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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 nanometal 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, TiO_2, CuO and Co_3O_4 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: $TiO_2 < Co_3O_4 < ZnO < CuO$. The toxicity is due not only to ROS-induced cell death, but also to damages to cell and mitochondrial membranes.

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). 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 (TiO_2), zinc oxide (ZnO), copper oxide (CuO) and cobalt oxide (Co_3O_4) are some of the most common industrial NP additives for various applications. With greater surface area per unit weight than their bulk counterparts, these nanometal oxides have superior performance. TiO_2 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-Co_3O_4 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* 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 TiO_2 NPs (20–30 nm) in rat tissues following intravenous injection and discovered that they were primarily accumulated in the liver. The level of TiO_2 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

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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 nanometal 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. Nanometal oxides

Metal oxide NPs including ZnO, TiO_2, CuO and Co_3O_4 were purchased from Sigma-Aldrich (St. Louis, MO). Nanoparticle size characterizations were determined with TEM in our lab and are reported along with the 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 min 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 l 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 $MgSO_4$; 0.44 mM KH_2PO_4; 0.33 mM Na_2HPO_4; 5.0 mM $NaHCO_3$, pH 7.6) without Ca^{2+}. 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 $CaCl_2$ 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 hemocytometer 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 IU/ml penicillin, 100 μg/ml streptomycin, 5 mM $NaHCO_3$, and 0.5% ITS (insulin–transferrin–selenium, Gibco). After 24 h, the medium was changed and the cultured cells were ready for the following experiments of NP exposure.

2.3. Human HepG2 cell culture and treatment protocol

The HepG2/2.2.1 (CRL-11997™) 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% CO_2 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 respective wells. 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 μl 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 μM 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 of incubation in 5%

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_3O_4	1308-06-1	2310-071409	51–132[a]	78.3[a]	Rhomboid/quadrate	Unavailable	99.5
CuO	1317-38-0	11118LE	17–45[a]	28[b]	Spherical	33	Analytical grade
TiO_2	13463-67-7	07811LE	17–64[a]	42.3[b]	Rhomboid/spherical	35.5	99.9
ZnO	1314-13-2	10822BE	47–106[a]	71[b]	Spherical/rhomboid/rod-shaped	15	Analytical grade

[a] Data collected from the TEM experiments.
[b] Data provided by NP supply vendors.