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Angie S. Morris
University of Iowa

Brittany E. Givens
University of Kentucky, brittany.givens@uky.edu

Aaron Silva
University of Iowa

Aliasger K. Salem
University of Iowa

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Copper Oxide Nanoparticle Diameter Mediates Serum-Sensitive Toxicity in BEAS-2B Cells

*Angie S. Morris, Brittany E. Givens, Aaron Silva, and Aliasger K. Salem**

Copper oxide (CuO) nanoparticles (NPs) are abundant in manufacturing processes, but they are an airway irritant. In vitro pulmonary toxicity of CuO NPs has been modeled using cell lines such as human bronchial epithelial cell line BEAS-2B. In 2D in vitro culture, BEAS-2B undergoes squamous differentiation due to the presence of serum. Differentiation is part of the repair process of lung cells in vivo that helps to preserve the epithelial lining of the respiratory tract. Herein, the effects of serum on the hydrodynamic diameter, cellular viability, cellular differentiation, and cellular uptake of 5 and 35 nm CuO NPs are investigated, and the mean cell area is used as the differentiation marker for BEAS-2B cells. The results demonstrate that the hydrodynamic diameter decreases with the addition of serum to the culture medium. Serum also increases the mean cell area, and only affects dose-dependent cytotoxicity of 35 nm CuO NPs, while simultaneously having no effect on intracellular Cu^{2+}. This study presents evidence that both NP size and the presence of serum in culture media influence the relative viability of BEAS-2B cells following CuO NP exposure and highlights a critical need for carefully designed experiments and accurately reported conditions.

1. Introduction

Nanoparticles (NPs) are abundantly used in commercial processes and consumer products. Among the most common NPs are CuO NPs, which are used in cosmetics, sensors, catalysis, and antimicrobials.[1,2] CuO NPs belong in the metal oxide NP class, which are known to readily dissociate into their component ions in aqueous media.[3–6] Copper dust causes toxicity when inhaled, and there are reports that copper in cigarette smoke causes inflammatory responses in the lungs.[7] The ability for CuO NPs to elicit the same response as copper dust is still under investigation. Previous research findings include that CuO exposure can induce reactive oxygen species and induce double strand breaks in DNA, which may lead to cancer.[6,8–11]

Although CuO NP toxicity has been widely studied in vitro, discrepancies between studies still exist. These discrepancies result from the cell line, cell culture method, and NP dissociation into ions.[1,6,9,12–18] The most common cell line used for toxicological investigations is A549 cells, which proliferate quickly and are an established lung toxicity model.[4,15,18,19] A549 cells were derived from an adenocarcinoma of alveolar cells. In contrast, BEAS-2B bronchial epithelial cells were obtained from a noncancerous source and are therefore considered a strong alternative to A549 cells for toxicity testing. BEAS-2B cells readily undergo squamous differentiation in the presence of as little as 2% serum, and therefore must be cultured in media that contain little to no fetal bovine serum (FBS).[20,21] Even so, many studies with

Dr. A. S. Morris, Prof. B. E. Givens, A. Silva, Prof. A. K. Salem
Department of Pharmaceutical Sciences
College of Pharmacy
University of Iowa
115 S. Grand Avenue, S228 PHAR, Iowa City, IA 52242, USA
E-mail: aliasger-salem@uiowa.edu

Dr. A. S. Morris, Prof. A. K. Salem
Department of Chemistry
College of Liberal Arts and Sciences
University of Iowa
115 S. Grand Avenue, S228 PHAR, Iowa City, IA 52242, USA

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/anbr.202000062.

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Prof. B. E. Givens, Prof. A. K. Salem
Department of Chemical and Biochemical Engineering
College of Engineering
University of Iowa
115 S. Grand Avenue, S228 PHAR, Iowa City, IA 52242, USA

Prof. B. E. Givens
Department of Chemical and Materials Engineering
College of Engineering
University of Kentucky
Lexington, KY 40506, USA

A. Silva, Prof. A. K. Salem
Roy J. Carver Department of Biomedical Engineering
College of Engineering
University of Iowa
115 S. Grand Avenue, S228 PHAR, Iowa City, IA 52242, USA

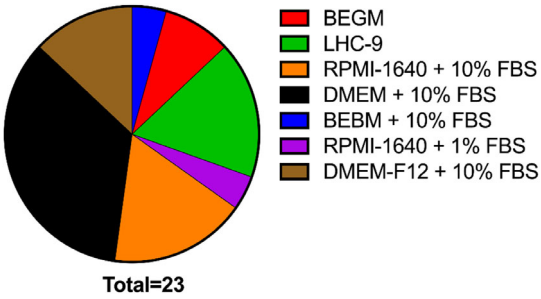

Figure 1. Media used for BEAS-2B NP investigations in 2019. Obtained from PubMed search of "Beas-2B" and "Nanoparticle". BEGM: bronchial epithelial growth medium; RPMI: Roswell Park Memorial Institute; DMEM: Dulbecco's Modified Essential Medium; BEBM: bronchial epithelial basal medium.

BEAS-2B cells and nanotoxicity utilize over 2% serum in the cell culture medium, potentially causing conflicting results (**Figure 1**).[22–44]

In the current study, we sought to address these discrepancies with NP toxicity in BEAS-2B cells by utilizing CuO NPs of two different sizes and varying amounts of FBS in the cell culture media. We investigated if these discrepancies were a result of changes in hydrodynamic diameter or differences in intracellular uptake of CuO NPs due to the presence of serum. All studies were conducted with LHC-9 medium, which was designed specifically for BEAS-2B cell culture.[21] We also used the mean cell area as a differentiation marker by culturing cells in LHC-9 medium containing FBS independent of CuO NP exposure. Through these investigations, we noted that there are NP size–dependent effects on cell viability and NP uptake as well as serum effects on the aforementioned parameters.

2. Results

2.1. Serum Influenced NP Size Distributions in Aqueous Solution

Two differently sized CuO NPs were characterized using transmission electron microscopy (TEM) and X-ray diffraction (XRD) for size and elemental analysis. These CuO NPs were also characterized in serum-free and serum-containing media using dynamic light scattering and laser Doppler velocimetry. The manufacturer specifications for the CuO NPs reported the sizes as 4 and 24 nm; however, micrographs and ImageJ analyses revealed that the measured particle sizes were 5 ± 1 and 35 ± 19 nm, respectively (**Figure 2**).

CuO NP aggregation in the cell culture medium with varying concentrations (%v/v) of FBS was assessed with data from a Malvern Zetasizer (**Table 1**). The hydrodynamic diameters of small (5 nm) versus large (35 nm) NPs in LHC-9 medium alone (0% FBS) were significantly different from one another ($p < 0.0001$), with values indicating that particles of 5 and 35 nm have different aggregation behavior in the cell culture medium. The same was true when 10% FBS was present in the LHC-9 medium ($p < 0.05$).

The 5 nm NPs had significantly different hydrodynamic diameters ($p < 0.01$) between 0% FBS and 10% FBS, but none of the

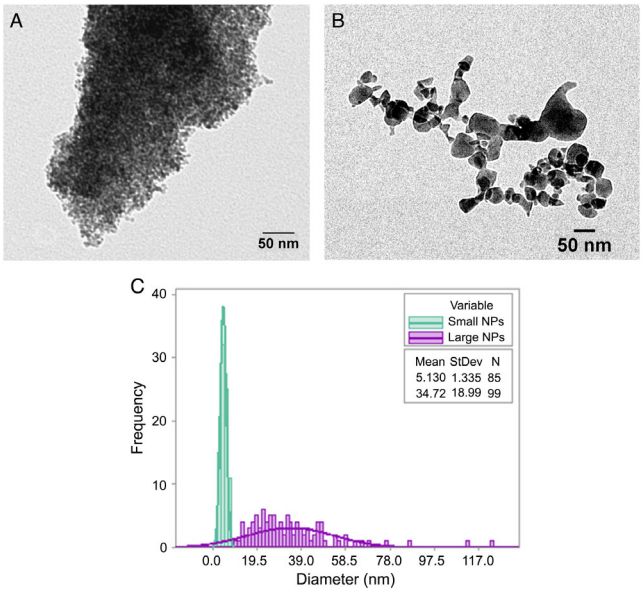

Figure 2. TEM images of A) 4 nm and B) 24 nm CuO NPs and C) their size distribution. The size distribution was obtained by analyzing images using ImageJ software. Upon obtaining these results, it was discovered that the 4 nm particles had a mean size of 5 nm and 24 nm particles had a mean size of 35 nm. These measured mean sizes are used for reference in this article. This figure has been previously published as part of a Ph.D. dissertation. Reproduced with permission.[58] 2017, ProQuest.

smaller incremental changes were significantly different. The aggregate diameter had an inverse relationship with the FBS concentration. As FBS increased, the hydrodynamic diameter decreased. These diameters ranged from 15 times (10% FBS) to 65 times (0% FBS) the diameter of an individual particle. The 35 nm NPs had significantly lower hydrodynamic diameters ($p < 0.05$) between 0% FBS and all other FBS concentrations. Unlike the 5 nm NPs, the 35 nm NPs decreased in hydrodynamic diameter from 0% FBS to 1% FBS, and then the hydrodynamic diameters remained fairly consistent at all other FBS concentrations.

In addition to the hydrodynamic diameter, the PDI for each sample was measured to ascertain the spread of the data. PDI close to 1 indicates that samples are polydisperse and consist of a wide range of sample variability. The PDI was highest in the absence of FBS for both 5 and 35 nm CuO NPs. The PDI in the presence of FBS was relatively consistent for the 35 nm CuO NPs at all percentages of FBS. For the 5 nm CuO NPs, 5% and 10% FBS had similar PDIs, which were lower than the PDI with 1% FBS.

2.2. Serum Did Not Alter the 2D Mean Cell Area after In Vitro Culture

The impact of serum on BEAS-2B squamous differentiation was assessed using mean cell area comparisons before and after incubation with FBS in the cell culture medium. Cells were incubated in LHC-9 medium containing 0% FBS (control) and 1–10% FBS for up to 72 h (**Table 2**). Changes in mean cell area were not

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Table 1. The hydrodynamic diameter and the polydispersity index (PDI) of 5 and 35 nm CuO NPs in culture media with varying levels of FBS. The average and standard deviation are reported for $n = 3$. Statistical significance was assessed by comparing to LHC-9 with 0% FBS.

		5 nm CuO NPs		35 nm CuO NPs	
		Mean diameter ± SD [nm]	PDI ± SD [nm]	Mean diameter ± SD [nm]	PDI ± SD [nm]
% FBS	0	326 ± 27***[a)]	0.70 ± 0.20	648 ± 56***[a)]	0.30 ± 0.10
	1	232 ± 96	0.60 ± 0.20	284 ± 50***[b)]	0.23 ± 0.03
	5	210 ± 117	0.40 ± 0.10	297 ± 44***[b)]	0.27 ± 0.05
	10	74 ± 28**[a),b)]	0.40 ± 0.10	300 ± 138***[a),b)]	0.23 ± 0.05

[a)]Significant differences between 5 and 35 nm particles. Replicative symbols indicate level of significance with: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $***p < 0.0001$. A summary of exact *p*-values is presented in Table S1, Supporting Information; [b)]Significantly different from 0% FBS.

significant, although the increase peaked at 48 h and declined at 72 h for most FBS concentrations. Specifically, from 0 to 48 h, FBS concentrations of 1% and 5% resulted in an increase in mean cell area and resulted in a decrease in mean cell area from 48 to 72 h. Cells incubated with 10% FBS exhibited the largest drop in mean cell area in the last 24 h, whereas 1% and 2% FBS in the cell culture media resulted in the largest increase in the mean cell area within the first 24 h. The only concentration with an increase in mean cell area between 48 and 72 h of incubation was 0% FBS, the control group.

2.3. Culturing with Serum Affected the Relative Cell Viability for 35 nm CuO NPs

At a constant Cu^{2+} concentration of 40 μg mL^{-1}, the effects of serum concentration on relative cell viability could readily be examined. Increasing the concentration of FBS in the LHC-9 media decreased the relative cell viability for 35 nm NPs, but did not for 5 nm CuO NPs or copper nitrate (Figure 4). In fact, the relative cell viability for 5 nm CuO NPs and copper nitrate decreased to 71% for NPs and 56% for copper nitrate with no FBS and increased back to ≈101% for 5 nm CuO NPs in just 1% FBS–containing media and 101% for copper nitrate in 2% FBS–containing media.

Table 2. The normalized mean cell size determined from image analysis of BEAS-2B cells upon incubation with FBS (0–10% v/v) from 0 to 72 h. Data were normalized to time zero, with increases in mean represented as values greater than one and decreases as values between zero and one. The mean ($n = 3$) and standard deviation are reported from three experimental replicates with each replicate averaged from a minimum of 100 cells. Statistical analysis was performed using nested one-way ANOVA grouping based on time. No significant differences were observed. (Please refer to Table S2, Supporting Information, for summary of *p*-values.).

% FBS [v/v]	0 h	24 h	48 h	72 h
0	1 ± 0	0.94 ± 0.27	0.92 ± 0.27	1.08 ± 0.14
1	1 ± 0	1.14 ± 0.10	1.18 ± 0.14	1.14 ± 0.09
2	1 ± 0	1.17 ± 0.06	1.14 ± 0.06	1.18 ± 0.15
5	1 ± 0	1.02 ± 0.15	1.10 ± 0.19	1.05 ± 0.18
10	1 ± 0	1.02 ± 0.11	1.08 ± 0.21	0.91 ± 0.25

The dose-dependent response for Cu^{2+} was investigated for cells cultured in LHC-9 medium with 0% FBS or 10% FBS (**Figure 3**). When there was no FBS in the culture medium, neither the 5 nm CuO NPs nor the 35 nm CuO NPs reduced the relative cell viability to or below 50%. For cells cultured in LHC-9 medium with 10% FBS, the 5 nm CuO NPs reduced cell viability to 51% at 100 μg mL^{-1} Cu^{2+}, and the 35 nm CuO NPs reduced cell viability to 9% at 20 μg mL^{-1} Cu^{2+} and 4–5% for 40–100 μg mL^{-1} Cu^{2+}. In the absence of FBS, copper nitrate appeared to have a positive effect on the relative cell viability, raising it above 100% up to 20 μg mL^{-1} Cu^{2+}, and then decreasing it sharply down to 54% at 40 μg mL^{-1} Cu^{2+} and further to 13% at 100 μg mL^{-1} Cu^{2+}. In the presence of 10% FBS, copper nitrate increased the relative viability above 100% up to 40 μg mL^{-1} Cu^{2+} and then down to 34% at 60 μg mL^{-1} and 6% at 100 μg mL^{-1}.

2.4. Intracellular Cu^{2+} Was Altered by NP Diameter and Serum

The amount of internalized copper was measured using inductively coupled plasma mass spectrometry (ICP-MS) to determine the influence of the NP diameter and serum concentration on NP uptake by BEAS-2B cells. The observed intracellular Cu^{2+} was ≈200 times larger for BEAS-2B cells exposed to 35 nm NPs compared with 5 nm CuO NPs. The addition of serum significantly increased intracellular Cu^{2+} for 5 nm CuO NPs from 0% to 3% serum ($p = 0.0028$) and for 35 nm CuO NPs from 3% to 10% serum ($p < 0.0001$). All other *p*-values from multiple comparisons are provided in Table S6, Supporting Information.

3. Discussion

The BEAS-2B cell line was initially developed by infecting primary human bronchial epithelial cells (HBECs) with adenovirus 12-SV40 to increase the lifespan to assess aging, differentiation, and carcinogenesis of normal HBECs in the 1980s[20,21,45,46] and is currently a common choice as an in vitro lung model of nanotoxicity.[47–49] BEAS-2B cells were observed to undergo squamous differentiation in the presence of 2% FBS, spurring the development of serum-free culture media such as LHC-9 and BEGM.[21] Even so, many reports utilize RPMI[31,50–52] or DMEM[32,41,53,54] with up to 10% FBS. These FBS concentrations raise concerns

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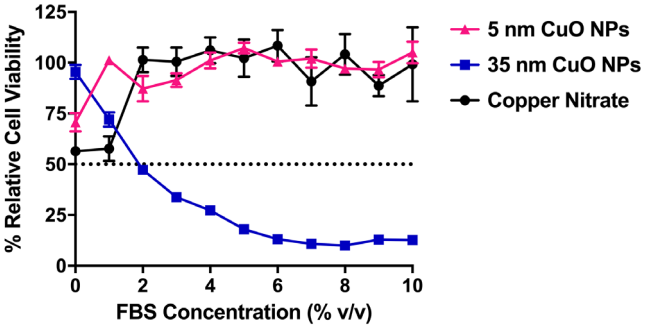

Figure 3. Effect of varying FBS concentration in LHC-9 medium on BEAS-2B toxicity induced by CuO NPs following 24 h of exposure. The data points represent the mean ($n = 8$) and error bars are standard deviations of the mean; two-way ANOVA was performed with multiple comparisons of means amongst a single FBS concentration across particle types (Table S3, Supporting Information). Significant differences between 5 nm CuO NPs and $Cu(NO_3)_2$ were only noted at 1% FBS ($p < 0.0001$). Statistical differences between 35 nm CuO NPs and 5 nm CuO NPs were always significant ($p < 0.0001$ at and above 2% FBS) and between 35 nm CuO NPs and $Cu(NO_3)_2$ were noted only for 1% FBS ($p > 0.05$); otherwise differences were significant to $p < 0.0001$.

about squamous differentiation in BEAS-2B cells.[43,45,55] In addition, when addressing NP toxicity, cells cannot differentiate between primary particle size and aggregate particle size. That is, the cell "sees" the aggregate size, regardless of what the primary particle size may be.[1,18,56,57] As such, the NPs used in this study were characterized not only according to their primary particle size (Figure 2), but also according to the hydrodynamic diameter in the LHC-9 medium with varying concentrations of FBS (Table 1).

Our investigations led to the discovery that serum-containing media only influence the cellular responses of BEAS-2B cells when exposed to 35 nm CuO NPs, and not for 5 nm CuO NPs. The NPs were first characterized according to their primary diameter and elemental composition. The 5 nm NPs had a greater tendency toward aggregation than the 35 nm NPs, as evidenced by the micrographs (Figure 2).[58] The same 5 nm NPs also exhibited a uniform, spherical shape, whereas the 35 nm NPs lacked a distinct morphology. Previously reported X-ray photoelectron spectroscopy (XPS) indicated that copper was in the same state, Cu(II), for both the small and large NPs, and XRD indicated that both NPs consisted of a single tenorite phase.[18] These characterizations confirm that the differences in the small and large CuO NPs used in this study are restricted to size and morphology, but not elemental composition.

The aqueous, or hydrodynamic, diameter and PDI were used to assess the average NP aggregation and dispersity under varying concentrations of FBS in the culture medium (Table 1). The results indicated that the 5 nm CuO NPs consistently decreased in hydrodynamic diameter with increasing FBS concentration, whereas the 35 nm CuO NPs only decreased in hydrodynamic diameter between 0% and 1% FBS. These results are consistent with the existing literature that serum decreases NP aggregation,[59–62] indicating that regardless of the serum concentration, the CuO NP aggregate size exposed to the cells is ≈300 nm.

This then suggests that the observed relative cell viability of BEAS-2B cells exposed to 35 nm CuO NPs was independent of the changes in perceived NP diameter (**Figure 4**). Our previous work indicated that the smaller NPs were capable of dissolving into component ions in as little as 1 h, whereas the larger NPs took 24 h to reach the same Cu^{2+} concentrations in the

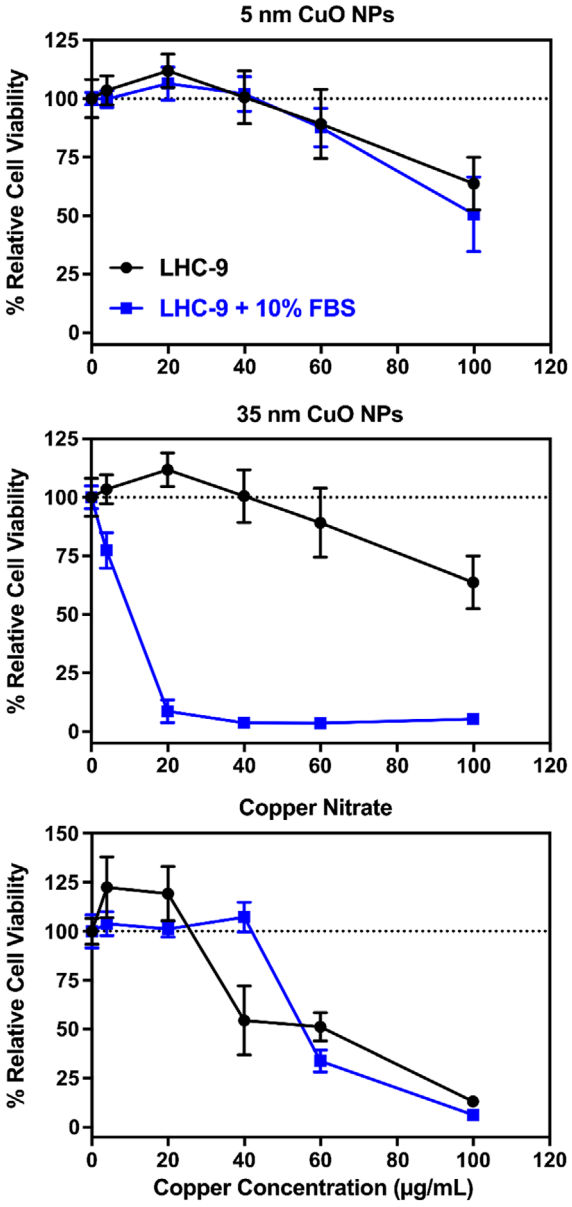

Figure 4. Effects of NP size and dose on BEAS-2B viability after 24 h of exposure in 0% or 10% FBS to 5 nm CuO NPs (top), 35 nm CuO NPs (middle), and copper nitrate (bottom). Data points represent the mean ($n = 8$) and error bars are the standard deviations of the mean. Statistical analysis was performed using a two-way ANOVA and multiple comparisons of means at the same copper concentration and varying particle type, and the actual p-values are reported in Table S4–S6, Supporting Information. Significant differences were noted between LHC-9 and LHC-9 + 10% FBS for 5 nm CuO NPs = 39.95 μg mL^{-1}, 35 nm CuO NPs ≥ 3.99 μg mL^{-1}, and $Cu(NO_3)_2$ from 3.99 to 59.92 μg mL^{-1} (inclusive).

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RPMI-1640 medium with 10% FBS.[18] Increases in the FBS concentration in LHC-9 medium resulted in decreased relative cell viability for 35 nm CuO NPs. Both the 5 and 35 nm NPs had hydrodynamic diameters of ≈300 nm with 10% FBS in the culture medium; however, the resultant relative cell viability was only 13% compared with the control for 35 nm NPs and 105% compared with the control for 5 nm NPs. Similar observations were made for varying copper II concentrations and comparing 0% FBS and 10% FBS in the culture medium (Figure 3). The copper nitrate and the 5 nm CuO NPs followed similar trends, in that increasing copper concentration decreased the relative cell viability at approximately equal rates with both 0% FBS and 10% FBS. In contrast, BEAS-2B cells were far more sensitive to the 35 nm CuO NPs when serum was present in the culture medium. These observations that increased serum increases the relative viability of cells treated with copper nitrate seems to indicate that copper ions are more toxic to BEAS-2B cells at increasing serum concentrations. Similarly, it was observed that CuO NPs were more toxic to BEAS-2B cells when there was BSA present in the culture medium compared with no BSA.[63] To eliminate the potential effects of NP settling time and delivered dose,[5,64–66] consistent concentrations of copper II were used for 5 nm CuO NPs, 35 nm CuO NPs, and copper nitrate. Therefore, all observed effects are expected to be a result of NP size and serum concentration. One additional step that could be taken to further improve the study would be to use an air–liquid interface (ALI) cell culture system for BEAS-2B cells.[38] These in vitro systems are particularly advantageous for lung cell models because they allow for apical exposure of epithelial cells to air, thus more closely representing the in vivo situation for epithelial cells in the respiratory tract.[67]

Potential differentiation of BEAS-2B cells was an important consideration in analyzing these results, in particular in determining if differentiation in the presence of serum-impacted relative cell viability. One measure of squamous cell differentiation, which can occur in response to FBS, is the mean cell area (Table 2).[20,43,68] This differentiation has additional impacts in vivo because it functions as the routine repair mechanism of epithelial defects to assure that the epithelial lining of the respiratory tract remains intact.[69] Generally, the trends in mean cell size, which was used as a differentiation marker in this study, increased over the first 48 h of incubation. Previous studies have noted a four-time increase in cell area in the presence of FBS, particularly due to transforming growth factor-β found in FBS.[45] In the three days of our study, the control group increased the mean cell area between 48 and 72 h; however, all of the cells cultured in FBS exhibited a decreased mean cell area between 48 and 72 h despite an increase in the first 48 h of the study. One possible explanation is that confluency impacted the mean cell area.

The quantity of intracellular copper was measured using ICP-MS (**Figure 5**). These results indicated that greater amounts of copper were present within cells that were exposed to 35 nm CuO NPs compared with 5 nm CuO NPs, regardless of the serum concentration. The lack of statistically significant differences as a function of serum amongst the 5 nm CuO NPs suggests that any of the observed differences in cytotoxicity as a function of serum was not due to the amount of Cu^{2+} within the cells. In addition, the 5 nm CuO NPs did not exhibit differences in relative cell viability above 2% FBS. The amount of internalized copper, which may be the amount of copper in the NPs, likely influenced the cell viability differences observed between 5 nm CuO NPs and 35 nm CuO NPs as seen in Figure 3 and 4. Furthermore, as copper is a micronutrient, we believe that the lower intracellular copper observed for the 5 nm CuO NPs could explain the relative cell viabilities at and above 100% compared with the untreated control.

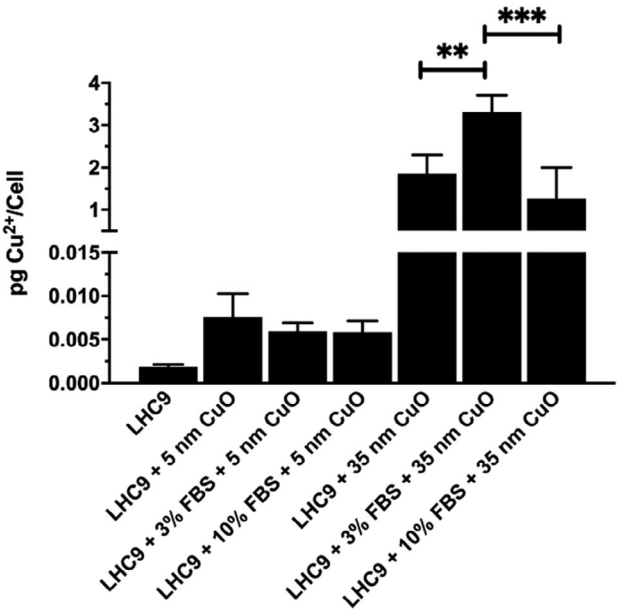

Figure 5. Intracellular Cu^{2+} measured using ICP-MS. The copper concentration reported is from lysed cells and was normalized to cell counts obtained by trypan blue exclusion and manual cell counting of a small sample of the cell digest. Values reported are the mean ($n = 3$) and error bars represent the standard deviations of the mean; statistical analysis was performed using one-way ANOVA with Tukey's posttest and comparing each mean to every other mean. Significant differences are noted as $^{**}p < 0.01$ and $^{***}p < 0.001$. Exact *p*-values for all comparisons are in Table S7, Supporting Information.

4. Conclusion

In this study, the interactions between serum and hydrodynamic diameter, cytotoxicity, and intracellular Cu^{2+} of 5 and 35 nm CuO NPs were investigated, as well as the squamous differentiation of BEAS-2B cells in the presence of serum (FBS). Using the mean cell area as the marker for differentiation, expected increases in cell area for cells cultured in serum-containing LHC-9 medium occurred during the first 48 h. Adding FBS to the LHC-9 medium resulted in a decrease in the hydrodynamic diameter of both 5 nm CuO NPs and 35 nm CuO NPs compared with the hydrodynamic diameter in serum-free media. The larger, 35 nm CuO NPs exhibited differences in relative cell viability with the addition of FBS. In contrast, the smaller, 5 nm CuO NPs did not elicit changes in the cell viability when FBS was added to the culture medium. Finally, the intracellular Cu^{2+} concentration was not affected by the serum concentration and was affected by the NP size where 35 nm CuO NPs resulted

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in much higher levels of intracellular Cu^{2+} than 5 nm CuO NPs. Overall, our investigation shows the importance of considering the use of serum for cytotoxicity of various NP sizes in studies of BEAS-2B cells.

5. Experimental Section

CuO NP Characterization and Aggregation: CuO NPs were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without any further modification. The morphology and average size of the particles were obtained using transmission electron microscopy (TEM, JEOL JEM-1230, JEOL Ltd., Akishima, Tokyo, Japan) and ImageJ.[70] TEM samples were prepared by suspending CuO NPs in water and depositing a small drop (≈5 μL) on a carbon-coated copper grid (Ted Pella, Inc., Redding, CA, USA). The water was removed after 1 min and samples were imaged immediately. X-ray diffraction (XRD) (Bruker, Billerica, MA, USA) was used for elemental analysis of CuO NPs, using methods reported previously.[18]

Aggregation of CuO NPs was assessed using the hydrodynamic diameter and polydispersity index (PDI) measured with a ZetaSizer Nano ZS (Malvern Instruments, Inc., Westborough, MA, USA). For these experiments, CuO NPs were suspended in LHC-9 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing varying concentrations (%v/v) of FBS (Atlanta Biologicals, Lawrenceville, GA, USA) from 0% to 10%.

Cell Maintenance: BEAS-2B cells were purchased from ATCC (Manassas, VA, USA) and maintained in LHC-9 medium. Cells were maintained in 75 cm^2 tissue culture flasks coated with 0.01 mg mL^{-1} fibronectin from human plasma (Sigma), 0.03 mg mL^{-1} bovine collagen type I (Sigma), and 0.01 mg mL^{-1} bovine serum albumin (BSA) (Sigma) in LHC-9 medium. Lyophilized fibronectin was dissolved in water at 1 mg mL^{-1} for 30 min at 37 °C. It was then aliquoted into 20 μL samples and stored at −20 °C until use. The coating solution was prepared in 3 mL stocks, and 1 mL was added to each T75 flask. After 24 h of incubation at 37 °C, the flasks were kept at room temperature and protected from light until use. The coating was stable for up to 1 month. Prior to plating cells, the coating solution was aspirated, and flasks were washed with 5 mL of 1X Dulbecco's phosphate-buffered saline (DPBS). Cells were maintained in a 5% CO_2 humidified atmosphere at 37 °C (New Brunswick Galaxy 170S, Eppendorf, Hauppauge, NY, USA). Subculturing was performed using a trypsin/ polyvinylpyrrolidone (PVP) solution prepared by weighing PVP (Sigma) and calculating the necessary volume of 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, Life Technologies, Grand Island, NY, USA) to form a 0.5% (w/v) PVP/trypsin-EDTA mixture, with a target volume of 10 mL. The 0.25% trypsin-EDTA was added to PVP powder and was vortexed to completely dissolve the PVP. Cells were detached from flasks using 2 mL trypsin/PVP for 5 min at 37 °C and this was neutralized with 8 mL of LHC-9 medium. Cells were then centrifuged at 230 × *g* for 5 min to form a pellet. The cell pellet was completely suspended in fresh LHC-9 medium, counted using trypan blue exclusion, and seeded at 1.1×10^5 cells/T75 flask or 4.4×10^4 cells/60 mm petri dish.

Cell Differentiation: BEAS-2B cells were passaged into 60 mm petri dishes that had been coated as described earlier. Cells were allowed to grow overnight prior to treatment with FBS-containing LHC-9 medium. Images were obtained using an inverted fluorescent microscope (EVOS FL cell imaging system, Thermo Fisher Scientific, Waltham, MA, USA) at 0 h (prior to changing the media), 24 h, 48 h (media refreshed), and 72 h. The area of cells was obtained from each image using a MATLAB script encoding Otsu's method for image processing using a minimum of 100 cells.[71] The mean cell area for each FBS concentration at each time was normalized against the mean cell area at time zero. The mean ratio and mean standard deviation are reported.

Cytotoxicity of CuO NPs In Vitro: BEAS-2B cells were seeded at 1×10^4 cells/well and 100 μL/well in a 96-well plate coated as described earlier and incubated overnight. The medium was removed, and 200 μL of CuO NP suspension or copper nitrate trihydrate (Strem Chemicals, Newburyport, MA, USA) in LHC-9 medium (40 μg mL^{-1} copper), with varying amounts of FBS, was added to the plate. After 24 h of NP exposure, the medium was removed and replaced with 100 μL of fresh LHC-9 medium. Then, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium reagent (CellTiter 96 Aq_{ueous} One solution, Promega Corporation, Madison, WI, USA) was added to each well and plates were incubated for 1–4 h at 37 °C and 5% CO_2 to allow for dye conversion. After incubation, plates were centrifuged at 500 × *g* for 20 min to sediment any remaining NPs, and 70 μL of medium was removed from the top of the wells and placed into a new 96-well plate. The absorbance of each sample at 490 nm was obtained using a SpectraMax plate reader (Molecular Devices, Inc., Sunnyvale, CA, USA). The absorbance was averaged to the control group without any NPs or FBS in the LHC-9 medium.

Uptake of CuO NPs In Vitro: BEAS-2B cells were seeded at 2×10^5 cells/dish in 60 mm petri dishes (coated as previously described). Cells were maintained at 37 °C and 5% CO_2 for 24 h, prior to adding 4 mL of NP suspension (10 μg mL^{-1}) in LHC-9 medium with varying amounts of FBS. After treatment for 24 h, the medium was removed, and cells were gently washed with 1X DPBS (Gibco) three times to remove any NPs in suspension. Cells were removed from the petri dishes using trypsin/PVP and centrifuged at 230 × *g* for 5 min. The supernatant was removed, and cells were resuspended in 1 mL 1X DPBS. Small portions of each sample (30 μL) were removed for cell counts using 0.2% trypan blue exclusion (Sigma). After counting, cells were centrifuged again at 230 × *g* for 5 min. The PBS was removed from the cell pellet and replaced with 100 μL of concentrated nitric acid for overnight digestion. The next day, samples were diluted with 5 mL of 1% nitric acid and analyzed alongside a standard curve of copper nitrate dissolved in 1% nitric acid (0–500 ppb). The copper concentration was measured using ICP-MS (Thermo X-series II, Thermo Fisher Scientific) with indium as the internal standard.

Statistical Analysis: The reported data are expressed as the mean ± standard deviation for independent trials, with no preprocessing. All the data were analyzed using ANOVA with Tukey's posttest using GraphPad Prism software (GraphPad, Inc., La Jolla, CA, USA). A two-way ANOVA multiple comparisons of means was used for cell viability, a nested one-way ANOVA to compare the mean values at each FBS concentration was performed for mean cell area, and an ordinary one-way ANOVA was used to compare the mean values for intracellular Cu^{2+} measurements.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Keywords

BEAS-2B cells, copper oxide nanoparticles, cytotoxicity, inductively coupled plasma mass spectrometry, serum

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