

Zinc Oxide Nanoparticles Induced Apoptosis and Necrosis in Human Neuroblastoma and Astrocytoma Cells

James C.K. Lai¹, Anurag K. Balaraju², Aishwarya Neti³, Maria B. Lai⁴, Satya M. Tadinada⁵, Vinay K. Idikuda⁶, M. Ratan M. Singh⁷, Krishnaveni Mukka⁸, Jean Pfau⁹, Alok Bhushan¹⁰, and Solomon W. Leung¹¹

¹Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, and Biomedical Research Institute, Division of Health Sciences, Idaho State University, Pocatello, ID 83209, USA Fax: 208-282-4305; Tel: 208-282-2275; email: lai@pharmacy.isu.edu

²Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, and Biomedical Research Institute, Division of Health Sciences, Idaho State University, Pocatello, ID 83209, USA Fax: 208-282-4305; Tel: 410-294-3056; email: kakkanur@pharmacy.isu.edu

³Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, and Biomedical Research Institute, Division of Health Sciences, Idaho State University, Pocatello, ID 83209, USA Fax: 208-282-4305; Tel: 832-322-1887; email: netiaish@isu.edu

⁴Department of Biomedical & Pharmaceutical Sciences, College of Pharmacy, and Biomedical Research Institute, Division of Health Sciences, Idaho State University, Pocatello, ID 83209, USA Fax: 208-282-4305; Tel: 208-716-0008; email: Maria.Lai07@gmail.com

⁵Department of Biomedical & Pharmaceutical Sciences, College of Pharmacy, and Biomedical Research Institute, Division of Health Sciences, Idaho State University, Pocatello, ID 83209, USA Fax: 208-282-4305; Tel: 208-282-4408; email: tadisaty@pharmacy.isu.edu

⁶Department of Biomedical & Pharmaceutical Sciences, College of Pharmacy, and Biomedical Research Institute, Division of Health Sciences, Idaho State University, Pocatello, ID 83209, USA Fax: 208-282-4305; Tel: 208-705-2081; email: idikvina@pharmacy.isu.edu

⁷Department of Biomedical & Pharmaceutical Sciences, College of Pharmacy, and Biomedical Research Institute, Division of Health Sciences, Idaho State University, Pocatello, ID 83209, USA Fax: 208-282-4305; Tel: 208-282-2275; email: singrata@pharmacy.isu.edu

⁸Department of Biomedical & Pharmaceutical Sciences, College of Pharmacy, and Biomedical Research Institute, Division of Health Sciences, Idaho State University, Pocatello, ID 83209, USA Fax: 208-282-4305; Tel: 208-282-2275; email: krishna@pharmacy.isu.edu

⁹Department of Biological Sciences, College of Science and Engineering, and Biomedical Research Institute, Idaho State University, Pocatello, ID 83209, USA Fax: 208-282-4305; Tel: 208-282-3914; email: pfaujean@isu.edu

¹⁰Department of Pharmaceutical Sciences, Jefferson School of Pharmacy, Thomas Jefferson University, Philadelphia, PA 19107, USA Fax: 215-503-3082; Tel: 215-503-5039; email: Alok.Bhushan@jefferson.edu

ABSTRACT

Because of their escalating uses in industrial and biomedical applications, humans are increasingly exposed to nanoparticles in occupational and other environmental settings. However, the environmental safety and health impact of such particles have not been elucidated. Previously, we have shown that many nanoparticles of metallic and non-metallic oxides exert cytotoxicity in human and other mammalian neural and non-neural cells. In this study, we have investigated the hypothesis that apoptosis is one mechanism underlying the toxicity of zinc oxide (ZnO) nanoparticles in human neuroblastoma SK-N-SH (neurons-like) and astrocytoma U87 (astrocytes-like) cells. Our results demonstrate the nanoparticles induced dose-related decreases in survival of both cell types. Furthermore, flow cytometric findings reveal that at higher treatment concentrations of 20-50 µg/mL of ZnO nanoparticles, both SK-N-SH and U87 cells were undergoing apoptosis and necrosis. However, significant proportions of SK-N-SH cells were at the late apoptotic state after treatment with the nanoparticles at 5, 10 and 25 µg/mL. Thus, our results strongly suggest both apoptosis and necrosis are cell death mechanisms underlying the differential cytotoxicity of ZnO nanoparticles in human neural SK-N-SH and U87 cells. As such they may have pathophysiological implications in the biocompatibility and health hazard of ZnO nanoparticles.

Key words: zinc oxide nanoparticles, cytotoxicity of zinc oxide nanoparticles, human neuroblastoma and astrocytoma cells, apoptosis & necrosis, biocompatibility, nanotoxicity

1 INTRODUCTION

Different nanoparticles have been increasingly employed in various household and other common industrial applications [1, 2]. Similarly, nanoparticles of metal oxides have also found their way into diverse biomedical applications such as drug delivery, fluorescence tagging to biosensors in robotics and bioengineering [1-6]. As their industrial applications are escalating, humans are more and more exposed to such nanoparticles in industrial and other occupational settings [1-6]. Nevertheless, nanoparticles of metallic and non-metallic oxides are known to exert cytotoxicity in human and other mammalian neural and non-neural cells [3-9].

Previous studies from our group have demonstrated several types of nanoparticles of metallic and non-metallic

oxides exert cytotoxicity in human and other mammalian neural and non-neural cells [3-9]. This study extends our previous work [3-9] as part of a systematic investigation of biocompatibility of nanoparticles of metallic and non-metallic oxides by elucidating the putative cytotoxicity of zinc oxide (ZnO) nanoparticles in neural cells employing human neuroblastoma SK-N-SH and astrocytoma U87 cells as environmental safety and health impact of such particles have not been elucidated.

We hypothesized that apoptosis is one mechanism underlying the putative toxicity of ZnO nanoparticles in these neural cells. This study, therefore, was initiated to investigate this hypothesis.

2 MATERIALS AND METHODS

2.1 Materials

Dulbecco's minimum essential medium (DMEM) for cell growth and other chemicals (usually of analytical grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Zinc oxide (ZnO) nanopowder (from Sigma-Aldrich, St. Louis, MO, USA; Cat. #544906; nanopowder, <100 nm particle size) was suspended in 100 mL of sterile saline in a sealed conical flask and the suspension stirred at ambient temperature overnight before being used to be diluted to the specified concentrations for treatment of cells (see below).

2.2 Cells and Culture Conditions

Human astrocytoma U87 (astrocytes-like) and human neuroblastoma SK-N-SH (neurons-like) cells were obtained from ATCC (Manassas, VA, USA) and were cultured in DMEM, supplemented with 10% (v/v) fetal bovine serum and were incubated at 37°C and 5% (v/v) CO₂ as described previously [3-6].

2.4 MTT Assay

Cellular viability was determined using the modified MTT assay [4]. Cells were seeded at a density of 2,500 or 3,000 cells per well in 96-well plates and allowed to attach to the bottom of each well. Cells were then treated with specified concentrations of ZnO nanoparticles for 48 hours at 37°C. MTT dye (0.5% (w/v) in phosphate-buffered saline) was added to each well and the plates (set one) were incubated for another 4 or 12 hours at 37°C. Purple-colored insoluble formazan crystals in viable cells were dissolved using

dimethyl sulfoxide (DMSO, 100 μ L per well). The absorbance of the content of each well in each plate was then measured at 567 nm using the multi-detection microplate reader (Bio-Tek Synergy HT, Winooski, VT, USA). To prevent ZnO nanoparticles from interfering with this assay (data not shown), the formazan material dissolved in DMSO in each well of each plate was quantitatively transferred to an empty well in another plate (set two) while the material in DMSO from a well with nanoparticles only (i.e., without cells) served as the corresponding control. The absorbance of the contents of each well in each plate (set two) was again measured after transfer using the same method as depicted above [4].

2.5 Other Methods

The assay of lactate dehydrogenase (LDH) release from cells (a marker of necrotic damage or necrotic cell death) was carried out as we had described previously [5,6]. Apoptosis and necrosis were assessed employing a flow cytometer. Cells were treated with specified concentrations of nanoparticles and after 48 hours of treatment, the cells were collected and treated with propidium iodide (PI; which stains necrotic cells) alone, Annexin V (AV; which stains apoptotic cells) alone or with both dyes. The distribution of apoptotic and necrotic cells were determined based on the scatter plots obtained from the flow cytometer.

2.6 Statistical Analysis of Data

Results are presented as mean \pm standard error of the mean (S.E.M.) of 6 determinations in each experiment. Replicate experiments showed essentially the same patterns of results. Data analysis was carried out by one-way ANOVA, followed by Tukey test for multiple comparisons using the software KaleidaGraph version 4 (Synergy Software, Reading, PA, USA). Significance level was set at $p < 0.05$.

3 RESULTS AND DISCUSSION

Table 1.		IC ₅₀
Nanoparticles	U87 cells	SK-N-SH cells
ZnO	~ 12 μ g/ml	~ 20 μ g/ml

Employing the MTT assay, we compared the effects of treatment of human astrocytoma U87 (astrocytes-like) and human neuroblastoma SK-N-SH (neurons-like) cells with ZnO nanoparticles at 0.1-100 μ g/ml. Treatment with ZnO nanoparticles induced concentration-related decreases in survival of both cell types at concentrations higher than 5 μ g/ml (data not shown). The concentrations of ZnO nanoparticles that induced 50% decreases in survival in U87 and SK-N-SH cells (i.e., the IC₅₀ values) were, respectively, ~ 12 and 20 μ g/ml (Table 1). These findings

suggested that the nanoparticles were somewhat more potent in lowering the survival of U87 cells than that of SK-N-SH cells.

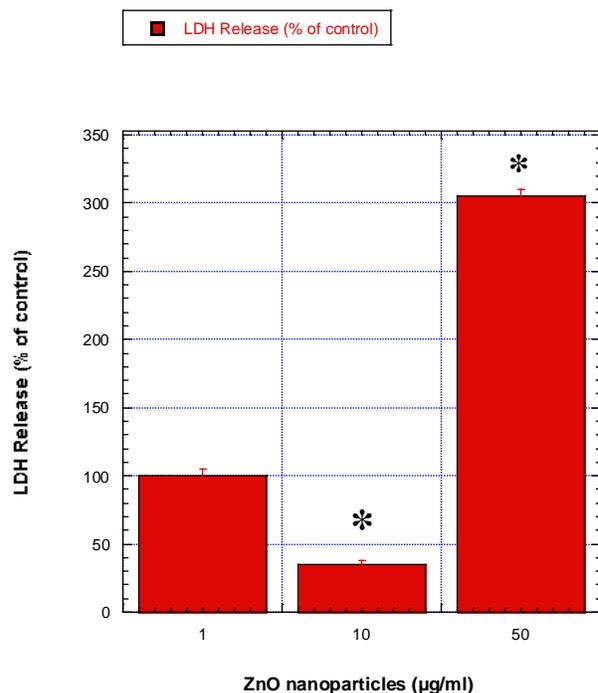


Figure 1: Treatment with ZnO nanoparticles on lactate dehydrogenase (LDH) release from human astrocytoma (astrocytes-like) U87 cells into the medium. U87 cells were treated with ZnO nanoparticles at the specified concentrations for 48 hours; at the end of the specified time, the medium was collected and kept frozen at -70°C until they were used to assay the LDH activity therein. The LDH released into the medium was normalized with respect to that obtained from control (i.e., the untreated U87 cells). Values are the mean \pm S.E.M. of at least three separate determinations; * $p < 0.05$ versus control.

Treatment with ZnO nanoparticles at concentrations of higher than 10 μ g/ml induced increases in LDH release from U87 cells into the culture medium, suggesting that the treatment resulted in necrotic damage and/or necrotic cell death in the treated cells (Fig. 1). However, this effect of the nanoparticles on SK-N-SH was much less marked compared to those in U87 cells and was only detected in SK-N-SH cells treated with the nanoparticles at 50 μ g/ml (data not shown), suggesting the necrotic effect of the nanoparticles in SK-N-SH cells was less pronounced than those in U87 cells with the same treatments.

To further delineate the mode of cell death induced by ZnO nanoparticles in the two neural cell types, we stained the nanoparticles-treated U87 and SK-N-SH cells with propidium iodide (PI) or Annexin V (AV) alone or in combination and then analyzed the stained cells with a flow cytometer. The distributions of apoptotic and necrotic cells

as determined based on the scatter plots obtained from the flow cytometer (data not shown) allowed us to draw the following conclusions. The flow cytometer data demonstrated that after treatment with ZnO nanoparticles at 10 and 25 µg/ml, the majority of U87 cells were exhibiting necrosis, a finding consistent with the internalization of the nanoparticles. While significant proportions of U87 cells were at the late apoptotic state after treatment with ZnO nanoparticles at 25 and 50 µg/ml, significant proportions of SK-N-SH cells were at the late apoptotic state after treatment with ZnO nanoparticles at 5, 10 and 25 µg/ml. Thus, these results strongly suggested that ZnO nanoparticles exerted some differential cytotoxic effects on the two human neurotumor cell types investigated.

4 CONCLUSIONS

Treatment with zinc oxide (ZnO) nanoparticles induced concentration-related decreases in survival of human astrocytoma U87 (astrocytes-like) and neuroblastoma SK-N-SH (neurons-like) cells at concentrations from 5-100 µg/ml, with IC₅₀ values of ~12 and 20 µg/ml, respectively. Treatment with these nanoparticles, especially at levels higher than the IC₅₀ values, also induced both cell types to release lactate dehydrogenase into the medium indicating the presence of necrotic damage induced by the nanoparticles. Results from flow cytometric studies revealed that after treatment with ZnO nanoparticles at 10 and 25 µg/ml, the majority of U87 cells were exhibiting necrosis, a finding consistent with the internalization of the nanoparticles. While significant proportions of U87 cells were at the late apoptotic state after treatment with ZnO nanoparticles at 25 and 50 µg/ml, significant proportions of SK-N-SH cells were at the late apoptotic state after treatment with ZnO nanoparticles at 5, 10 and 25 µg/ml. These results strongly suggested that ZnO nanoparticles exerted differential cytotoxic effects on the two human neurotumor cell types investigated. Thus, our results may assume pathophysiological importance in determining how exposure to ZnO nanoparticles may impact on the structure and function of neural cells, especially because there is evidence that nanoparticles readily penetrate the blood-brain barrier.

5 ACKNOWLEDGEMENTS

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