Toxic Potential of Food-Relevant Nanoparticles in the Intestinal Epithelium

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ABSTRACT

The food industry is becoming increasingly interested in the use of nanoparticles in food. This study investigated the toxic potential of the food-relevant nanoparticles silica (SiO_2), titanium dioxide (TiO_2), and zinc oxide (ZnO) on intestinal epithelial C2BBe1 cells. Despite nanoparticle internalization by cells, as visualized by TEM, toxicity was not observed after 24-hour exposure of cells to nanoparticles. SiO_2 and TiO_2 nanoparticles were incubated with digestive solutions to mimic gastric and intestinal digestion to which nanoparticles would be exposed *in vivo* (ZnO dissolved and could not be recovered). Exposure of cells to the "digested" nanoparticles also did not cause toxicity. To more accurately simulate consumer exposure to nanoparticles in foods, cells were treated with nanoparticles repeatedly and evaluated for toxicity for up to 40 nanoparticle exposures. Only ZnO caused slight toxicity. Proliferation of cell populations exposed long-term to nanoparticles was unaffected.

Keywords: cytotoxicity, SiO_2, TiO_2, ZnO, food

1 INTRODUCTION

Nanoparticle use in foods has been an interest of the food industry for several years and some food products already incorporate nanomaterials. The food additives SiO_2 and TiO_2, used as anti-caking and whitening agents, respectively, have been shown to contain a nano-sized component [1,2]. Other nanoparticle applications being explored include sensors for bacteria responsible for food spoilage or included in packaging for freshness or antimicrobial properties, and encapsulation to increase bioavailability of nutrients or provide controlled release or flavor masking [3-5].

Despite the increased interest in food applications for nanoparticles, the FDA currently has no specific regulations for the use of nanoparticles in foods. A 2012 draft guidance suggested that food manufacturers investigate the safety of foods incorporating nanoparticles, but as of now many of the inorganic nanoparticles are generally regarded as safe for use in foods [6]. Because of the large surface area of nanoparticles, a much larger proportion of atoms are exposed on their surface than on that of their larger counterparts, which causes nanoparticles to be more reactive [7,8]. Thus, it is imperative to evaluate the effects of ingested nanoparticles before assuming they are safe for consumption.

There is currently incomplete knowledge of the toxicity of food-relevant nanoparticles encountered by the gastrointestinal (GI) tract. The literature to date includes several *in vitro* and *in vivo* studies that describe toxicity in response to food-relevant nanoparticle exposure [9-14]. However, there remains a need for studies conducted using well-characterized nanoparticles, especially investigating the effects of long-term nanoparticle exposure on the intestines.

In the current investigation we tested the hypothesis that nanoparticle toxicity is dependent on particle size, chemistry, and surface charge. Studies were designed to assess potential toxic effects of food-relevant nanoparticles on intestinal epithelial cells in response to both short-term and long-term exposure.

2 METHODS AND RESULTS

These experiments were conducted with commercially available TiO_2, SiO_2, and ZnO nanoparticles. Although food-grade SiO_2 and TiO_2 may be more relevant to foods, using well-characterized nanoparticles provided a way to focus strictly on the nano-fraction of the size distribution in the food-grade materials.

2.1 Nanoparticle Characterization and Toxicity in C2BBe1 cells

SiO_2, TiO_2, and ZnO nanoparticles were characterized by dynamic light scattering to measure particle size and by zeta potential analysis to measure surface charge (Table 1). In cell culture media (DMEM, 10% FBS), TiO_2 had a size of approximately 200 nm, probably due to aggregation, and SiO_2 and ZnO were approximately 100 nm (Table 1).

C2BBe1 cells, originally cloned from the human colon cancer-derived Caco-2 line, were chosen to represent intestinal epithelial cells. Cells were treated with a 10 $\mu g/cm^2$ dose of nanoparticles, centrifuged briefly to promote contact of cells with nanoparticles, and incubated for 24 hours. TEM was performed on treated cells to demonstrate nanoparticle internalization. The images showed that SiO_2, TiO_2, and ZnO were internalized by cells (Figure 1). Many nanoparticles were clustered within membranes, suggesting localization within vesicles (Figure

	TiO_2		SiO_2		ZnO	
	Size	**Charge**	**Size**	**Charge**	**Size**	**Charge**
DMEM/FBS	227 nm	-11 mV	96 nm	- 10.7 mV	112 nm	- 19.3 mV
Pepsin, pH 2	238 nm	+ 14 mV	3039 nm	+ 0.7 mV	**Dissolves**	**Dissolves**
Pancreatin, pH 7	223 nm	- 39 mV	109 nm	-38 mV		
Bile Salts, pH 7	207 nm	- 35 mV	109 nm	- 29 mV		

Table 1: Nanoparticle characterization after digestive treatment
Size and charge of TiO_2, SiO_2, and ZnO nanoparticles in culture medium and digestive enzyme solutions as measured by dynamic light scattering and zeta potential analysis.

1A, 1C), while others appeared free in the cytoplasm (Figure 1B). Following nanoparticle treatment, cellular toxicity was evaluated using several different toxicity assays. Sytox Red is a nuclear dye that binds to DNA, but can only enter cells if the membrane is damaged. Thus, Sytox Red staining and analysis by flow cytometry was used to examine necrotic cell death or late apoptosis in cells. Annexin V binds to phosphatidylserine, which is exposed on the outer surface of cell membranes in apoptosis. Hence, staining with FITC-conjugated Annexin V was used to assay apoptosis. Flow cytometric analysis demonstrated that 24-hour exposure to nanoparticles induced neither necrotic cell death nor apoptosis (Figure 2A, 2B). Necrotic cell death was also evaluated by measuring lactate dehydrogenase (LDH) release from cells into the culture media, which occurs when cell membranes are damaged. The viabilities of cells calculated from measurement of LDH release were again comparable between untreated control and nanoparticle-treated cells, suggesting no induced cell death (Figure 2C).

2.2 *In Vitro* Digestion of Nanoparticles

To simulate the digestion process as would be encountered in the GI tract, particles were sequentially incubated with pepsin (pH 2; stomach), then pancreatin and bile salts (pH 7; small intestine) for one hour each. Particles were characterized by DLS and zeta potential measurements following each treatment (Table 1). While incubation in pepsin at pH 2 caused SiO_2 to lose its surface charge and TiO_2 to become positively charged, both were once again negatively charged after incubation with pancreatin and bile salts. Sizes of both particles were similar to the starting size after pancreatin/bile salt treatment. The strong negative charge (-30 to -40 mV) observed on SiO_2 and TiO_2 nanoparticles after incubation with pancreatin and bile salts may be indicative of protein adsorption to the nanoparticles. ZnO particles dissolved during the pepsin treatment, so they were not subjected to digestive treatments for subsequent studies.

Cells were incubated for 24 hours with "digested" SiO_2 and TiO_2 nanoparticles at a concentration of 10 μg/cm^2, and assayed for toxicity as described above. Flow cytometric analysis demonstrated that 24-hour exposure to "digested" nanoparticles induced neither necrotic cell death nor apoptosis (Figure 2A, 2B). In the LDH assay, digested SiO_2 and TiO_2 consistently showed significantly lower LDH release (higher viabilities; $p<0.01$) than the untreated control cells, which suggests that the digestive solutions may interfere with the assay (Figure 2C).

2.3 Long-term Nanoparticle Exposure in C2BBe1 cells

Nanoparticle incorporation into common foods will lead to repeated exposure of consumers to these nanoparticles. Thus, long-term studies were conducted in which cells were exposed to nanoparticles repeatedly. Cells were plated, treated with nanoparticles for 24 hours, washed, and then

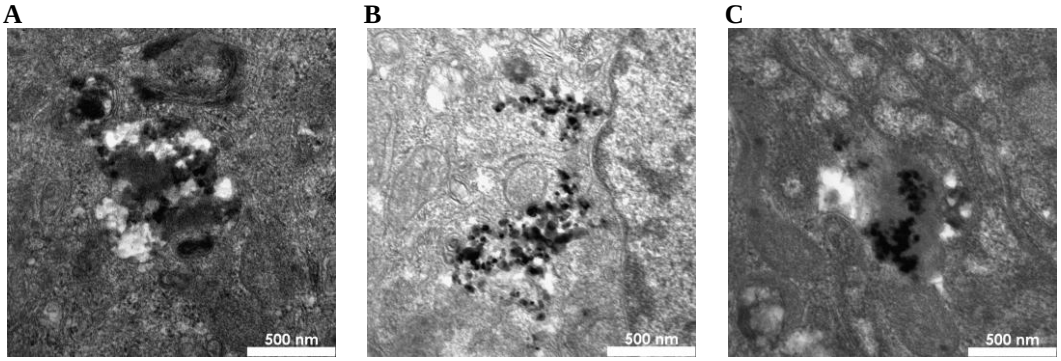

Figure 1: Uptake of nanoparticles by C2BBe1 cells
Representative TEM images of C2BBe1 cells. Cells were treated with nanoparticles for 24 hours before harvest, fixation in glutaraldehyde, and processing for TEM. A) SiO_2-treated cell. B) TiO2-treated cell. C) ZnO-treated cell.

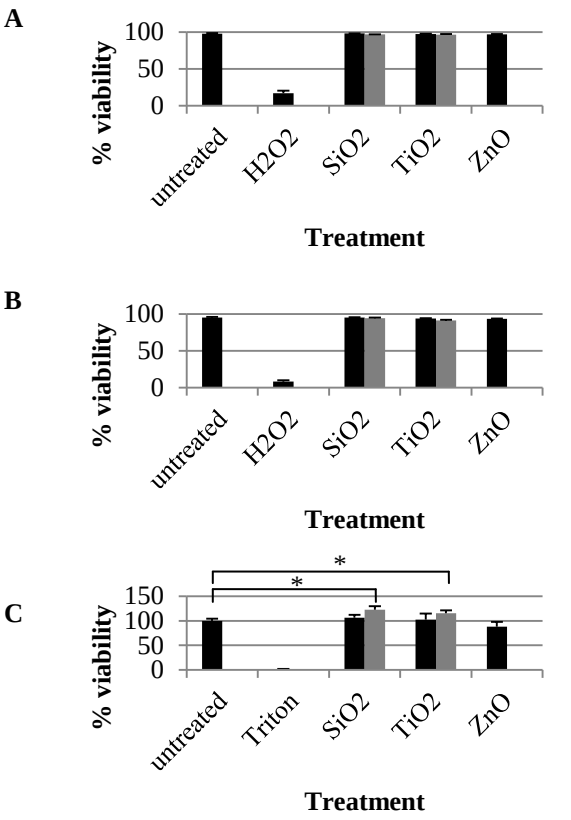

Figure 2: Toxicity of 24-hour nanoparticle treatment on C2BBe1 cells

C2BBe1 cells were treated with nanoparticles for 24 hours before performing toxicity assays. Gray bars represent nanoparticles incubated with digestive enzyme solutions before treating cells. Cells were stained with A) Sytox Red or B) FITC Annexin V and analyzed by flow cytometry. Data are presented as percent viability based on the percent of positively stained cells measured by flow cytometry. C) Toxicity was analyzed by LDH assay. Data were normalized to untreated control cells as 100% viability and Triton X-100-treated cells as 0% viability. Statistical significance was determined by Student's *t*-test ($p<0.01$).

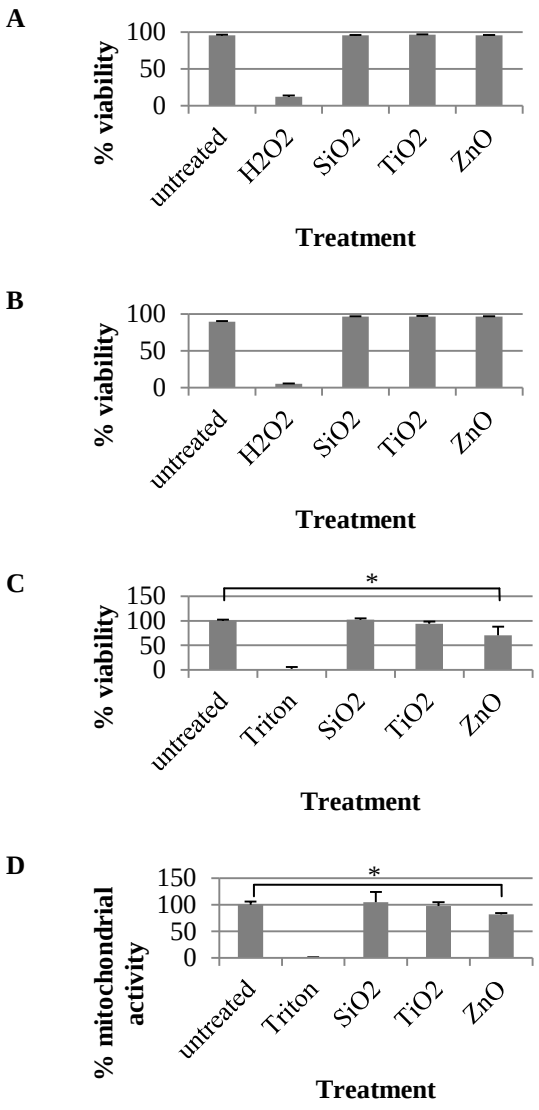

Figure 3: Toxicity of long-term nanoparticle exposure on C2BBe1 cells

C2BBe1 cells were repeatedly exposed to nanoparticles and toxicity was analyzed 24 hours after the most recent nanoparticle treatment. Cells were stained with A) Sytox Red and B) FITC Annexin V, and analyzed by flow cytometry. C) Toxicity was analyzed by LDH assay. Percent viability of cells was normalized to untreated control cells as 100% viability and Triton X-100-treated cells as 0% viability. D) Mitochondrial activity of cells was assessed using the MTT assay. Percent viability of cells was normalized to untreated control cells as 100% viability. Statistical significance was determined using Student's *t*-test ($p<0.01$).

cell culture media was replaced. Cells were grown to confluence, passed into new plates, and re-treated with nanoparticles for 24 hours. This process was repeated after each successive cell passage (approximately weekly). Toxicity was assayed as described above primarily between 20-40 nanoparticle exposure cycles. Cells were evaluated for toxicity directly after 24-hour nanoparticle treatment. Flow cytometric analysis demonstrated that repeated exposure to TiO_2 or SiO_2 induced neither necrotic cell death nor apoptosis (Figure 3A, B). In 3 out of 9 experiments, ZnO treatment caused a decrease to less than 80% viability by Sytox Red staining, suggesting slight cell death induced by ZnO. LDH assay revealed no changes in viability for SiO_2- and TiO_2-treated cells, but a consistent, approximately 30% decrease in cell viability ($p<0.01$) for ZnO-treated cells (Figure 3C). This suggests that ZnO causes slight toxicity in cells treated long-term. The MTT assay was used on the long-term exposure cells to analyze the mitochondrial activity of these cells based on reduction of a tetrazolium dye by mitochondrial enzymes. This assay

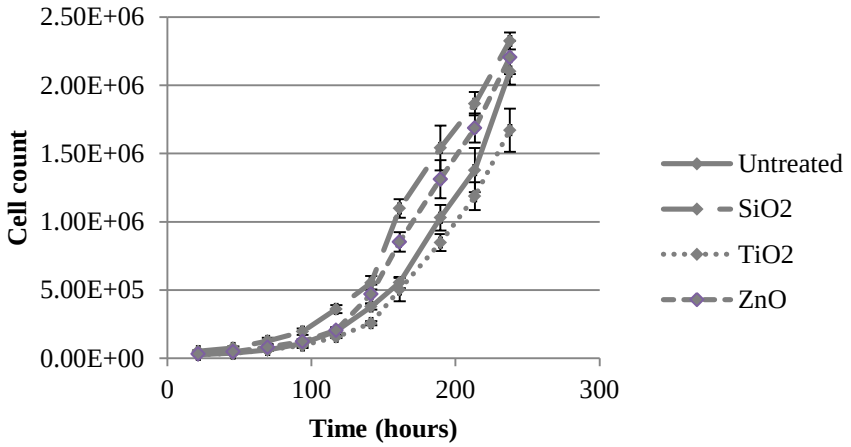

Figure 4: Growth curve after 11 long-term nanoparticle exposure cycles
C2BBe1 cells which had been repeatedly exposed to nanoparticles (11 exposures) were plated at 5×10^4 cells/well at time 0 and counted daily over a period of 10 days using a Beckman Coulter Counter. These data are representative of several curves.

revealed no change in mitochondrial activity after treatment with SiO_2 and TiO_2, but a significant ($p<0.01$), approximately 20% decrease in mitochondrial activity in cells treated with ZnO (Figure 3D). This trend was observed in 5 out of 8 experiments.

To assess the impact of nanoparticle exposure upon cell proliferation, growth curves were plotted with daily cell counts following repeated nanoparticle exposure. Data generated by these experiments demonstrated no changes in proliferation (Figure 4).

3 CONCLUSIONS

Based on the single 24-hour exposure studies as well as the long-term exposure studies, TiO_2 and SiO_2 do not seem to be directly toxic to intestinal epithelial cells. Incubating these nanoparticles with digestive enzyme solutions did not alter the toxicity to the C2BBe1 cells. However, the ZnO did appear to cause slight toxicity to cells based on decreases in cell viability seen with the LDH assay and Sytox Red staining as well as a decrease in mitochondrial activity in the MTT assay. This was especially true after repeated ZnO exposures in the long-term study. These studies suggest that nano-scale SiO_2, TiO_2, and ZnO in foods is not directly toxic to the intestinal epithelium. However, the slight toxicity of ZnO suggests the need for additional study.

Although we did not observe direct nanoparticle toxicity, we did observe nanoparticle internalization by cells. Because one function of intestinal epithelial cells is to absorb nutrients and transport them to the circulation, it is likely that nanoparticles taken up by these cells may likewise be transported across the epithelium and into the circulation. This could lead to nanoparticle accumulation in the liver or other organs and the potential for tissue damage or dysfunction. Thus, the fate of ingested nanoparticles *in vivo* needs to be further investigated.

REFERENCES

[1] Weir A, Westerhoff P, Fabricius L, et al. Environ Sci Technol. 2012;46(4):2242-2250.
[2] Dekkers S, Krystek P, Peters RJ, et al. Nanotoxicology. 2011;5(3):393-405.
[3] Sozer N, Kokini J. Trends Biotechnol. 2009;27(2): 82-89.
[4] Chaudhry Q, Castle L, Watkins R, editors. Nanotechnologies in Food. Cambridge: RSC Publishing; 2010.
[5] Chaudhry Q, Scotter M, Blackburn J, et al. Food Addit Contam. 2008;25(3):241-258.
[6] US FDA Foods Generally Recognized as Safe (GRAS). [online] 2013 [cited 2013 January 31] http://www.fda.gov.
[7] Auffan M, Rose J, Bottero JY, et al. Nat Nanotechnol. 2009;4:634-641.
[8] Nel A, Xia T, Mädler L, et al. Science. 2006;311(5761):622-627.
[9] Gerloff K, Pereira DIA, Faria N, et al. Nanotoxicology. 2012;[epub. online ahead of print].
[10] De Angelis I, Barone F, Zijno A, et al. Nanotoxicology. 2012;[epub. online ahead of print].
[11] Koeneman BA, Zhang Y, Westerhoff P, et al. Cell Biol Toxicol. 2010;26(3):225-238.
[12] Pasupuleti S, Alapati S, Ganapathy S, et al. Toxicol Ind Health. 2012;28(8):675-686.
[13] Onishchenko GE, Erokhina MV, Abramchuk SS, et al. Bull Exp Biol Med. 2012;154(2):265-270.
[14] Wang J, Zhou G, Chen C, et al. Toxicol Lett. 2007;168(2):176-185.