/L ZnO NPs and bulk ZnO suspension, the oxidative effects observed were caused partly by intracellular ROS generated by disturbances to cellular functions and partly by Zn2+ (Franklin et al., 2007; Heinlan et al., 2008) that may have induced shrinkage and loss of cell cyttoplasm without damaging the cell membrane (Fig. 5d–e). This was different from the 50 mg/L TiO2 NPs group, implying that the toxicity of TiO2 NPs was partly related to ROS generated under illumination. NPs ingested by zebrafish through diet may be deposited not only in the intestine (X. Zhang et al., 2007) but also may be transported to other organs in the fish by diffusion and blood circulation (Wang et al., 2004). NPs are able to generate ROS in both liver and gut tissue in the absence of light (Long et al., 2006; Savic et al., 2003). Consequently, in the present study, oxidative stress and tissue damage were induced in the liver and gut by NPs through superfluous ROS as indicated by the biomarkers SOD, CAT and GSH. All biomarkers measured in liver tissue in this paper were sensitive to 50 mg/L TiO2 NPs and 5 mg/L ZnO NPs suspension, as well as 50 mg/L TiO2 NPs in the dark (Fig. 4), demonstrating that the liver is the main target of NPs. Ultimately, the disruption of anti-oxidant systems would enhance the generation of ROS and produce more serious oxidative damage to tissues. Consequently, oxidative stress and oxidative damage must play an important role in nano-toxicity. As shown by the MDA and protein carbonyl contents of the liver and gut (Fig. 4), it is noteworthy that suspensions containing 5 mg/L of ZnO NPs and bulk ZnO induced lipid peroxidation in the liver but no peroxidation in gut tissue, which was opposite of the effects of exposure to the 50 mg/L TiO2 NPs and the 50 mg/L TiO2 NPs in the dark.

Therefore, NPs with different chemical compositions generated according kinds of ROS through distinct mechanisms that affected certain cell components. Additionally, the activities of diverse NPs in various tissues differed.

5. Conclusions

From the results of this study, it can be concluded that: (1) the acute toxicity of TiO2 NPs to zebrafish was significantly higher than that of bulk TiO2 while zinc oxides were equally toxic in NP and bulk formulations; (2) NPs could cause toxic effects despite that they formed aggregates in suspensions; (3) Metal ions released by ZnO contributed to toxicities but were not the main lethal mechanism of the ZnO NPs and bulk ZnO suspensions tested; (4) TiO2 NPs were able to cause toxicity effects without entering the cells, and the extracellular ·OH generated by TiO2 NPs could induce oxidative damage directly on the cell membranes of gill tissue. Thus, further research into the properties of NPs with different chemical compositions and the NP behaviors of environmental water chemistry as related to the toxicity mechanisms of NPs, are required to evaluate the aquatic eco-toxicity of metal-oxide NPs and to determine definitively whether their toxicity is caused by nano-effects.

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