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A study of the mechanism of *in vitro* cytotoxicity of metal oxide nanoparticles using catfish primary hepatocytes and human HepG2 cells

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Abstract

Nanoparticles (NPs), including nano metal oxides, are being used in diverse applications such as medicine, clothing, cosmetics and food. In order to promote the safe development of nanotechnology, it is essential to assess the potential adverse health consequences associated with human exposure. The liver is a target site for NP toxicity, due to NP accumulation within it after ingestion, inhalation or absorption. The toxicity of nano-ZnO, TiO₂, CuO and Co₃O₄ was investigated using a primary culture of channel catfish hepatocytes and human HepG2 cells as *in vitro* model systems for assessing the impact of metal oxide NPs on human and environmental health. Some mechanisms of nanotoxicity were determined by using phase contrast inverted microscopy, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, reactive oxygen species (ROS) assays, and flow cytometric assays. Nano-CuO and ZnO showed significant toxicity in both HepG2 cells and catfish primary hepatocytes. The results demonstrate that HepG2 cells are more sensitive than catfish primary hepatocytes to the toxicity of metal oxide NPs. The overall ranking of the toxicity of metal oxides to the test cells is as follows: TiO₂ < Co₃O₄ < ZnO < CuO. The toxicity is due not only to ROS-induced cell death, but also damages to cell and mitochondrial membranes.

Keywords

Cytotoxicity; nanoparticle; metal oxide; catfish; primary hepatocyte; HepG2 cell

1. Introduction

Nanotechnology is one of the fastest growing sectors of the high-tech economy. It is based on nanoparticles (NPs), both naturally occurring and man-made or engineered nanoparticles (ENPs). NPs, which are smaller than the size of human cells, are widely used in cosmetics, electronics, food and medicine. They have also been used in biological applications that require long-term, multi-target and highly sensitive imaging (Jaiswal and Simon, 2004).

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However, because of their high catalytic activity, concerns about their possible harmful effects on humans and the natural environments have been raised (Nel et al., 2006; Thill et al., 2006).

Titanium dioxide (TiO₂), zinc oxide (ZnO), copper oxide (CuO) and cobalt oxide (Co₃O₄) are some of the most common industrial NP additives for various applications. With greater surface area per unit weight than their bulk counterparts, these nano metal oxides have superior performance. TiO₂ is an opacifier used in paints, paper, plastic, and cosmetic products. ZnO NPs are included in personal care products such as toothpaste, beauty care products, sunscreens (Serpone et al., 2007), and textiles (Becheri et al., 2008). Nano-CuO has industrial applications in gas sensors and catalytic processes (Dutta et al., 2003). Nano-Co₃O₄ is one of the most important magnetic materials because of its role in catalysis, gas sensing, magnetism, and media tapes (Koshizaki et al., 1999; Papis et al., 2009).

Because of the increasing production and application of NPs, their release into the natural environment is inevitable. In the form of manufacturing and household wastes, metal oxide NPs will likely end up in natural water bodies and incorporated into biological systems *via* the food, medicine, and polluted water. Following ingestion, it is necessary to determine the distribution of the nanoparticles *in vivo* in order to identify potential targets for their toxicity (Wiesner et al., 2006; Robichaud et al., 2005). To date, there have been limited studies relevant to this topic. Fabian et al. (2008) determined the distribution of TiO₂ NPs (20–30 nm) in rat tissues following intravenous injection and discovered that they were primarily accumulated in the liver. The level of TiO₂ in the liver remained constant over the observation time, but decreased with time in other organs.

Isolated hepatocytes have widespread use in pharmacology and toxicology and have been employed to study the biotransformation and hepatotoxic and genotoxic effects of a myriad of chemicals. Cell cultures provide the best experimental system to study toxic mechanisms at the molecular and cellular levels by allowing the cells to be studied in a controlled environment in isolation from multiple physiological systems which regulate their activities *in vivo* (Castano et al., 2003). Most studies using hepatocytes require that the liver's physiological functions be maintained for data to be meaningful. Primary hepatocyte cultures appear to be the most powerful *in vitro* system, as the liver's specific functions and responses to toxic insults are retained for several days up to several weeks (Guillouzo, 1998). Boess et al. (2003) reported that the BRL 3A immortal rat liver cell line was selected as a convenient *in vitro* model to assess nanocellular toxicity. This cell line has been well characterized for its relevance to toxicity models.

The toxicity of metal oxide NPs has been reported for mammalian cell lines (Brunner et al., 2006; Chang et al., 2007; Ying and Hwang., 2010), bacteria (Adams et al., 2006; Huang et al., 2008; Hu et al., 2009), plants (Lin and Xing, 2008), and crustaceans (Lovern et al., 2007; Heinlaan et al., 2008). Research in fish has demonstrated that piscine models exhibited similar toxicological and adaptive responses to oxidative stress—a common toxicity imposed by NPs; therefore, piscine models may serve as a good surrogate for mammalian species (Kelly et al., 1998). A species of catfish was chosen as the model in this study because the catfish industry represents a significant segment of the economy in the state of Mississippi (Aker et al., 2008). We hypothesized that the nano metal oxides would exhibit toxicity to both human and piscine cells consistent with their respective sizes. In the study, we conducted a comparison between catfish primary hepatocytes and human HepG2 cells for metal oxide NP toxicity. Our objectives were: (1) to determine if there is a common mechanism of *in vitro* cytotoxicity of the selected nanometal oxides to human and piscine cells; and (2) to compare the toxicity of each in the two species.

2. Materials and Methods

2.1 Nano metal oxides

Metal oxide NPs including ZnO, TiO₂, CuO and Co₃O₄ were purchased from Sigma-Aldrich (St. Louis, MO). Nanoparticle size characterizations were determined with TEM in our lab and are reported along with manufacturers' characterizations in Table 1. The NPs were not coated. Stock solutions at the concentration of 1 mg/ml were prepared in MilliQ water by 30 minutes of sonication with a FS30 ultrasonic system (Fisher Scientific).

2.2 Preparation of catfish and cultured primary fish hepatocytes

Channel catfish (*Ictalurus punctatus*) of 150–200 g each were obtained from Simmons Catfish Farm located in Yazoo County, Mississippi. They were kept in 220 liter glass tanks with circulating water for 2 weeks (23 ± 0.5 °C) for acclimation. The fish were fed once daily with commercial pellet food.

The catfish were sacrificed and the culturing of primary catfish hepatocytes was carried out according to the method of Kim and Takemura (2003) and Zhou et al. (2006). The body was wetted with 75% ethanol for several seconds, then the liver was carefully excised from the abdominal cavity, transferred onto a plastic Petri dish, and rinsed twice with phosphate buffered saline (PBS: 136.9 mM NaCl; 5.4 mM KCl; 0.81 mM MgSO₄; 0.44 mM KH₂PO₄; 0.33 mM Na₂HPO₄; 5.0 mM NaHCO₃, pH 7.6) without Ca²⁺. The liver was dissected into small pieces with a scalpel and scissors, and the tissue was digested for 20 min at room temperature with PBS containing 0.1% collagenase (Sigma) on a shaker. The softened liver tissue was agitated and filtered through a 200 mesh nylon filter with pore size of 89 µm (Ted Pella Inc.; Redding, CA). The resulting cell suspension was transferred to a 50 ml sterilized centrifuge tube (Falcon, NJ) and centrifuged three times for 5 min each at 90×g in PBS buffer containing 1.5 mM CaCl₂ at 10°C. After the last wash, the cell pellets were re-suspended in Leibovitz's L-15 medium (L-15, Gibco). Cells were counted using a haemocytometer based on the trypan blue exclusion method; and only those cultures with more than 90% cell viability were used for further experiments. The isolated hepatocytes were seeded at a density of 6×10^5 per ml (100 µL: 60,000 per well) in a 96-well "Primaria" plate (Falcon) at room temperature. The culture medium contained L-15, 100 i.u./ml penicillin, 100 µg/ml streptomycin, 5 mM NaHCO₃, and 0.5% ITS (insulin-transferrin-selenium, Gibco). After 24 h, the medium was changed and the cultured cells were prepared for the following experiments of NP exposure.

2.3 Human HepG2 cell culture and treatment protocol

The HepG2/2.2.1 (CRL-11997TM) cell line was purchased from American Type Culture Collection (ATCC; Manassas, Virginia). The cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium with 2.5 mM L-glutamine, 15 mM HEPES, 0.5 mM sodium pyruvate and 1.2 mg/ml sodium bicarbonate, and supplemented with 0.4 mg/ml G418 and 10% fetal bovine serum at 37 °C in a 95% air/5% CO₂ humidified incubator (Isotemp; Fisher Scientific, Houston, TX). After 24 h, they were seeded in 96-well or 24-well plates. Cells were treated with a range of concentrations of NPs suspended in MilliQ water for 48 h. After 48 h of exposure, the various toxicity end points were measured in control and nanoparticle-exposed cells.

2.4 MTT assay

Preparations and tests were performed exactly as previously described (Gong and Han, 2006). Cells were plated onto a 96-well culture plate in 100 µl of culture medium. After incubation for 24 h, NPs at various concentrations were added to each well. The cells were then cultivated for an additional 48 h, followed by the addition of 25 µl of MTT solution (5

mg/ml) to each well and further incubation of 4 h. The supernatants were removed before adding 100 μ l of DMSO to dissolve the formazan crystal at 37 °C for 30 min. The absorbance was measured with a Triad LT microplate reader (Dynex Technologies, Chantilly, VA) at 560 nm.

2.5 Reactive oxygen species (ROS)

The method was a slightly modified version of the method reported by Wang and Joseph (1999). To measure ROS generation, a fluorometric assay using intracellular oxidation of 2, 7-dichlorofluorescein diacetate (DCFH-DA, Sigma, St. Louis, MO) was performed. Cells were incubated with 100 μ M DCFH-DA for 30 min. After incubation, cells were washed with PBS and incubated in fresh medium containing various concentrations of metal oxide NPs. The fluorescence intensity was measured using a microplate spectrofluorometer (Dynex Technologies, Chantilly, VA) with excitation and emission wavelengths of 485 and 530 nm, respectively. After 24 h or 48 h incubations in 5% CO₂/95% air at 37 °C, the fluorescence from each well was measured again. The percentage of increase in fluorescence per well was calculated with the formula $[(F_t - F_{t_0})/F_{t_0} \times 100]$, where F_{t_0} is the fluorescence at time zero and F_t is the fluorescence at 24 h or 48 h in the presence of NPs.

2.6 Flow cytometric assay

Annexin V-PI (Propidium Iodide) staining combined with flow cytometry (Han et al., 2008; Gong and Han, 2006) is commonly used to differentiate between cell apoptosis and necrosis. Briefly, 24 h after seeding cells into a twenty-four-well plate, cells were exposed to various concentrations of NPs for 48 h. Thereafter, cells were trypsinized and pelleted by centrifugation at 1500 rpm for 8 min followed by washing twice in PBS. For flow cytometry, cells were resuspended in assay buffer at the density of 10⁶ cells/ml and incubated at 5 μ g/ml PI and 2 μ g/ml Annexin V for 15 min in the dark. Finally, the cell solution was diluted fourfold with assay buffer and analyzed using a FACScan flow cytometer (Becton Dickson). All samples were treated gently to reduce mechanical damage to the cells. Viable cells were negative for both PI and annexin V (LL area). Early apoptotic cells were positive for annexin V and negative for PI (LR area), whereas late apoptotic dead cells displayed both high annexin V and PI labeling (UR area). Non-viable cells, which underwent necrosis, were positive for PI and negative for annexin V (UL area).

2.7 External cell morphology determination

The Olympus Model CKX41 Phase contrast microscope was used to assess external cell morphology.

2.8 Statistical analysis

Data were expressed as means $\pm$ standard deviation (SD) using 2003 Microsoft Excel. All experiments were conducted at least 3 times, and each treatment had four replicates or more. We used the regression calculation to determine the IC₅₀ (concentration that inhibits cellular activity by 50% compared to the control group) value and the correlation between cell viability and the concentration of the test metal oxides. The SAS System for Windows, V9.0 (SAS Institute, Gary, NC) was used for statistical evaluations. Differences among treatment and control groups were tested by one-way analysis of variance (ANOVA), followed by pair-wise comparisons between groups using Duncan's test. Differences at $p < 0.05$ were considered significant. Histograms in selected figures labeled with different letters represent significant differences.

3. Results

3.1 Characterization of cell morphology treated with NPs

Phase contrast microscopy light micrographs of the HepG2 cells following exposure to selected concentrations of metal oxide NPs are presented in Fig. 1. The HepG2 control cells were intact and remained spindle-shaped (Fig. 1A). HepG2 cells exposed to metal oxide NPs exhibited altered morphology, and most of them changed into a spherical shape and lost adhesion to the containment vessel. Exposure to three metal oxide NPs resulted in extensive cell mortality as evidenced by cell debris and significant decrease in the number of cells (Fig. 1B, 1D and 1E). For cells treated with TiO₂, no significant changes in morphology or cell quantity were seen (Fig. 1C). This result is similar to those of the micrographs of HepG2 cells exposed to TiO₂ at 25, 100 and 200 mg/l (pictures not shown).

Fig. 2 shows light micrographs of the catfish primary hepatocytes treated with metal oxide NPs at selected concentrations. There was a significant decrease in the number of cells after exposure to nano-CuO and ZnO at the concentration of 200 mg/l (Fig. 2B, 2D), but no significant change at lower concentrations (pictures not shown). Meanwhile, cell debris was observed in the primary hepatocytes treated with CuO NPs. No significant decrease in the number of cells treated with nano-TiO₂ and Co₃O₄ was observed despite the fact that numerous particles were wrapped around or attached to the surface of the primary cells.

3.2 MTT assay

Cell viability was determined by MTT assay following exposure to metal oxide NPs for 48 h (Fig. 3). For HepG2 cells (Fig. 3A), all metal oxide NPs except nano-TiO₂ reduced cell viability, even at low concentrations. Nano-CuO exhibited the highest cytotoxicity among the four metal oxide NPs, with 48.1% inhibition at 5 mg/l. IC₅₀ values for nano-CuO, ZnO and Co₃O₄ are 4.69, 30.8 and 24.2 mg/l, respectively (R^2 for each calculation: 0.97, 0.99 and 0.93, respectively). Instead of inhibition, some enhancement of cell viability by exposure to nano-TiO₂ (eg., for the 100 mg/l group) was observed. As shown in Fig. 3B, the metal oxide NPs exhibited lower cytotoxicity to the catfish primary hepatocytes than to the HepG2 cells. Nano-CuO, the most potent NP to the HepG2 cells, only has 51% inhibition against the catfish hepatocytes at the concentration of 200 mg/l. The IC₅₀ values of nano-CuO and ZnO are 181.8 and 275.6 mg/l, respectively (R^2 for each calculation: 0.95 and 0.98, respectively). Co₃O₄ showed a stimulatory effect at 25 and 50 mg/l and inhibitory effect at 100 and 200 mg/l, respectively, towards the primary cells, while TiO₂ exhibited no significant toxicity.

3.3 TEM images of cells treated with metal oxide NPs

TEM images in Fig. 4 reveal that NPs experience different fates and induce different consequences when encountering living cells. These views serve to facilitate identification of basic cell components. The cells shown in Fig. 4A are the primary liver cells of catfish which have been fixed by glutaraldehyde and osmium tetroxide (8,000 $\times$). The large nucleus is centrally located with two prominent nucleoli. Between the nucleus and the cell surface, the cytoplasm contains numerous organelles. As shown in Fig. 4B, nano-ZnO can be seen from both the outside and the inside of the cell (arrows), and cause damages to the membrane of the primary hepatocytes. ZnO NPs accumulate in the nucleus or attach to the surface of microvilli and organelles. Fig. 4C shows that nano-TiO₂ did not penetrate or damage the cell membrane. Instead, a number of them were attached to or aggregated on the surface of the cell membrane. There are similar results in TEM images of HepG2 cells (Fig. 4D and 4E). As shown in Fig. 4D, ZnO NPs can be observed from both the inside and outside the cell, and the cell membrane was distinctly damaged. Fig. 4E shows TiO₂ NPs

inside the cell cytoplasm of a HepG2 cell with no discernible damage to the membrane. The Image of control HepG2 cells is shown in Fig. 4F for comparison.

3.4 Reactive oxygen species (ROS)

We investigated the effect of the metal oxide NPs on intracellular ROS generation at exposure time points of 12, 24, 36 and 48 h. The generation of intracellular ROS was measured with increased intensity of DCF fluorescence. The results show a similar trend in ROS generation due to exposure to NPs for each cell type at two different time points (Fig. 5). After incubation with nano-CuO and Co₃O₄, both HepG2 cells and the primary hepatocytes showed significant increase in intracellular ROS in a dose-dependent manner. Intracellular ROS, induced by nano- Co₃O₄ at 200 mg/l for 12 h, reached 142.7-fold of that of HepG2 control cells (results not shown). At low concentrations, exposure to nano-ZnO resulted in a slight increase in intracellular ROS. However, exposure at high concentrations did not result in any further significant increase in ROS. Nano-TiO₂ also resulted in a significant increase in intracellular ROS in both cell types, and the ROS level in the primary cells treated with TiO₂ NPs at 200 mg/l for 48 h is 2.2 fold of that of the primary control cells.

3.5 Flow cytometric assay

To clarify whether metal oxide NPs induce apoptosis, we used results of the flow cytometric assay to sort the ZnO-treated primary cells into intact cells, dead cells, early apoptotic cells or late apoptotic cells after staining with annexin V-FITC and PI. As shown in Table 2 and Fig. 6, higher concentrations of ZnO yielded larger numbers of necrotic primary hepatocytes, which are presented as percent (%) in the upper left (UL) quadrant, and the effect was most significant at the concentration of 100 mg/l. The main source of the increased number of necrotic primary hepatocytes as concentration increases is the decrease in population of the viable cells instead of the apoptotic cells.

4. Discussion

Due to the ever expanding usage of NPs in consumer products, the likelihood of their entrance into the environment is increasing. Their application in products such as washing machines, personal care items, and clothing will undoubtedly result in their introduction into sewage treatment plants, and ultimately into the natural aquatic environment. The toxicity of nano-ZnO, TiO₂, CuO and Co₃O₄ was investigated using a primary culture of catfish hepatocytes and human HepG2 cells as the *in vitro* model systems. The biological effects of these NPs upon the two cell species were directly compared, and the mechanism of their cytotoxicity was investigated.

Primary cells, being more differentiated, may differ from cell lines in their cytotoxic responses. Segner and Schüürmann (1997) demonstrated that isolated trout hepatocytes were much more sensitive to iron (II) sulphate than the continuous trout liver cell line, R1. They speculated that this difference might be due to a higher level of unsaturated fatty acids in the hepatocyte membranes, leading to a particular sensitivity to iron-catalyzed oxidative stress. Farkas et al. (2010) reported that silver NPs were highly cytotoxic to rainbow trout hepatocytes due to the reduction of metabolic activity and membrane integrity at low concentrations. Calculated half maximal effective concentration (EC₅₀) values were between 2.5 mg/l and 4.9 mg/l, and were lower than those of the rat liver derived cell line BRL 3A (Hussain et al., 2005). However, our results were opposite to those reports. The modified method using collagenase proved to be successful for isolation of the cells. This was determined by electron microscopy, as the isolated catfish hepatocytes retained their structural integrity, with the organelles intact and virtually no swelling or rupturing (Fig.

4A). Hepatocytes exposed to nano-ZnO and CuO at high doses for 48 h exhibited a distinct cellular change, which was visualized using the inverted light microscope. These microscopic observations correlated well with the cytotoxicity test of nano-ZnO and CuO using the MTT assay. The cytotoxicity varied in a concentration-dependent manner, with cellular viability decreasing by almost 50% at the highest dose of 200 mg/l. By comparing the cytotoxicity of metal oxide NPs to the primary hepatocytes vs. HepG2 cells, it was noted that there was a marked difference in the respective cellular viabilities at equal test concentrations. Nano-ZnO and CuO showed significant toxic effect on HepG2 cells at low concentrations, with cellular viability decreasing by approximately 90% at the concentration of 50 mg/l. These results would suggest that HepG2 cells are more sensitive than the primary hepatocytes to the cytotoxicity of nano-CuO, ZnO and Co₃O₄. Meanwhile, we investigated and compared the cytotoxicity of nano-ZnO, TiO₂, CuO and Co₃O₄ using the catfish primary hepatocytes and MSTO-211H cells, and the results were similar (results not shown).

The isolated hepatocyte retains the major ingredients and features of an intact cell (functional organelles, enzyme interactions, physiological cofactors and metabolite concentrations, etc.) without the complexity of the intact animal. Primary cultures of fish hepatocytes have the additional advantage of higher biotransformation capacity when compared to existing cell lines (Lee et al., 1993). They also appear to be more stable during *in vitro* incubations (Segner, 1998; Naicker et al., 2007). Cells of RTG-2 and R1 cell lines were compared with gill and liver cells of rainbow trout on the basis of cytotoxicity induced by chemicals of the Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC) group, and the correlation coefficients (R^2) were 0.58 and 0.69, respectively; i.e. the correlations between primary cells and cell lines were slightly worse than those observed between different cell lines. A major factor contributing to this difference between primary cells and cell lines might be the higher or complicated metabolic capacity of the primary culture of fish cells. However, reports supporting this assumption are scarce (Castano et al., 2003). Thus, it justifies the contribution of this study to the aforementioned research topic.

Particle dimensions are recognized as being fundamental to nano-toxicity. This was derived from the fact that NPs have been consistently demonstrated to be capable of eliciting more pronounced toxicity than their larger (microparticulate) counterparts (Johnston et al., 2010). Hence, particle characterization prior to toxicity study is important (Murdock et al., 2008), and accordingly, in this study, the particle sizes in the media were determined with TEM. The measured characteristics of the selected NPs were found to be similar to the information provided by the suppliers and are summarized in Table 1.

The medium used in a cell line study can influence how NPs are dispersed and how this may impact their subsequent toxicity. The serum used in our experiments contains a variety of ingredients, and the individual constituent responsible for the improved dispersal of NPs within the experiments is unknown. The importance of considering the solutions in which NPs are suspended was demonstrated by Foucaud et al. (2007) who indicated that dipalmitoyl phosphatidylcholine (DPPC) and bovine serum albumin (BSA) were able to better disperse carbon black NPs. When compared to particles contained within saline only, this arrangement increased the ability of carbon black NPs to induce production of ROS within monocytic MonoMac 6 cells. In addition, Sager et al. (2007) reported similar results in their study. These findings indicate that the composition of NP dispersing solutions is able to affect NP toxicity, thus it is important to ensure their composition is relevant to the expected exposure scenario (Johnston et al., 2010).

In this study, the overall ranking of the toxicity of metal oxides NPs to the test cells is as follows: TiO₂ < Co₃O₄ < ZnO < CuO (Fig. 1, 2, 3), which does not show a relationship

consistent with their relative sizes (Table 1). This result is supportable by previous studies of metal oxide nanoparticles conducted by Adams et al. (2006) and Puzyn et al. (2011). Their reports indicated that within the study range [e.g., 15–90 nm in Puzyn et al., (2011)], size is not a critical factor for determining the variation in nanotoxicity. Therefore, other factors must also influence the end-point cytotoxicity of these NPs *in vitro*.

A common mechanism of the toxicity of NPs is thought to be mediated *via* oxidative stress (Kohen and Nyska, 2002) that damages lipids, carbohydrates, proteins and DNA (Kelly et al., 1998). Lipid peroxidation is considered most dangerous as it leads to alterations in cell membrane properties which in turn disrupt vital cellular functions (Rikans and Hornbrook, 1997). Recently, several studies demonstrated that ROS was a common inducer of cell death (Gong and Han, 2006; Imai and Nakagawa, 2003; Kroemer et al., 1998). Fahmy and Cormier (2009) reported the impact of SiO₂, Fe₂O₃ and CuO NPs on respiratory epithelial cells, and demonstrated that there is significant variation among different metal oxide NPs regarding their ability to generate oxidative stress and promote cell death. Our results were similar to those reports. Highly toxic nano-CuO induced the generation of intracellular ROS and resulted in the accumulation of ROS. Conversely, less toxic nano-TiO₂ induced low concentrations of ROS. In addition, our results from the flow cytometric assays showed that ZnO NPs promote cell death in a concentration-dependent manner. Specifically, increasing concentrations of ZnO NPs result in increased apoptosis of cells with a 1.37% increase in comparison to control at the concentration of 50 mg/l (Table 2 and Fig. 6). By comparison, CuO NPs caused a 6% increase in the apoptosis of cells at the concentration of 50 mg/l (detailed data not shown). Numerous publications have reported H₂O₂ induced apoptosis in cells in a concentration-dependent manner (Nair et al., 2004; Tang et al., 2005). Thus, we speculate that metal oxide induced ROS would play an important role in the induction of cytotoxicity of NPs.

In this study, ZnO-induced ROS was lower than that of CuO and Co₃O₄ at equivalent concentrations. Meanwhile, anomalously, ZnO at low concentrations led to the formation of higher concentration of ROS than ZnO at higher concentrations. Therefore, the mechanism of cytotoxicity of NPs is complicated and the possibility of effects from metal ions has been proposed. Wood et al. (1996) reported Ag⁺ toxicity towards aquatic organisms such as fish is caused by the disturbance of ionic regulation. Silver ions were taken up into gill cells via proton-coupled Na⁺ channels (Bury and Wood, 1999) and may then block the ion transporter Na⁺/K⁺ ATPase. Almofti et al. (2003) found that Ag⁺ toxicity in rat liver mitochondria was caused by an increase of the mitochondrial membrane permeability and the consequent release of cytochrome c, generated by the interaction of Ag⁺ with SH groups of mitochondrial membrane proteins. Ag NPs were also found to cause impairment of mitochondrial function in rat liver cells mainly by changing mitochondrial membrane permeability (Palmeira, 2008). In our study, the cytotoxicity of ZnO NPs was also evidenced by the appearance of morphological changes in HepG2 cells. Loss of normal morphology started to occur in 24 h at 100 mg/l. With a consequent increase in exposure time, cells retracted into spherical shape and formed clusters in media after detachment from the surface. These results were in agreement with the previous reports. For example, it was reported that ZnO-exposed Neuro-2A cells reflect abnormal morphology, cellular shrinkage, and detachment from the surface of the flask at doses >100 µg/ml as well as decreased mitochondrial function and significantly increased LDH at concentrations of 50–100 µg/ml after 24–48 h exposure (Jeng and Swanson, 2006). Meanwhile, our TEM image showed that ZnO NPs accumulated inside the primary cells with breakage in cell membranes (Fig. 4B, 4D). Therefore, we speculate that the breakage of cell and mitochondrial membranes is one of the most important causes of cell death.

5. Conclusions

In conclusion, we found that nano-CuO and ZnO showed measurable toxicity to both HepG2 cells and catfish primary hepatocytes but with some marked difference. The aforementioned nanoparticles showed significant toxicity to HepG2 cells at low concentrations, but required much higher concentrations to show significant toxicity to the fish primary hepatocytes. This clearly demonstrated that HepG2 cells are more sensitive than catfish primary hepatocytes to the toxicity of these metal oxide NPs and suggested that the size-dependent toxicity of NPs is mitigable by other factors. Moreover, the main mechanism of NP cytotoxicity may differ, depending on the types of NPs and cells involved.

The results not only revealed the difference in toxicity of NPs to different cells, but in having done so, may also help us develop rapid and efficient test strategies to assess the impact of these emerging materials on human health and the natural environment. Cell cultures can serve as experimental systems both to assess the potential for toxicity of engineered nanoparticles and to study their mechanisms of toxicity at the molecular and cellular levels by allowing the cells to be studied in a controlled environment in isolation from multiple physiological systems that help determine their fate *in vivo*. Based on this screening study with cell cultures, we are able to propose the possible mechanisms responsible for the observed nanotoxicities. Whether this can be extrapolated to the organismal level or to the natural environment can only be answered by *in vivo* studies with populations of catfish.

Highlights

1. We conducted a nanotoxicity study by using catfish primary hepatocytes and human HepG2 cells.
2. It was studied by using phase contrast inverted microscopy and MTT/ROS/flow cytometric assays.
3. HepG2 cells are more sensitive to the toxicity of metal oxide NPs.
4. The overall ranking of the toxicity of metal oxides is as follows: $\text{TiO}_2 < \text{Co}_3\text{O}_4 < \text{ZnO} < \text{CuO}$
5. The toxicity is caused by ROS-induced cell death and damages to cell and mitochondrial membranes.

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Fig. 1. Phase contrast microscopy light micrographs of HepG2 control cells (A); HepG2 cells treated with 50 mg/l ZnO NPs (B) and 50 mg/l TiO₂ NPs (C) for 48 h; HepG2 cells treated with 25 mg/l CuO NPs (D) and 25 mg/l Co₃O₄ NPs (E) for 48 h.

Fig. 2. Phase contrast microscopy light micrographs of the catfish control primary hepatocytes (A); catfish primary hepatocytes treated with 200 mg/l ZnO NPs (B) and 200 mg/l TiO₂ NPs (C) for 48 h; the catfish primary hepatocytes treated with 200 mg/l CuO NPs (D) and 200 mg/l Co₃O₄ NPs (E) for 48 h.

Fig. 3. MTT activities of HepG2 cells (A) and primary hepatocytes (B) treated with metal oxide NPs of various concentrations for 48 h (replicate number ≥ 4). Figure only reflects the data points of effective concentrations. Histograms labeled with different letters represent significant differences ($p < 0.05$).

Fig. 4.

TEM images of the control cells of catfish primary hepatocytes, (A, 8,000X); the primary hepatocytes treated with ZnO (B, 20,000X) and TiO₂ (C, 60,000X) at the concentration of 200 mg/l for 48 h, respectively; HepG2 cells treated with ZnO (D, 10,000X) and TiO₂ (E, 15,000X) at the concentration of 200 mg/l for 48 h, respectively; and the control HepG2 cells (F, 60,000X). The arrows indicate where the nanoparticles are located.

Treatment with metal oxide of various concentrations

Treatment with metal oxide of various concentrations

Fig. 5.

The accumulation of intracellular ROS in HepG2 cells induced by metal oxide NPs of various concentrations for 24 h (A), 48h (B) and the catfish primary hepatocytes treated with metal oxide NPs of various concentrations for 24 h (C), 48 h (D).

Fig. 6.

Bivariate plots of ZnO-induced effects on the primary hepatocyte cells as determined by flow cytometric assay. Control cells (A); the cells treated with ZnO for 48 h at the concentration of 25 mg/l (B); the concentration of 50 mg/l (C); the concentration of 100 mg/l (D); the concentration of 200 mg/l (E). In each plot, Upper Left (UL) depicts non-viable cells or PI positive cells, Upper Right (UR) depicts apoptotic and necrotic cells or annexin V and PI positive cells, Lower Left (LL) depicts live cells or annexin V and PI negative, Lower Right (LR) depicts annexin V positive cells or early apoptotic cells.

Table 1

Characterization of Metal oxide NPs

Metal Oxide	CAS No.	Batch No. (Lot No.)	Size Range (nm)	Average Size (nm)	Shape	Specific Surface Area (m ² /g)	Purity (%)
Co ₃ O ₄	1308-06-1	2310-071409	51–132 *	78.3 nm *	Rhomboid/quadrate	Unavailable	99.5
CuO	1317-38-0	11118LE	17–45 *	28 nm **	Spherical	33	Analytical Grade
TiO ₂	13463-67-7	07811LE	17–64 *	42.3 nm **	Rhomboid/spherical	35.5	99.9
ZnO	1314-13-2	10822BE	47–106 *	71 nm **	Spherical/rhomboid/rod-shaped	15	Analytical Grade

* Data collected from the TEM experiments

** Data provided by NP supply vendors.

Table 2

ZnO-induced effects on the primary hepatocytes as determined by flow cytometric assay.

Primary cell	Damaged or dead cells	Apoptotic cells	Viable cells	Early Apoptotic Cells
Control	2.11%	1.56%	95.21%	1.07%
25 mg/l ZnO	3.21%	3.32%	88.57%	4.90%
50 mg/l ZnO	12.75%	2.93%	82.5%	1.82%
100 mg/l ZnO	26.13%	3.41%	68.84%	1.62%
200 mg/l ZnO	23.03%	4.46%	71.39%	1.12%