Published in final edited form as:

Toxicol In Vitro. 2015 April ; 29(3): 502–511. doi:10.1016/j.tiv.2014.12.023.

Toxicity of copper oxide nanoparticles in lung epithelial cells exposed at the air-liquid interface compared with *in vivo* assessment

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Abstract

The toxicity of spark-generated copper oxide nanoparticles (CuONPs) was evaluated in human bronchial epithelial cells (HBEC) and lung adenocarcinoma cells (A549 cells) using an *in vitro* air-liquid interface (ALI) exposure system. Dose-response results were compared to *in vivo* inhalation and instillation studies of CuONP. Cells were exposed to particle-free clean air (controls) or spark-generated CuONPs. The number median diameter, geometric standard deviation and total number concentration of CuONPs were 9.2 nm, 1.48 and 2.27×10^7 particles/cm³, respectively. Outcome measures included cell viability, cytotoxicity, oxidative stress and proinflammatory chemokine production. Exposure to clean air (2 or 4 hr) did not induce toxicity in HBEC or A549 cells. Compared with controls, CuONP exposures significantly reduced cell viability, increased lactate dehydrogenase (LDH) release and elevated levels of reactive oxygen species (ROS) and IL-8 in a dose-dependent manner. A549 cells were significantly more susceptible to CuONP effects than HBEC. Antioxidant treatment reduced CuONP-induced cytotoxicity. When dose was expressed per area of exposed epithelium there was good agreement of toxicity measures with murine *in vivo* studies. This demonstrates that *in vitro* ALI studies can provide meaningful data on nanotoxicity of metal oxides.

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Conflict of Interests

The authors declare they have no competing financial interests.

Authors' contributions

XFJ participated in the study design; performed cell culture, *in vitro* exposure studies and biological assays; assisted with data analysis; and assisted in drafting the manuscript. JHP conducted NPs generation and characterization of nanoaerosols. TMP made contribution to the experiment design and coordinated NPs generation and characterization. PST conceived of and supervised the overall study; designed the experiments; performed data analysis; participated in drafting, revising and editing the manuscript; and authored the final manuscript. All authors read and approved the submitted manuscript.

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Keywords

Air-liquid interface; copper oxide nanoparticles; *in vitro* exposures; human bronchial epithelial cells; N-acetylcysteine; oxidative stress

1. Introduction

The increasing production and large-scale applications of metallic nanoparticles (NPs) have led to major concerns regarding the potential environmental and human health risks. Copper oxide nanoparticles are widely used in a variety of established and emerging technologies that include catalysts, printed electronics, wood protection, solar energy conversion, magnetic storage and antimicrobial products (Doong et al., 2013; Evans et al., 2008; Kaur et al., 2014; Lee et al., 2008; Pandey et al., 2012; Ren et al., 2009). People living near or working among sources of copper particles emission such as copper smelters, refineries, and processing facilities may be in danger of high levels of exposure. Adverse health effects of inhalation exposure to copper fumes in humans has been reported in workers involved in cutting brass pipes with electric cutting torches (Armstrong et al., 1983). With widespread applications of CuONPs, it is necessary to clearly understand the biological consequences of CuONP exposure in relation to human health. Recently, a number of studies, including two from our group, have investigated the toxic effects of CuONPs on airway cell lines *in vitro* and their pulmonary toxicity in animals (Elihn et al., 2013; Fahmy and Cormier, 2009; Kim et al., 2011; Kumar and Nagesha, 2013; Pettibone et al., 2008). Following whole-body inhalation exposure to CuONPs, inflammatory responses in mice were induced, including elevated cytokine production in bronchial lavage fluid, increased recruitment of inflammatory cells to the lung, and perivascularitis and alveolitis in lung (Kim et al., 2011; Pettibone et al., 2008). CuONPs possess microbiocidal properties that have various antimicrobial applications. Ren et al. (2009) reported that CuONPs in suspension showed activity against a range of bacterial pathogens, including methicillin-resistant *Staphylococcus aureus* and *Escherichia coli*. However, CuONPs were found to be highly toxic compared to other metal oxide NPs (Gunawan et al., 2011; Karlsson et al., 2008). Significant toxicity and broad-spectrum bioactivity of CuONPs make them a high priority for further study.

There is an urgent need to establish highly predictive, streamlined approaches for toxicity screening and assessment of engineered NPs. Cell-based *in vitro* methods, which are simple, fast and cost-effective, have been evaluated for toxicity screening of new NPs. Most *in vitro* studies of NP toxicity are based upon the exposures of submerged cell cultures to particle suspensions. However, submerged exposure has limited predictive power compared with *in vivo* exposure at environmentally-relevant conditions due to limitations such as surface coating of particles with medium components, changes of particle dissolution and agglomeration processes. Particles deposition driven by diffusion and sedimentation in submerged systems is greatly different from deposition in the lung, complicating comparison of the dose of particles on the cells to the dose in inhalation studies (Mühlfeld et al., 2008; Paur et al., 2011; Volckens et al., 2009). To avoid these undesirable “matrix effects” of submerged exposures, *in vitro* exposure systems at an ALI have been developed

where airborne NPs are deposited directly onto the cells without first having to penetrate a thick layer of cell culture media (Blank et al., 2006; Kim et al., 2013; Lenz et al., 2009, 2013; Rothen-Rutishauser et al., 2009; Raemy et al., 2012; Savi et al., 2008). In these studies, NP aerosols were generated by flame spray pyrolysis or NP suspensions were sprayed or nebulized into micron-sized droplets and subsequently deposited onto cells at the ALI. However, it was unknown whether the aerosol particles maintained similar physicochemical properties to the original particles. To better replicate exposure to the metal-based engineered NPs, we sought to generate fresh NPs continuously and consistently while characterizing their physicochemical properties and determining their appropriate dose metrics.

The first aim of this study was to characterize CuONPs generated by a spark discharge system (SDS) which simulates the metal fume from the heating of metallic copper. The spark discharge technique is flexible with respect to tested material, the particle size distributions are narrow and can be controlled via the supplied current, particles with fixed characteristics can be produced continuously over many hours, and scale-up is possible. This technique was introduced in 1988 by Schwyn *et al.* (1988) and has been applied to produce ultrafine particles by other research groups (Bitterle et al., 2006; Kim and Chang, 2005; Roth et al., 2004). However, none of these studies has integrated a SDS with an ALI exposure system to investigate the toxicity of metal based NPs.

The second aim of this study was to evaluate and compare the toxicity of spark-generated CuONPs in HBEC and A549 cells, in order to select a better possible cell model for toxic screening of inhaled NPs. Most *in vitro* studies carried out previously have used cancerous or transformed epithelial cell lines (Kim et al., 2013; Lenz et al., 2009; Savi et al., 2008). Toxicological data of CuONPs on primary human airway cells is sparse. A549 cells, derived from carcinoma cells of type II alveolar cells, behave like bronchial epithelium. We sought to determine if A549 cells response to CuONPs exposure in the similar way as HBEC. We further evaluated the role of oxidative stress in the cytotoxicity of CuONPs by introducing a ROS scavenger, N-acetylcysteine (NAC). A third aim was to compare this novel *in vitro* approach to our prior *in vivo* inhalation toxicology studies of the same nanomaterials performed in mice.

2. Materials and methods

2.1. Generation of CuONPs and exposure of cells

As shown in Figure 1, the experimental setup consists of a clean air supply, a SDS, a charge neutralizer, a carbon dioxide (CO₂) supply, an *in vitro* exposure system, a gas monitor or an alternative unit for measurement/sampling. The air supply system is composed of an oil trap, a diffusion dryer, and a high efficiency particulate air (HEPA) filter to remove oil contaminants, humidity, and particles, respectively. The dry, clean air, controlled by a mass flow controller (GFC37, Aalborg Instruments, Orangeburg, USA), is delivered to the SDS. A spark discharge is formed in an air environment between two electrodes. The electrical circuit includes a resistance of 0.5 M Ω (two 1-M Ω resistors arranged in parallel), a capacitance of 1nF, a loading current of 0.25 mA, and an applied voltage of 5 kV. Test electrodes are copper rods (alloy 101, Cu 99.99%, McMaster-Carr Elmhurst, USA). Aerosol

(4.75 L/min) from the SDS is passed through an aerosol neutralizer (3087, TSI Inc., USA) and CO₂ (0.25 L/min) is added to the particle-laden air to maintain 5% CO₂ atmosphere and physiologic pH for the cells.

Cells were exposed to clean air or CuONP aerosol as previously described by Kim *et al.* (2013), except that the aerosol distribution system was optimized by using highly developed (HD) distributor (Vitrocell®, Waldkirch, Germany) instead of a glass manifold. Briefly, spark-generated CuONPs were evenly distributed into two HD distributors which were connected with the ALI exposure chamber (6-CF module, Vitrocell®, Waldkirch, Germany) holding six cell-covered transwell inserts. The CuONP aerosol was delivered to the cells at 5 mL/min flow rate regulated by six mass flow controllers (GFC17, Aalborg Instruments, Orangeburg, USA) downstream of the exposure chamber. Each compartment contained 16 mL of phenol-free complete medium (exposure medium). The ALI exposure system was maintained at 37°C by circulating temperature-controlled water through the baffles within the HD distributors and module. The particle-free clean air used for sham control was obtained by filtering CuONP aerosol with a HEPA filter. The incubator control cells (control) used for calculating relative changes in cell responses were incubated at the ALI under standard culture conditions (5% CO₂, 95% humidity, and 37°C). Doses were adjusted by varying the exposure duration (0, 2 or 4 hr) to ensure consistency of particle size distribution and low emission of secondary gases.

2.2. Characterization of spark-generated CuONPs

The size distribution of the particles was measured using a scanning mobility particle sizer (SMPS; 3936, TSI Inc., USA) consisting of a classifier controller (3080, TSI Inc., USA), aerosol neutralizer (Kr⁸⁵; 3077, TSI Inc., USA), differential mobility analyzer (DMA; 3085, TSI Inc., USA), and a condensation particle counter (CPC; 3776, TSI Inc., USA). The SMPS measured particles from 1.95 to 63.8 nm mobility equivalent diameter at a sampling flow rate of 1.5 L/min.

The CuONPs were also sampled onto transmission electron microscope (TEM) nickel grids (200-mesh, carbon layer, 01840N-F, Ted Pella, Inc., USA). A high resolution TEM (HRTEM, JEM-1230, Jeol Ltd., Japan) was used to evaluate the projected area (PA) diameter (d_{PA}) and morphology of particles. The d_{PA} is defined as the diameter of the circle having the same projected area as the particle's two-dimensional silhouette. A total number of 100 particles and agglomerates were counted. PA was obtained using ImageJ software (version 1.47, NIH, USA) and d_{PA} was calculated as: $d_{PA} = 2 \times (PA/\pi)^{0.5}$. A gas monitor (PortaSens II, ATI, USA) was used for monitoring ozone (O₃) and nitrogen oxides (NO_x) when the SDS was operating.

For X-ray diffraction (XRD) analysis, particles were collected onto a polyvinyl chloride membrane filter (5 µm-pore, 37 mm-diameter, SKC Gulf Coast Inc., USA) at 4 L/min for 200 hr. The phases of the agglomerated particles were determined by XRD (D8 Advance, Bruker, USA) using Cu K α radiation ($\lambda = 1.54056 \text{ \AA}$) at a step size of $0.02^\circ 2\theta$. The XRD patterns were matched against the international crystallography diffraction database containing standard patterns using the Eva 3.1 software (Bruker, USA) to identify the phases present in these particles.

2.3. Epithelial cell culture

A549 cells (ATCC[®] CCL-185[™], ATCC, USA) were cultivated in Dulbecco's modified Eagle medium (DMEM, with 4.5 g/L glucose, 4 mM L-glutamine, Gibco[®] Life Technology, Grand Island, USA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, USA), 1% penicillin G/streptomycin (Gibco[®], 10000 units/mL/10000 µg/mL) and 20 mM HEPES (Gibco[®]) at 37°C in a humidified atmosphere containing 5% CO₂, subsequently referred to as DMEM complete medium. Before exposures, A549 cells were dissociated with 0.25% Trypsin-EDTA (Gibco[®]) and counted, then 0.5 mL of cell suspension was seeded onto the apical side of each transwell membrane insert (Cat. 3460, polyester, 12 mm diameter, 1.12 cm², 4 × 10⁶ pores/cm², 0.4 µm pore size, Corning, USA) at a density of 1.25 × 10⁵ cells/mL. Cells were grown to at least 90% confluence for 24 hr under submerged condition with 0.6 mL DMEM complete medium under the basal side of the transwell. Cultures were prepared for exposure by removing the apical medium and incubating cells for an additional 12 hr. Cells on transwells were washed twice with calcium and magnesium-free Dulbecco's phosphate-buffered saline (DPBS, Gibco[®]), and transferred to the ALI culture compartments, where the cells were supplied with 16 ml exposure medium on the basal side of the transwell.

Primary HBEC cultures were provided by the *In Vitro* Models and Cell Culture Core of the University of Iowa. HBEC were grown on a collagen-treated 12 mm transwell insert (Corning, USA). The HBEC cultures were drawn from two cadaver bronchi. Donors 16-13 and 17-13 were young male Caucasian adults with a smoking history of 1/2 pack/day for five years. Donor 16-13 died of head trauma and donor 17-13 died of meningitis. The differentiated HBEC were seeded at a density of 2.5 × 10⁵ cells/cm² and maintained under the ALI condition with culture medium (1:1 ratio of DMEM and Ham's F-12 supplemented with 2% Ultrosor G, penicillin (100 units/mL), streptomycin (100 µg/mL), gentamycin (50 µg/mL), fluconazole (2 µg/mL), and amphotericin B (1.25 µg/mL) on the basal side of the transwell.

2.4. Cellular responses assay

2.4.1. Cell viability—After 0 (control), 2 and 4 hr exposure to clean air or CuONP aerosol in the ALI system, cell viability of exposed cells and control cells were assessed using the Alamar Blue Assay (Sigma, St. Louis, USA). Cellular mitochondrial enzyme activity was demonstrated by resazurin reduction capacity and quantified using fluorescence intensity (relative fluorescence units, RFU) (AL-Nasiry et al., 2007). Cells on transwells were washed with DPBS on both sides of membranes, 500 µL of cell wash solution was collected and spun at 14,000 × g for 15 min three times and then stored at -80°C for analyses of LDH and cytokines. After washing, 450 µL of fresh phenol-free medium containing 50 µM Alamar Blue was added to the apical side of the cells and incubated for 75 min in a 37°C, 5% CO₂ incubator, then media with Alamar Blue were withdrawn and added into 96-well flat bottom plates (200 µL/well) (Corning, USA). Duplicate wells were assayed and fluorescence was quantified at 570 nm (excitation) and 590 nm (emission) using a microplate reader (SpectraMax M5, Molecular Device, USA).

2.4.2. Cell membrane integrity and cytotoxicity—LDH is a stable cytoplasmic enzyme that converts lactate to pyruvate. LDH released from damaged cell was measured using a Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer. The absorbance of the reaction product was measured at 490 nm using a microplate reader (SpectraMax M5) with a reference wavelength above 600 nm. L-LDH standard (Roche) serial dilutions were used to calculate the LDH activity in cell wash solutions (1:4 diluted in DPBS, starting at 27.5 U/mL).

2.4.3. Oxidative stress—The generation of intracellular ROS was measured using 5-(and-6)-carboxy-2', 7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA, C 400, Invitrogen, Carlsbad, USA). The carboxy-H2DCFDA passively enters the cell where it reacts with ROS to form the highly fluorescent compound (2', 7'-dichlorofluorescein). Cells on transwells were washed twice with 250 μ L of DPBS, 500 μ L of Hanks' balanced salt solution (HBSS with Ca^{2+} and Mg^{2+}) containing 25 μ M carboxy-H2DCFDA was added to the apical side of the cells and incubated in darkness for 30 min at 37°C, then HBSS solution was removed and cells were washed twice again. After washing, cells were lysed with 450 μ L of 0.1% Triton X-100 in PBS for 30 min at 37°C, all the lysates were collected into black microcentrifuge tubes and centrifuged at 14,000 $\times$ g for 15 min at 4°C. Supernatant fluorescence intensity was measured in duplicate with excitation at 485 nm and emission at 530 nm (SpectraMax M5).

2.4.4. Proinflammatory mediator quantification—The inflammatory response after exposure was determined by quantifying the amount of released IL-8, which is a potent chemoattractant for neutrophils. The level of IL-8 secreted by epithelial cells in cell wash solution was detected via enzyme-linked immunosorbent assay (ELISA) using human IL-8 ultrasensitive ELISA kit (Cat.KHC0084, Life Technologies, USA) with a standard curve covering the range of 0.39–25 pg/mL.

2.4.5. Effects of NAC on cytotoxicity induced by CuONPs—NAC, a direct scavenger of ROS, is commonly used to inhibit ROS and identify ROS inducers (Sun, 2010). NAC (Cat. A9165, Sigma, USA) was dissolved in DPBS to make 1M stock solution and freshly added to the exposure medium at a final concentration of 5 mM. A549 cells on transwells were exposed to CuONPs for 4 hr coincident with or without NAC treatment. Cells used as controls were incubated at the ALI with basal medium in the presence or absence of 5 mM NAC in 37°C, 5% CO_2 incubator. Cell viability and intracellular ROS were measured as described above.

2.5. Statistical Analyses

Data were analyzed using SAS (Version 9.3, SAS Institute, Inc., Cary, USA). Data were expressed as mean $\pm$ standard deviation (SD) of three independent experiments unless otherwise noted, and p-values less than 0.05 were considered statistically significant.

Generalized linear models (GLM) were fit to the data to test each of the study hypotheses. For the main hypotheses regarding the effect of CuONP exposure, the dose-dependent responses, and whether there is difference in that effect between HBEC and A549 cells, four

GLMs were fit each with one of the following dependent variables: viability, ROS, LDH, or IL-8 concentration. The independent variables in each case were cell (A549 or HBEC), dose (control, 2 hr exposure, or 4 hr exposure), and treatment (air or CuONPs). In each case the initial model was run with all three independent variables, all corresponding two-way interactions as well as the three-way interaction. If the p-value associated with the Type III Sums of Squares three-way interaction was greater than 0.1, a subsequent model was reduced and run including only the independent variables and their two-way interactions. The same criterion was used to determine whether any of the two-way interactions could be eliminated from the model. For the experiment involving NAC treatment, two GLMs were fit with group (control, NAC, CuONPs with NAC, or CuONPs) as the only independent variable and either viability or ROS as the dependent variable. In the case of each GLM, pair wise differences between the least square means and their associated p-values were used to explore the size and direction of observed effects.

3. Results

3.1. Characterization of spark-generated CuONPs

The size distribution and total concentration of the CuONPs obtained by SMPS are shown in Figure 2a and Table 1. The number median diameter (NMD), geometric standard deviation (GSD) and total number concentration (TNC) were 9.2 nm, 1.48 and 2.27×10^7 particles/cm³, respectively, based on particle number. Table 1 also provides distribution data based upon surface area and mass. The deposited CuONPs were not spherical but exhibited clusters and chain agglomerates formed from smaller primary particles. The mean of d_{PA} estimated from TEM was 17.6 ± 12.0 nm, and the primary particle size was as small as 5 nm (Figures 2b and 2c). The large size estimate from TEM counting is likely due to the preferential deposition on the grids of agglomerated CuONPs. During the generation of CuONPs, potentially harmful gases including O₃ and NO_x were detected at low concentrations (O₃: 0.24 ± 0.01 ppm, NO_x: 1.0 ± 0.0 ppm) in spark-generated CuONP aerosol.

As a result of very small primary particle size, the XRD pattern of CuONPs exhibited significant background interference. Nevertheless, the XRD pattern indicated that there were crystalline as well as amorphous materials present in the sample. In particular, the background subtracted XRD data (Figure 2d) indicated that the crystalline fraction of spark-generated particles consisted mainly of CuO and Cu₂O.

3.2. Responses of human lung epithelial cells to CuONP exposure

3.2.1. CuONPs induced cytotoxicity on A549 cells and HBEC—A preliminary experiment was conducted to test whether low-level generated O₃ and NO_x would cause toxic effects on exposed cells. A549 cells were exposed to clean air (Figure 1, flowpath A, SDS off), CuONP aerosol (flowpath A, SDS on) or filtered CuONP aerosol (particle-free air with generated O₃ and NO_x, flowpath B, SDS on) for 4 hr. The viability of cells exposed to filtered CuONPs was not significantly different compared with incubator control ($p = 0.206$) (see Table 2 and Supplementary File, Figure S1). This result indicated that the amount of O₃ and NO_x in the CuONP aerosol produced minimal cytotoxic effects on cells beyond that

attributable to clean air exposure. Thus, in subsequent exposures, cells exposed to particle-free clean air were used as sham controls for statistical comparison in each experiment.

As shown in Figure 3a, no significant difference was found in cell viability of air exposed groups compared with that of incubator controls. This result further confirmed that the conditions of the experimental setup (i.e. clean air exposure at a flow rate 5 mL/min) did not affect cell viability. This observation was also in agreement with previous findings (Bitterle et al., 2006; Kim et al., 2013).

Figure 3 summarizes the outcome measures for each exposure group. CuONP exposures severely decreased the viability of A549 cells in a dose-dependent manner. Cell viability expressed as percent of control was $75 \pm 15\%$ and $37 \pm 4.8\%$ for 2 hr and 4 hr CuONP exposure, respectively. Compared to clean air exposure groups, CuONP exposure significantly reduced the viability of A549 cells ($p < 0.001$ for both time points) (Figure 3a). In HBEC cells, CuONP exposure also resulted in a significant decrease of cell viability compared with air exposure (2 hr: $p < 0.05$, 4 hr: $p < 0.001$) in a dose-dependent manner. After exposure to CuONPs, the cell viability loss in HBEC (2 hr: 2%, 4 hr: 15%) was less than the loss in A549 cells (2 hr: 15%, 4 hr: 63%) ($p < 0.001$ for both time points). The effects of 4 hour exposure to CuONPs including loss of cells from the transwell are illustrated in the micrograph included in Figure S2 in the Supplemental File. From this comparison, it was observed that A549 cells are more sensitive to the toxicity of CuONPs than primary lung epithelial cells.

3.2.2. CuONPs induced LDH release from A549 cells and HBEC—Cytotoxicity of CuONPs was also evaluated by cell membrane integrity as indicated by LDH leaking from exposed cells. Figure 3b showed that clean air exposure did not change the LDH release from exposed cells compared to incubator controls. For A549 cells, CuONP exposures induced a dose-dependent increase of LDH release, which was significantly greater than clean air exposures (2 hr: $p < 0.01$, 4 hr: $p < 0.001$). CuONP-exposed HBEC showed a similar pattern of LDH release to A549 cells, but a significantly lower level of LDH than A549 cells after 4 hr exposure to CuONPs ($p < 0.001$). Overall, levels of LDH release confirmed the results of cell viability, CuONP exposure resulted in more membrane damage to A549 cells than to primary HBEC cells in a dose-dependent manner.

3.2.3. Toxicity of CuONPs is mediated by oxidative stress—Oxidative stress associated with the generation of ROS has been reported as a mechanism of toxicity of copper nanoparticle exposure (Ahamed et al., 2010; Fahmy and Cormier, 2009). We explored whether CuONP-induced cell viability loss was accompanied by a change in ROS generation in the exposed cells. Figure 3c shows intracellular ROS levels in A549 cells and HBEC after exposure to clean air or CuONPs. There was a small but significant increase of ROS after 4 hr exposure to air in A549 cells compared with control ($p < 0.001$) and with 2 hr exposure ($p < 0.01$). This was not observed in air-exposed HBEC. Two-hour exposure to CuONPs induced significantly higher level of ROS compared to control in A549 cells ($p < 0.01$) but not in HBEC ($p = 0.85$). Interestingly, 4 hr exposure to CuONPs significantly increased the ROS levels compared to control and clean air exposure in both cell types ($p <$

0.001), however, HBEC produced significantly higher levels of ROS ($270 \pm 39\%$ of controls) than A549 cells ($190 \pm 15\%$ of controls, $p < 0.001$) at this dose.

3.2.4. CuONPs induced proinflammatory chemokine release—To investigate the inflammatory response to CuONPs, the capacity of exposed cells to produce proinflammatory mediators was measured by quantifying the amount of IL-8 release. IL-8 plays a key role in the recruitment of inflammatory cells to the lung from the circulation (Pease and Sabroe, 2002). The levels of IL-8 released by cells exposed to clean air were not altered compared to incubator controls (Figure 3d) in either cell type. Compared with clean air exposure, CuONP exposure for 4 hr significantly increased the production of IL-8 in both cells types compared with incubator control, sham control and 2 hr CuONP exposure (all $p < 0.001$). The amount of IL-8 released from cells exposed to CuONPs for 2 hr or 4 hr were not significantly different between A549 cells and HBEC (both $p > 0.05$). This result indicated that A549 cells could release comparable amount of IL-8 with HBEC in response to CuONP exposure, even though there were fewer remaining viable A549 cells (37%) than HBEC (85%) after exposure to CuONPs.

3.3. Relationships between outcome variables and cell type, treatment and dose

GLMs were used to explore the relationships shown in Figures 3a to 3d and Table 3 between the outcome variables and cell type (A549, HBEC), treatment (air, CuONPs) and dose (0 (control), 2 and 4 hr exposure). Model sum of squares and p-values are provided in the Supplemental File (Table S1). This analysis showed that cell viability was highly significantly associated with all three independent variables, the two-way interactions (cell type*treatment; cell type*dose; treatment*dose) and the three-way interaction (all $p < 0.0001$). This indicates that the dose-dependent loss of viability induced by the CuONP treatment seen in both cell types was significantly enhanced in the A549 cells over the HBEC. Compared to HBEC, A549 cells demonstrated a 7.5-fold greater loss in viability at the low dose (2 hr) and a 4.2-fold greater loss at the high dose (4 hr). Thus, A549 cells represent a more sensitive *in vitro* model than human primary epithelial cells. Similar results were observed when the modeled dependent variable was LDH release from exposed cells or intracellular ROS production. IL-8 production from exposed cells was highly significantly related to treatment, dose and the interaction between treatment and dose (all $p < 0.0001$) but not to cell type, this model did not yield $p < 0.1$ for the three-way interaction nor for the two-way interactions involving cell type.

3.4. Effects of ROS inhibitor NAC on A549 cell toxicity induced by CuONPs

To determine whether the accumulation of intracellular ROS plays a major role in the loss of cell viability induced by CuONP exposure, we attempted to scavenge ROS in A549 cells with antioxidant NAC. As shown in Figure 4, A549 cells treated with 5 mM NAC but not exposed to CuONPs showed no reduction in cell viability but a small decrease in intracellular ROS level compared to incubator control ($p < 0.01$). Cells co-exposed to CuONPs together with 5 mM NAC for 4 hr were effectively protected from CuONP-induced toxicity compared to the cells exposed to CuONPs alone, with increased cell survival (from $38 \pm 3.9\%$ to $84 \pm 1.9\%$, $p < 0.001$) and decreased ROS production (from $190 \pm 13\%$ to 130

$\pm 2.7\%$, $p < 0.001$). These results demonstrated that NAC could blunt the toxic effects of CuONP exposure through reduction of the ROS generation in the exposed cells.

3.5. Comparison of *in vitro* with *in vivo* exposures to CuONP

Table 4 compares dose-response data for these *in vitro* studies with our prior murine studies of CuONPs (primary particle size (TEM) 12 ± 1 nm, crystalline phases (XRD) Cu, Cu₂O, CuO and surface area 12 ± 0.2 m²/g) that used subacute inhalation exposure (4 hr/day, 10 days) or intratracheal instillation exposure (24 hr). Although the CuONP was similarly sized (9.2–12 nm) the inhalation study used nebulization instead of the SDS for NP generation. Notwithstanding this limitation, there was good agreement in the toxicity measures (expressed throughout as percent of sham-exposed controls) for comparable doses expressed as ng/cm² of epithelial area. LDH release, an indicator of cell damage, yielded similar increases above sham-exposed controls for *in vivo* and *in vitro* studies. Release of IL-8 (CXCL8) from the human cells *in vitro* was comparable to the *in vivo* release of KC (CXCL1), the murine IL-8 homolog. Both chemokines are neutrophil chemoattractants released by macrophages and lung epithelial cells. Loss of cell viability in the *in vitro* studies was compared to a cytotoxicity index from histopathology examination of mouse lung tissue. The cytotoxicity index is the ratio of the ordinal lung cytotoxicity score for sham-exposed mice to CuONP-exposed mice multiplied by 100 to yield a percentage. Although a less direct comparison than LDH or chemokine production, there was good agreement for this outcome as well (Table 4).

4. Discussion

The adverse health effects elicited by Cu nanoparticles *in vivo* have previously been documented by our group. CuONPs induced inflammatory responses with increased recruitment of total cells and neutrophils to the lungs as well as increased total protein and LDH activity in bronchoalveolar lavage fluid (Kim et al., 2011; Pettibone et al., 2008). Many *in vitro* studies have investigated the toxic effects of CuONPs on airway cells in submerged culture (Ahamed et al., 2010; Fahmy and Cormier, 2009; Karlsson et al., 2008; Midander et al., 2009; Moschini et al., 2013) or at the ALI (Kim et al., 2013). Fahmy (2009) and Karlsson (2008) have reported that nano-sized CuO toxicity in human lung cells is higher than micron-sized CuO or other metal-based NPs.

In this work, we exposed human lung primary or epithelial adenocarcinoma cells to spark-generated CuONPs at the ALI to test the potential toxicity of these particles. We found that CuONP exposure significantly decreased the cell viability and increased LDH release from both cell types in a dose-dependent manner and CuONPs induced greater cytotoxicity in A549 cells than in HBEC. Here, the primary HBEC exhibit many properties comparable to the intact human airway epithelium including formation of a polarized epithelial membrane with tight junctions, surface microvilli, cilia and a covering of mucus (Karp et al., 2002). A549 cell monolayers without these structures were not protected from the toxic effects of CuONPs. Dose-dependent cytotoxicity of CuONPs has been reported in several other cell lines such as human cardiac microvascular endothelial cells (Sun et al., 2011) and hepatocarcinoma HepG2 cells (Piret et al., 2012).

Due to the interference of some NPs with the testing methods, it was suggested by Monteiro-Riviere (2009) that cytotoxicity of NPs needs to be assessed with more than one assay to validate the findings. In the evaluation of cytotoxicity of spark-generated CuONPs, we performed two different assays (Alamar Blue assay and LDH release detection) to enhance the strength of the viability data. LDH release results were in consort with the measures of cell viability. In our previous *in vitro* exposure study (Kim et al., 2013), manufactured CuONPs (particle size: 25 nm) were used for sequential exposure (4 hr exposure-2 hr break-4 hr exposure) of A549 cell at the ALI. It was observed that this exposure regimen induced 27% cell death, less than that of the present 4 hr exposure (62%). This difference may be due to the smaller nanoaerosol size in the present study.

The ability of NPs to induce oxidative stress in cells is a key factor in determining their toxicity, and also a fundamental mechanistic paradigm in nanotoxicology (Ahamed et al., 2010; Akhtar et al., 2013; Fahmy and Cormier, 2009). NPs may induce oxidative stress by triggering excessive generation of ROS in cells. ROS is a collective marker of hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and the hydroxyl radical ($HO^\bullet$). In this study, we found that intracellular ROS levels in both A549 cells and HBEC exposed to CuONPs were increased in a dose-dependent manner. Four-hour exposure to CuONPs significantly increased the ROS levels in both cell types, but HBEC produced significantly higher levels of ROS than A549 cells. CuONP exposure has been shown to increase intracellular ROS production leading to cell death in different types of exposed cells including *Escherichia coli* bacteria (Gunawan et al., 2011), human laryngeal epithelial cells (HEp-2) (Fahmy and Cormier, 2009), A549 cells (Akhtar et al., 2013; Kim et al., 2013), and HepG2 cells (Piret et al., 2012). Our results are in an agreement with these studies. We observed that the elevated intracellular ROS production was accompanied by increased cell viability loss and LDH release after 4 hr exposure to CuONPs, and the ROS level in HBEC was significantly higher than in A549 cells, while the loss of cell viability was significantly less than that of A549. Considering the ROS was produced and measured in viable cells, we normalized the ROS value of CuONPs exposed cells to its cell viability value to interpret the relation between oxidative stress and cytotoxicity. A549 cells had higher ratios (2 hr: 1.66, 4 hr: 5.26) of ROS to viability than HBEC cells (2 hr: 1.04, 4 hr: 3.12) after exposure to CuONPs. This suggests that the higher ROS measurement in HBEC after 4 hr CuONP exposure was likely due to higher cell viability compared with A549 cells. We demonstrated that filtered clean air did not produce cytotoxic effects on A549 cells (Figure 3a). There was, however, a significant increase of ROS after 4 hr exposure to particle-free clean air in A549 cells (Figure 3c, $p < 0.001$, compared with control). Thus, A549 cells also appear less resilient to particle-free clean air than HBEC.

NAC co-exposure with CuONPs significantly reduced cell death and intracellular ROS production. This result is supported by Piret *et al.* who stated that copper (II) oxide nanoparticles induced cellular toxicity in HepG2 cells and NAC treatment abolished the cytotoxic effect of CuONPs (Piret et al., 2012). We found that antioxidant treatment blunted the toxic effects of CuONPs and confirmed that oxidative stress plays an important mechanistic role in CuONPs-induced cytotoxicity. It has been proposed that there are different ways for NPs to generate ROS (Song et al., 2010). The NPs can induce ROS

generation on their surface due to their physiochemical characteristics; the NPs entering into cells can result in mitochondrial damage leading to the generation of free radicals; and the activation of NADPH-oxidase enzyme in phagocytic cells can induce ROS. These radicals can oxidize macromolecules (DNA, lipids and proteins) resulting in significant oxidative stress and cell death (Akhtar et al., 2013). Wang et al. (2012) confirmed that CuONPs were clearly located in both the cell nucleus and the mitochondria after being taken up by A549 cells through endocytosis, which produced DNA and mitochondrial damage resulting in apoptosis.

Escalation of oxidative stress may also induce production and release of proinflammatory chemokines. IL-8 is produced by alveolar macrophages, neutrophils, bronchial epithelial cells, and pulmonary microvascular endothelial cells. IL-8 responses in airway epithelial cells both *in vitro* and *in vivo* with exposure to a diverse set of inhaled particles were demonstrated by Duncan et al. (2014). In this study, we measured IL-8 due to its important role in the initiation and persistence of neutrophilic inflammation. Several studies have reported elevation of IL-8 expression or extracellular release as a result of CuONP exposure (Cho et al., 2012; Kim et al., 2013; Piret et al., 2012). We found a significant dose-dependent increase in IL-8 production in both cell types after exposure to CuONPs, and IL-8 production from exposed cells was highly significantly related to treatment and dose, but not to cell type.

After 4 hr exposure, the Cu concentration as measured by inductively coupled plasma-mass spectrometry was higher in the basal medium than that on/in the cells (data not shown). This may be due to longer exposure to CuONPs resulting in more cell death and leading to Cu release into medium. Meanwhile, loss of viability of adherent cells may provide an avenue for CuONPs to transit into the medium. Microscopic examination of the cells following 4 hr CuONP exposure revealed that some cells had detached from the transwell membrane. This result indicated that the measured cellular dose was probably influenced by the viability of the exposed cells and the solubility of CuONPs. The solubility of CuONPs is high and the presence of serum in exposure media may increase dissolution of CuONPs as suggested by Midander et al. (2009). Results from several other studies suggest many factors can influence the observed cellular doses of NPs exposure at the ALI including the size, shape, and solubility of the particles. Perhaps the most important factors are geometry and the flow conditions of the unit delivering the NPs to the cells (Bitterle et al., 2006; Kim et al., 2013; Lenz et al., 2009).

Comparison of these ALI *in vitro* studies to our prior murine inhalation and intratracheal instillation studies (Table 4) was enabled by exposure monitoring and the assessment of comparable measures of toxicity: LDH release, chemokine production and cytotoxicity. SMPS nanoaerosols data demonstrated that the SDS delivered 2.27×10^7 particles/cm³ to the exposure system with a NMD of 9.2 nm, a GSD of 1.48 and a mean of d_{PA} of 14.6 nm. With the density of Cu/Cu₂O/CuO particles of 7.5 g/cm³, a flow rate of 5 mL/min and 50% measured deposition efficiency at the ALI; we calculated a nominal dose delivered to the cells of 74 ng/cm² for 2 hr exposure and 148 ng/cm² for 4 hr exposure. This falls within the range of doses in our *in vivo* murine studies of 4 to 147 ng/cm². This dosimetry calculation uses the literature approximation of 680 cm² for the surface area of the mouse epithelium

(Pinkerton et al., 1992) and 1.12 cm^2 for the surface area of epithelial cells grown to confluence on the transwells. In our *in vivo* sub-acute inhalation study (Kim et al., 2011), mice were exposed 4 hr/day for 10 days and we delivered an estimated CuONP dose of $32 \mu\text{g}/\text{mouse}$ ($47 \text{ ng}/\text{cm}^2$) which induced LDH release, very high chemokine production and cytotoxicity. Mice exposed to three doses of CuONP by instillation exhibited dose-dependent increases in these outcomes measured 24 hr after dosing. While comparison of these *in vivo* data to the *in vitro* exposures represents an approximation due to variance in the underlying assumptions, it does support the utility of the ALI *in vitro* studies for hazard identification and for ranking potencies of one nanomaterial to another. Donaldson et al. (2008) previously found value in comparing neutrophils in bronchoalveolar lavage from rat inhalation studies of micron-sized TiO_2 and BaSO_4 to IL-8 production from A549 cells *in vitro*. We found greater benefit in employing similar toxicity measures in both *in vitro* and *in vivo* studies, that is, comparing LDH release, chemokine production and cytotoxicity across three study designs, inhalation, instillation and ALI *in vitro*.

5. Conclusions

Spark-generated CuONP exposure induced cytotoxicity, oxidative stress and IL-8 release in lung epithelial cells in a dose-dependent manner. A549 cells were more susceptible to CuONPs toxic effects than primary HBEC. Antioxidant NAC reduced CuONPs induced cytotoxicity by lowering the generation of intracellular ROS. These results support the use of A549 cells to evaluate NP toxicity *in vitro* with the caveat that these cells have enhanced susceptibility to cytotoxicity and oxidative stress over human primary bronchial epithelial cells. Comparison of nanotoxicity measures between *in vitro* and *in vivo* studies supported use of our integrated *in vitro* exposure system as a promising test platform for the evaluation of the impact of engineered nanoparticles on pulmonary health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institute for Environmental Health Sciences through Environmental Health Sciences Research Center in University of Iowa (NIEHS P30 ES005605). The authors thank Dr. Andrea Adamcakova-Dodd for helping with the experimental set up and reading the manuscript and making valuable suggestions, Sarah Perry for performing the statistical analyses and made contributions to the interpretation of the data, Mr. Tobias Krebs for technical support of ALI exposure system, Dr. Imali Mudunkotuwa and Dr. Daniel Unruh from the laboratory of Dr. Vicki Grassian for the XRD analysis, and Mr. Philip Karp for his technical assistance with the primary human airway epithelial cultures.

Abbreviations

ALI	Air-liquid interface
CuONPs	Copper oxide nanoparticles
CI	Confidence Interval
CO ₂	Carbon dioxide

H2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
d_{PA}	Projected area diameter
DMEM	Dulbecco's modified eagle medium
DPBS	Dulbecco's phosphate-buffered saline
ELISA	Enzyme-Linked Immunosorbent Assay
GLM	Generalized linear model
GSD	Geometric standard deviation
HBEC	Human Bronchial Epithelial Cells
HBSS	Hanks balanced salt solution
HEPA	High efficiency particulate air
hr	Hour
IL	Interleukin
LDH	Lactate dehydrogenase
LS Means	Least Squares Means
NAC	N-acetylcysteine
NP	Nanoparticle
NMD	Number median diameter
NO_x	Nitrogen oxides
O₃	Ozone
PA	Projected area
RFU	Relative fluorescence units
ROS	Reactive oxygen species
SD	Standard deviation
SDS	Spark discharge system
SE	Standard error
SMPS	Scanning mobility particle sizer
TEM	Transmission electron microscope
TNC	Total number concentration
XRD	X-ray diffraction

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Highlights

A stable nanoparticle aerosol for air-liquid interface exposure is generated.

A promising *in vitro* test platform for the evaluation of nanotoxicity is proposed.

A549 cells are susceptible to CuONP toxicity over primary bronchial epithelial cells.

A dose-dependent consistency of toxicity is comparable between *in vitro* and *in vivo*.

Figure 1.
Schematic of the experimental setup including a clean air supply, a spark discharge system (SDS), a charge neutralizer, a CO₂ supply, aerosol sampling devices (SMPS and TEM grids) and a gas (O₃/NO_x) monitor, and the *in vitro* exposure system (Vitrocell®) consisting of HD distributors, an ALI culture chamber and mass flow controllers. Flowpath A is for CuONPs exposures and flowpath B inserted a HEPA filter ahead of the HD distributor inlet for particle-free clean air exposures.

Figure 2. Characterizations of CuONPs from the SDS

(a) NPs size distribution showing NMD of 9.2 ± 0.17 nm and GSD of 1.48 ± 0.01 . (b) High resolution TEM showing primary particles and agglomerates. (c) NPs size evaluation from TEM projected area diameter indicating a mean size of 17.6 ± 12.0 nm. (d) X-ray diffraction showing the predominance of CuO and Cu₂O. (Voltage and current of SDS: 5 kV, 0.25 mA). All pictures showed are representative picture from three independent experiments.

Figure 3. Cell responses after exposure to clean air or CuONPs at the ALI

CuONP exposure induced a dose-dependent: (a) loss of cell viability that was lower in HBEC than in A549 cells ($p < 0.001$); (b) increase in LDH release, also lower in HBEC than in A549 cells after 4 hr exposure ($p < 0.001$); (c) increase in intracellular ROS production (2 hr exposure to CuONPs induced significantly higher level of ROS in A549 cells ($p < 0.01$) but not in HBEC ($p = 0.85$) than that in control cells, and 4 hr exposure significantly increased the ROS levels in both cell types ($p < 0.001$)); and (d) increase in IL-8 production where no significant difference was found between two cell types. Exposure to clean air for 4 hr produced a small increase in ROS but no change in viability, LDH or IL-8. Values are presented as percent of the incubator control and expressed as mean $\pm$ SD and analyzed using GLM. Data are representative of three independent experiments. Color lines in each figure indicate significant difference between cell types (red), treatments (brown), and doses (green), all the lines without asterisks denote *** $p < 0.001$, otherwise marked with * $p < 0.05$ or ** $p < 0.01$.

Figure 4. Antioxidant N-acetylcysteine (NAC) partially protected A549 cells from the toxic effects of CuONP exposure

A549 Cells co-exposed to CuONPs together with 5 mM NAC for 4 hr were protected from CuONPs induced toxicity with reduced cell death ($p < 0.001$), decreased ROS production ($p < 0.001$) and IL-8 release ($p < 0.001$) compared to the cells exposed to CuONPs alone.

Values were presented as percent of the incubator control. The data were expressed as mean $\pm$ SD of three independent experiments and analyzed using GLM procedure. Lines above the bars indicate significant difference between groups, the lines without asterisk denote *** $p < 0.001$, otherwise marked with ** $p < 0.01$.

Table 1
The size distribution and total concentration of CuONPs based on number, surface area and mass.

Parameter	Number base	Surface area base	Mass base
Median (nm)	9.2±0.2	12.7±0.4	14.7±0.4
Geometric Standard Deviation	1.48±0.01	1.49±0.01	1.50±0.02
Total concentration	$2.3 \times 10^7 \pm 1.2 \times 10^6$ particles/cm ³	$8.3 \times 10^9 \pm 7.6 \times 10^8$ nm ² /cm ³	170 ± 19 µg/m ³ $^{\dagger}86 \pm 17$ µg/m ³

[†] Measured by a gravimetric method

Table 2

LS Means and 95% CI of A549 cell viability after exposure to air, filtered CuONPs and CuONPs compared with incubator controls.

Group	N	LS Means	95% CI
Control	3	100.0	95.1, 104.9
Air	3	95.7	90.8, 100.6
Filtered CuONPs	3	91.5	86.7, 96.4
CuONPs	3	39.7	34.8, 44.6

Table 3

Least squares means (LS Means) and 95% confidence intervals (CI) for cell viability, ROS, and LDH analyzed using generalized linear models (GLM)

Cell type	Treatment	Dose	Viability (% of control)		ROS (% of control)		LDH (% of control)	
			LS Means	95% CI	LS Means	95% CI	LS Means	95% CI
A549	Air	Control	100.0	95.5, 104.5	100.0	88.7, 111.3	100.0	74.9, 125.1
		2 hr	106.7	100.4, 113.0	112.7	101.4, 124.0	83.6	48.1, 119.0
		4 hr	100.4	94.1, 106.7	138.9	124.3, 153.5	108.0	72.5, 143.5
	CuONPs	Control	100.0	95.5, 104.5	100.0	90.4, 109.6	100.0	74.9, 125.1
		2 hr	74.6	69.2, 80.1	123.5	110.9, 136.2	158.1	122.6, 193.5
		4 hr	37.2	31.7, 42.6	184.8	170.2, 199.4	288.6	253.1, 324.1
HBEC	Air	Control	100.0	95.5, 104.5	100.0	89.7, 110.4	100.0	74.9, 125.1
		2 hr	107.5	101.2, 113.8	104.6	90.0, 119.2	109.4	73.9, 144.9
		4 hr	109.1	102.8, 115.4	115.9	101.3, 130.5	104.4	69.0, 139.9
	CuONPs	Control	100.0	95.5, 104.5	100.0	89.7, 110.4	100.0	74.9, 125.1
		2 hr	97.9	91.6, 104.2	101.8	87.2, 116.4	116.9	81.4, 152.4
		4 hr	85.5	79.2, 91.8	66.9	252.3, 281.5	165.8	138.3, 193.2

Table 4Comparison of *in vivo* inhalation studies to *in vitro* studies.

Study	Exposure				Outcomes, % of Sham-exposed Mice or Cells			
	Model	Time, hr	CuO mass	CuO mass/area [‡] ng/cm ²	LDH	Chemokine Release [‡]	Cytotoxicity Index	Cell Viability
<i>In Vivo</i>	Inhalation	40	32 µg/mouse	47	336	4944	50	
<i>In Vivo</i>	Instillation	24	3 µg/mouse	4	118	204	100	
		24	35 µg/mouse	51	145	898	50	
		24	100 µg/mouse	147	209	3696	40	
<i>In Vitro</i> ALI [*]	ALI - A549	2	83 ng/well	74	167	299		55
		4	166 ng/well	148	373	1110		35
	ALI - HBEC	2	83 ng/well	74	152	225		90
		4	166 ng/well	148	306	1719		81

^{*} There is a separate sham-exposed control for each cell type and duration of exposure. ALI is air liquid interface exposure system.

[‡] Epithelial surface area for mice is 680 cm² and for cells on the transwells is 1.12 cm².

[‡] Pro-inflammatory chemokines: IL-8 for *in vitro* human cells and KC for the murine exposures *in vivo*