HeLa Cells' Interaction with Nanoparticles Zinc Oxide and Titanium Dioxide

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Interest in nanoparticles has grown significantly as concerns of their potential toxicity to human cells and aquatic life have been raised. Harmful impacts of engineered nanoparticles are of great interest as they are used in many common products from pharmaceuticals to sunscreen. Nanoparticles are materials with a diameter less than 100 nanometers, which makes their penetration of cell membranes quick and efficient. This work explored the impacts of zinc oxide (ZnO) and titanium dioxide (TiO_2) nanoparticles on living HeLa cells. The goal of the experiment was to determine whether cell mortality would increase with increasing nanoparticle concentration, and if a crucial nanoparticle concentration exists that results in increased cell mortality. Cells were prepared, cultured, subcultured, and analyzed observing strict aseptic technique. After one month of successful HeLa cell replication, ZnO and TiO_2 nanoparticle dispersions were prepared at a range of dilutions and introduced to the HeLa cells. The nanoparticles' effects on cells were established using hemocytometry to determine cell viability and cell mortality. The results suggest that cell mortality increased monotonically with higher concentrations of TiO_2. In contrast, lower concentrations of ZnO increased cell viability, while increased cell mortality was observed at the higher concentrations of ZnO. Given that results with ZnO were less conclusive, more research is needed to address the impact of ZnO on HeLa cells. Future studies will aid in understanding the implications of nanoparticles on living cells.

Keywords: Nanoparticles, HeLa Cells, HeLa Cells and Nanoparticles, Titanium Dioxide and HeLa Cells, Zinc Oxide and HeLa Cells, Health Implications and Nanoparticles, Nanoparticle Effects, Environmental Impacts and Nanoparticles, Toxicity of Engineered Nanoparticles

Introduction

Interest in nanoparticles and their effect on aquatic life and human cells has led to increased research on the environmental toxicity of nanoparticles [1]. Nanoparticles are defined as particulate materials with a diameter between one and 100 nanometers and may contain a wide variety of compounds, including metals, inorganic oxides, and organic materials. While some nanoparticles occur naturally, most concern is centered on the environmental health impacts of engineered nanoparticles that are commonly used in a wide range of products, from industrial coatings to cosmetics to pharmaceuticals [2]. Two widely used engineered nanoparticles are titanium dioxide (titania, TiO_2) and zinc oxide (ZnO); these materials are frequently used in toothpaste, sunscreen, and various types of paints and plastics [3]. Nanoparticles can reflect and disperse UV light making them ideal for use in sun protection products [3]. Nanoparticle titania has further uses in orthopedic implants, oncological medicines, the manufacturing of electronics, and water decontamination. According to Brandão et al. [4], the International Agency for Research on Cancer (IARC) classifies TiO_2 as a Group 2B carcinogen, considered "possibly carcinogenic to humans." TiO_2 specifically has been seen to increase free radical production and tissue inflammation [4].

Past research involving nanoparticles has centered on using phagocytes, macrophages, monocytes, and dendritic cells as models for understanding nanoparticle effects on human cells [5]. According to Brandão et al. [4], the following four human cell lines are good models for genotoxicity research: neurons, lung cells, glial cells, and liver cells. In previous studies, human cell lines of the respiratory system were the focus of experimentation, as concern about nanoparticle toxicity originally focused on risks due to

inhalation [4]. In more recent studies, scientists have observed how nanoparticles enter human cells after being ingested orally or applied topically [6]. Human cell lines derived from lung, brain, kidney, spleen, and liver tissue are becoming central to nanoparticle genotoxicity research as understanding of nanoparticle toxicity increases [4].

Liposomes are another important and influential human cell model used to investigate the health impacts of nanoparticles. According to Wang and Wang [7], the liposome was the first platform used to study human cell interaction with nanoparticles. Liposomes are widely used in research because therapeutic drugs can easily enter their outer membranes [7]. Copper nanoparticles in conjunction with serum albumin have been widely successful for use in chemotherapy treatments used to target aggressive breast cancer [8]. Copper nanoparticles are ideal for use in oncological medicines because of their ability to enter and disrupt cancer cells with negligible harm to healthy cells [8]. Nanoparticles are also being used to detect pathogens (surface antigens on invaders are detected through the magnetic properties of nanoparticles) and for passive targeting in cancer treatments, a technique where tumors are identified through nanoparticle collection in cancerous tissue [7].

The small scale of nanoparticles makes their penetration of cell membranes fast and efficient, making them preferred for use in nanomedicine and biomedical treatments. For example, nanoparticles are widely used to treat aggressive glioblastoma brain cancer cells [3], [9]. Nanoparticles are used to treat transmissible disease and illnesses associated with the cardiovascular and respiratory systems [5]. According to McMillan et al. [5], nanoparticles have been successful used as medicinal implants and in the restoration of damaged tissue and joints. They are also increasingly useful for identifying issues within the central nervous system.

Of particular interest in studying nanoparticle-cell interaction are HeLa cells. HeLa cells have been used in many experiments involving nanoparticles as they provide an excellent human cell model for conducting research [10]. HeLa cells are an immortalized human cell line derived from cervical cancer cells that are widely used in biomedical research. HeLa cells are currently being used in research settings to create a vaccine for HIV and have contributed to many scientific advances in understanding leukemia, AIDS, and various types of cancer [11]. HeLa cells proved instrumental in developing a vaccination for polio [12] and in better understanding immune system activity after vaccination for COVID-19 [13].

This work investigated the effect of titania and zinc oxide nanoparticle dispersions on HeLa cell cultures in vitro. The experiment examined the cell toxicity of different concentrations of TiO_2 and ZnO. A goal of the experiment was to determine whether cell death would increase with increasing nanoparticle concentration, and whether there was a critical nanoparticle concentration of either TiO_2 or ZnO which indicated the onset of cell mortality.

Experiment

Research was performed in the biosafety level 2 labs of the University of Minnesota Nano Center from January 2022 to May 2022. Biosafety level 2 (BSL-2) containment was required for protection against human disease agents and human papilloma virus associated with HeLa cells.

Materials and Equipment

The He-La cell line employed in this study was obtained from the American Type Culture Collection (ATCC). Cells were kept frozen at -196°C for ongoing use in the Nano Center lab until harvested and cultured. The HeLa cells used in the study were derived from this in-house frozen stock and were thawed using a warm water bath. Cells were then cultured in a CO_2 incubator (Thermo Scientific) using T75 culture flasks. Cell subcultures were transferred to 6-well plates with 2 mL wells for nanoparticle exposure and cell imaging. Separation of cell suspensions was done using a Beckman XR-30 refrigerated centrifuge. All HeLa cell handling was carried out in a BSL-2 biosafety cabinet. Nanoparticle dispersions of zinc oxide (ZnO) and titania (TiO_2) were obtained from Sigma Aldrich (St. Louis, MO) and were diluted to achieve physiological pH and a low nanoparticle concentration, as described below. Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS), obtained from Fisher Scientific, were the chosen growth media. Washing and dilution was carried out using 1 X phosphate buffered saline (PBS). Trypsin was used to separate cells adhered to the culture flasks during subculturing. Trypan blue was the staining agent used for hemocytometry and cell counting.

For nanoparticle dilution preparation and administration, a pH probe and six 50 mL sealed test tubes for dilution storage were used. A Leica DMI series inverted microscope with Leica imaging software were utilized for cell analysis, cell observation, cell photography, and cell counting. Hemocytometry slides, coverslips, and worksheets to perform desired calculations were used for cell counting.

Methods

Cell Thawing and Cell Preparation

Complete growth media was prepared using DMEM + 10% FBS and warmed to 37°C in a water bath. Two vials of HeLa cells were removed from the liquid nitrogen storage and thawed to 37°C observing proper sterilization and aseptic handling techniques. The thawed cell suspensions were transferred into 15 mL sterile centrifuge tubes, into which 10 mL of complete growth media was slowly introduced. The cell suspension was spun for three minutes at 500 rpm and the supernatant was removed in the biosafety cabinet. The concentrated cell contents were placed in two separate T75 flasks, and 15 mL of complete growth media was added to each flask. The flasks were incubated at 37°C with 5% CO_2. Confluence checks were performed every 48-72 hours. The HeLa cell cultures grew for six days before confluence was high enough (75% +) for the first media change.

Well Plate Preparation

A solution of DMEM + 10% FBS, PBS, and trypsin was warmed to 37°C. The DMEM/FBS solution was removed from each T75 flask with a 10 mL pipette and discarded. The cells were then washed with 3 mL of PBS to remove the FBS, which acts as a trypsin inhibitor. Three mL of trypsin were added to each flask and incubated for 10 minutes at 37°C to detach the HeLa cells from the surface of each flask. After incubation, 7 mL of complete growth media was added

to the flask. A micropipette was used to remove a small volume of the suspended cells from the T75 flask. The pipetted cells from the T75 flask were then placed into each well of a fresh 6-well plate, along with 2 mL of complete growth media. The well plates were rocked gently back and forth to mix components and were incubated at 37°C for 48-72 hours.

Cell Culture Maintenance

Cell cultures in the T75 flasks and 6-well plates were checked biweekly to monitor cell viability and to calculate confluence. Cells were also carefully monitored to ensure that no contamination had occurred. Each time confluence reached approximately 75%, a media change was performed, as follows. A solution of DMEM/FBS and PBS was brought to 37°C. The spent growth media from each T75 flask or 6-well plate was removed and added to the biowaste container housed in the biosafety cabinet. The remaining cells were washed one to two times with 0.5 mL PBS (for the 6-well plates) or 3 mL PBS (for the T75 flasks). The PBS rinse was discarded and growth media was added to each 6-well plate (2 mL) and each T75 flask (15 mL). The refreshed cultures in the plates or flasks were incubated at 37°C until confluent, after which they were subcultured to allow for controlled growth.

ZnO and TiO_2 Dispersion Preparation

The initial pH values of the ZnO and TiO_2 dispersions were measured using a Mettler pH probe. As received, the ZnO and TiO_2 dispersions were pH 8.318 and pH 3.428, respectively. Due to the fact that these pH values were outside the physiological pH of 7.0, it was determined adverse effects on HeLa cells could occur. To mitigate the influence of unwanted variables, the dispersions were diluted to a pH of 7.0 to limit unwanted cell damage. Dilutions were kept in sealed 50 mL test tubes and refrigerated for use throughout the study. The 50 mL test tubes were rocked back and forth before each application of use to ensure even distribution of nanoparticles when introduced to HeLa cells. The diluted dispersions of ZnO and TiO_2 were prepared at three different concentrations. (See Table 1.)

Nanoparticle	Nanoparticle Mass	Media Volume
ZnO (x 1)	0.5 µg	26.999 mL DMEM + 10% FBS
ZnO (x 3)	1.5 µg	26.991 mL DMEM + 10% FBS
ZnO (x 5)	2.5 µg	26.998 mL DMEM + 10% FBS
TiO2 (x 1)	0.5 µg	26.995 mL DMEM + 10% FBS
TiO2 (x 3)	1.5 µg	26.999 mL DMEM + 10% FBS
TiO2 (x 5)	2.5 µg	26.997 mL DMEM + 10% FBS

Table 1: Nanoparticle ZnO and TiO_2 dispersion preparation with nanoparticle mass (µg) in relation to media volume of DMEM/FBS.

The HeLa cell cultures in the 6-well plates were checked for confluence before adding the diluted nanoparticle dispersions. The existing growth media in the 6-well plates was removed and the cells were washed one to two times with 0.5 mL PBS. Two mL of each dispersion listed in Table 1 was added to each well in each 6-well plate. The cell-nanoparticle mixtures were then incubated at 37°C for four days to allow for growth.

Cell Characterization

The cell cultures treated with nanoparticle dispersions were imaged every four days using a Leica DMI inverted microscope with Leica Application Suite (LAS) software. The images were used as a visual indicator of cell viability and cell mortality.

Cell culture viability and mortality were measured using hemocytometry. To analyze cultures from the T75 flasks and 6-well plates, the old growth media was removed and the cells were washed with PBS. Trypsin was added to the cultures in each flask or well and the trypsinized cells were incubated for 10 minutes at 37°C to detach the cells from the flask or well surfaces. New complete growth media was then pipetted into each flask or well. The complete growth media and trypsinized cells were transferred to a microcentrifuge tube along with 100 µL of the Trypan blue staining agent and gently agitated. A small volume (15-20 µL) of this mixture was transferred from the microcentrifuge tube to a hemocytometry slide. A coverslip was placed over the hemocytometry slide to seal the sample, and the slide was placed on the stage of the inverted microscope. Cell counting was performed manually to quantify dead and viable cells.

Based on hemocytometer images collected with the microscope, the following metrics were obtained: number of viable cells, number of dead cells, percent viability, number of cells per square, and cell concentration (cells/mL). Calculations dictated the amount of growth media and trypsinized cells (in µL) to be added to each well of a 6-well plate after trypsinization in preparation for exposure to nanoparticles. The calculation sought to determine V_1 (the amount of trypsinized cells to be added to each well for exposure to nanoparticles) using the following calculation: $V_1 = C_2V_2/C_1$. The number of viable cells (the number of living cells counted through hemocytometry) was assigned the initial concentration (C_1). The final concentration was held constant across trials at 200 cell/mL (C_2). The final volume of growth media and trypsinized cells was also held constant at 2000 µL (V_2). With these three known variables, the volume of growth media + trypsinized cells to be added to each well (V_1) was established. After V_1 was determined and the proper amount of cell suspension added to each well, 2 mL of fresh growth media were then pipetted into each well and incubated at 37°C for 48 hours. Subculturing of the T75 flask was then performed through removing 9.5 mL of the growth media and trypsinized cells. A fresh 14.5 mL of new growth media was then added to the T75 flask and the flask was incubated at 37°C for 48 hours.

Other calculations were also performed following hemocytometry to determine percent viability. Assessment of total viable cells and total non-viable cells dictated the percent viability through a simple calculation: total viable cells/total cells x 100%. (The total cells equaled the number of viable and non-viable cells combined.) The average number of cells per square of each hemocytometer slide, the dilution factor, and the total concentration of cells per T75 flask or 2 mL well were also found using the previously cited calculations to reach conclusions.

Results

HeLa cells were exposed to ZnO and TiO_2 dispersions through identical trials over eight weeks. Hemocytometry and cell counting were conducted biweekly after HeLa cell exposure to nanoparticle dispersions. Preliminary results suggest that increasing concentrations of TiO_2 increased cell mortality monotonically. Percent viability of HeLa cells exposed to TiO_2 concentrations decreased from 54.69 % under 0.5 μg of TiO_2 to 46.56 % under 1.5 μg of TiO_2 and to 38.46 % under 2.5 μg of TiO_2. Concentrations of ZnO at 1.5 μg *increased* cell viability (62.79 % viability) while the highest concentration of ZnO at 2.5 μg increased cell mortality (49.73 % viability). Concentrations of 0.5 μg of ZnO had little effect on HeLa cells with a percent viability of 54.67 %, mirroring the percent viability of HeLa cells exposed to 0.5 μg of TiO_2. (See Table 2 for a graphical representation of results.)

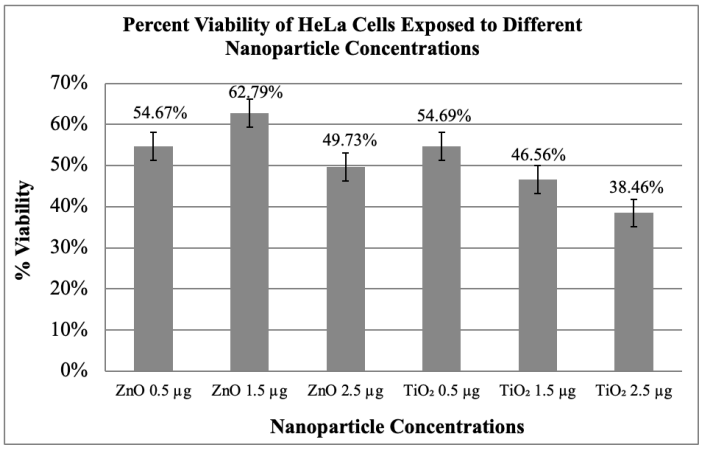

Table 2: HeLa cell viability under 0.5 μg ZnO, 1.5 μg ZnO, 2.5 μg ZnO, 0.5 μg TiO2, 1.5 μg TiO2, and 2.5 μg TiO2.

On week five of the study, a large cluster of cells were observed, indicating cell splitting of the HeLa cells or a possible increase in viability for the cells exposed to the ZnO and TiO_2 nanoparticles. Large masses of HeLa cells were observed under the microscope again at week six, indicating further cell aggregation. Outside of cell splitting or increases in viability, it is possible that the HeLa cells were disturbed during transfer to the incubator, as abrupt agitation can cause cell clumping. No definitive conclusion was reached concerning the aggregated cells. At the end of the study, a positive control treatment tested the impact of actinomycin D on HeLa cells. As expected, actinomycin D caused complete cell death of the HeLa cells.

Discussion

The study sought to quantify the effects of ZnO and TiO_2 nanoparticles on HeLa cells. The objective of the experiment was to determine whether cell death would increase with increasing nanoparticle concentration, and whether there was a critical nanoparticle concentration of either TiO_2 or ZnO which indicated the onset of cell mortality. The experiment showed that exposure of HeLa cells to TiO_2 had a significant toxic impact on the cells in the study, while applications of ZnO affected HeLa cells to a lesser degree. Cell mortality increased monotonically in the presence of increasing TiO_2 concentrations, but no such trend was observed for cell exposure to ZnO. The latter finding suggests the need to explore a wider range of ZnO concentrations through research, with the goal of establishing the relationship (or lack thereof) between ZnO nanoparticle exposure and HeLa cell mortality. Specifically, more studies are needed to understand the counterintuitive increase in cell viability of HeLa cells exposed to ZnO concentrations at approximately 1.5 μg.

The observed increase in HeLa cell mortality at higher levels of TiO_2 suggests the need to investigate whether TiO_2 nanoparticles may have carcinogenic effects on human cells or human tissue. Likewise, observed increases in HeLa cell mortality at the highest levels of ZnO nanoparticle exposure also indicates that future research needs to determine whether there might be a threshold value of zinc oxide nanoparticle exposure above which cell mortality accelerates.

This study highlighted only two of the many nanoparticle formulations that are used in industry and consumer products, and to which workers, consumers, and the environment are being exposed to regularly. Zinc oxide and titanium dioxide were deemed a practical choice in beginning research on nanoparticle toxicity due to the fact that concerns of the impact of ZnO and TiO_2 nanoparticle exposure are already established and growing in the scientific and medical fields. However, research is required to identify the implications of biological exposure to all types and compositions of nanoparticles. Future studies that utilize zinc oxide, titanium dioxide, and other common nanoparticles will aid to understand the comprehensive consequences of nanoparticles on human cells, human tissue, and ultimately, the environment.

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*The isolation and immortalization of HeLa cells changed the scientific landscape in the 20th century. HeLa cells, named for Henrietta Lacks, were first collected by two Johns Hopkins researchers in February, 1951, and through the efforts of later researchers, were established as a standard for cell laboratory propagation and studies [14]. The original HeLa cells were obtained from 31-year-old patient Henrietta Lacks, who was being treated for cervical cancer at Johns Hopkins Hospital [10]. The cells from Henrietta Lacks were obtained without her knowledge, as permission was not required for cell harvesting in 1951. Henrietta Lacks later died from the cancer on October 4, 1951.

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