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Copper Oxide Nanoparticles Induce Oxidative Stress and Cytotoxicity in Airway Epithelial Cells

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Abstract

Metal oxide nanoparticles are often used as industrial catalysts and elevated levels of these particles have been clearly demonstrated at sites surrounding factories. To date, limited toxicity data on metal oxide nanoparticles are available. To understand the impact of these airborne pollutants on the respiratory system, airway epithelial (HEp-2) cells were exposed to increasing doses of silicon oxide (SiO₂), ferric oxide (Fe₂O₃) and copper oxide (CuO) nanoparticles, the leading metal oxides found in ambient air surrounding factories. CuO induced the greatest amount of cytotoxicity in a dose dependent manner; while even high doses (400 µg/cm²) of SiO₂ and Fe₂O₃ were non-toxic to HEp-2 cells. Although all metal oxide nanoparticles were able to generate ROS in HEp-2 cells, CuO was better able to overwhelm antioxidant defenses (e.g. catalase and glutathione reductase). A significant increase in the level of 8-isoprostanes and in the ratio of GSSG to total glutathione in cells exposed to CuO suggested that ROS generated by CuO induced oxidative stress in HEp-2 cells. Co-treatment of cells with CuO and the antioxidant resveratrol increased cell viability suggesting that oxidative stress may be the cause of the cytotoxic effect of CuO. These studies demonstrated that there is a high degree of variability in the cytotoxic effects of metal oxides, that this variability is not due to the solubility of the transition metal, and that this variability appears to involve sustained oxidative stress possibly due to redox cycling.

Keywords

Nanoparticles; HEp-2 cells; CuO; Oxidative stress; Resveratrol

Introduction

The massive increase in manufacturing and utilization of metal oxide nanoparticles has lead to major concerns regarding the potential health impact of these particles on the pulmonary system. Since these particles have a small aerodynamic diameter (<0.1 µm), they can escape air filters, contaminate ambient air, penetrate deep into the lungs, reach the alveolar region and evoke adverse pulmonary effects (Oberdorster *et al.*, 2005).

Many epidemiological studies have demonstrated a correlation between the level of nanoparticles (PM_{0.1}) in ambient air and a significant increase in pulmonary disease including

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Declaration of interest:

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exacerbation of bronchial asthma (Penttinen *et al.*, 2001; Weichenthal *et al.*, 2007). Since the chemical composition of airborne $PM_{0.1}$ varies significantly according to the location and time of sample collection, the exact particle responsible for adverse pulmonary effects has remained elusive. Experimental studies have supported the epidemiological findings and have provided evidence suggesting a role for oxidative stress in these events (Yang *et al.*, 2009). Oxidative stress generated in cells exposed to nanoparticles may stimulate inflammatory responses, oxidize lipids or even lead to cell death. Chemical analysis of different populations of $PM_{0.1}$ has demonstrated the elevated presence of metal oxide nanoparticles at sites surrounding factories as compared to remote (i.e. “cleaner”) areas (Rogaczewska and Matczak, 1985). Despite the increase in the levels of these particles in ambient air, epidemiological studies rarely focus on the health impact associated with the exposure to these specific particles.

At present, metal oxide nanoparticles are used in manufacturing of hundreds of commercial products, and their industrial applications are expected to expand during the next decade. Silica which is composed of SiO_2 , is one of the most abundant oxides present in ambient air and comprises (up to 8%) of all total airborne nanoparticles (Balduzzi *et al.*, 2004), typically in crystalline (quartz) or amorphous form. The amorphous form of silica is widely used in many industries and applications such as fillers in the rubber industry, anti-caking agents in powder materials such as paints and cosmetics (Merget *et al.*, 2002). Copper oxide (CuO) nanoparticles are used in antimicrobial preparations, heat transfer fluids, semiconductors or intrauterine contraceptive devices (Aruoja *et al.*, 2009). Ferric oxide (Fe_2O_3) nanoparticles are used as catalysts and in the manufacture of pigments (Montes-Hernandez *et al.*, 2006).

Oxidative stress is often used to explain toxicity associated with particle exposure. Although the ability of crystalline silica to generate oxidative stress in pulmonary cells has been demonstrated (Fanizza *et al.*, 2007), little is known about the ability of amorphous silica nanoparticles to induce oxidative stress. Amorphous silica nanoparticles demonstrate less ability to induce pulmonary inflammation (Warheit *et al.*, 1995) and fibrosis (Reuzel *et al.*, 1991) as compared to quartz particles of the same size. Furthermore, silicosis is associated with the exposure to quartz but not amorphous silica particles suggesting that these particles may have different toxicity profiles (Reuzel *et al.*, 1991).

Both copper and iron ions are able to generate oxidative stress (Moriwaki *et al.*, 2008). Although, oral administration of copper oxide nanoparticles induces hepatotoxicity and nephrotoxicity in exposed rats (Lei *et al.*, 2008), it is not known whether this toxicity is mediated by the generation of oxidative stress in the liver and the kidney tissues. Data demonstrating the toxic effect of ferric oxide nanoparticles remain controversial. While exposure to ferric oxide nanoparticles does not produce inflammation in vascular endothelial cells *in vitro* (Gojova *et al.*, 2007), it significantly decreases cell viability in cancer cells (Choi *et al.*, 2009). Although inhalation is the primary source of exposure to metal oxides in ambient air, data demonstrating the effect of metal oxide nanoparticles on the pulmonary system remain scarce. Therefore, comparative toxicological assessments need to be conducted to better understand the role of metal composition in the observed adverse pulmonary effects associated with the exposure to $PM_{0.1}$. Because of their presence in airborne particulate matter, we choose to investigate the biological effects of three nanoparticles: amorphous silicon oxide (SiO_2), ferric oxide (Fe_2O_3), and copper (II) oxide (CuO).

We hypothesized that different metal oxide particles will have different abilities to generate oxidative stress and alter cell viability based on the transition metal. The aim of this study was to compare the *in vitro* responses of respiratory epithelial cells following exposure to two types of commercially available metal oxide nanoparticles and amorphous SiO_2 nanoparticles. In particular, we investigated the intrinsic ability of silicon oxide, ferric oxide and copper (II) oxide nanoparticles to decrease cell viability and generate oxidative stress in respiratory

epithelial cells. Human laryngeal epithelial cells (HEp-2) were chosen, since they are used in many pulmonary toxicological assays (Rudolf *et al.*, 2001; Kvolik *et al.*, 2005) and represent target cells which are usually subjected to significant amounts of airborne particles.

Materials and Methods

Reagents

2',7'-dichlorofluorescein-diacetate (H₂DCFDA), 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), H₂O₂, oxidized glutathione (GSSG), reduced glutathione (GSH), β -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH), sulfosalicylic acid (SSA), superoxide dismutase (SOD) determination kit and glutathione assay kit were all obtained from Sigma (St Louis, MO). Copper (II) oxide (CuO) particles (30 nm, # 45407) and ferric oxide (Fe₂O₃) particles (20–40 nm, # 45007) were purchased from Alfa Aesar (Ward Hill, MA) and silicon oxide (SiO₂) (80 nm, # 4830HT) was obtained from Nanostructured & Amorphous Materials, Inc (Los Alamos, NM). Resveratrol was purchased from Axxora (San Diego, CA) and Alamar Blue was obtained from Invitrogen (Carlsbad, USA). All organic solvents were of Fisher optima grade (Fisher Scientific, Hampton, NH).

Methods

Cell culture and treatment

Human laryngeal epithelial cells (HEp-2 cells) were purchased from ATCC (Manassas, VA) and were cultured in 75 cm² flask at the density of 2×10^4 cell/cm² in Dulbecco's Modified Eagle's Medium-Reduced Serum (DMEM-RS), supplemented with 2% heat inactivated fetal bovine serum (FBS) at 37°C in a humidified incubator containing 5% CO₂. At 85 % confluence, cells were harvested using 0.25% trypsin and were sub-cultured into 75 cm² flasks, 6-well plates or 96 well plates. Cells were allowed to recover for 2 days prior to treatment. Particles were suspended in cell culture medium by pulse sonication (30 s on, 30 s off) using 50% amplitude (Sonics and Materials Inc, CT, USA) for 4 min to avoid particle agglomeration, followed by vigorous vortexing for 1 min prior to administration to the cells. A serial dilution was established by mixing equal volumes of particle suspension and cell culture medium followed by vigorous vortexing. All experiments were performed using HEp-2 cells at passage 10–20 and were replicated with at least two independent cell passages.

Cell viability assay

The cytotoxic effect of particles on HEp-2 cells was determined by the Alamar blue assay as previously described (Baudouin *et al.*, 2007). Briefly, HEp-2 cells were cultured in 96 well plates at the density of 2×10^4 cell/cm² and then, incubated with particles (4 to 400 μ g/cm²), suspended in cell culture medium (200 μ l/well) for 5 h to establish a dose response curve. Cell viability was estimated by measuring the emitted fluorescence of the reduced alamar blue using a plate reader (ex/em: 530/590) and was normalized to medium only treated cells (100% viability) and 0.1% saponin (0% viability). Unlike other cell viability assays, the presence of nanoparticles does not interfere with the Alamar blue assay (Simon-Deckers *et al.*, 2008). A dose-response curve consisting of log doses of particles and percent cell viability associated with the exposure to each dose was plotted using the nonlinear fit (Third order polynomial; Graphpad Prism 5 software, La Jolla, CA). To investigate the influence of resveratrol, desferoxamine and D-penicillamine on the cytotoxic effect of copper oxide, cells were co-treated with 100 μ M of resveratrol, 100 μ M desferoxamine or 100 μ M D-penicillamine prior to assessment of cell viability and the data were compared to medium containing 100 μ M resveratrol, 100 μ M desferoxamine or 100 μ M D-penicillamine; respectively (100% viability) and 0.1% saponin (0% viability).

Measurement of cellular reactive oxygen species (ROS)

The production of reactive oxygen species in HEp-2 cells was measured by pre-loading the cells with 10 μM 2,7-dichlorofluorescein diacetate (H_2DCFDA) at 37°C for 40 min in the dark in 6-well plate. The cells were then washed and incubated with particle suspension (80 $\mu\text{g}/\text{cm}^2$; 125 $\mu\text{g}/\text{ml}$) for 30 min at 37°C. After the treatment, cells were washed, scraped, lysed, pulse sonicated for 15 s (1 s on, 1 s off) using 50% amplitude, and centrifuged at $12,000 \times g$ for 15 min at 4°C. The intensity of DCF fluorescence in the cell lysate was measured using a plate reader (ex/em: 485/530) and was normalized to protein content measured by BCA protein assay (Thermo Fisher Scientific Inc., Waltham, MA).

Antioxidant enzyme activity

After treating the cells for 4 h with particle suspension (80 $\mu\text{g}/\text{cm}^2$), cells were washed with PBS, scraped, lysed, sonicated for 15 s (1 s on, 1 s off) on ice and centrifuged at $12,000 \times g$ for 15 min at 4°C. The supernatant (cell lysate) was removed and the protein concentration was measured by the BCA method. The activities of different antioxidant enzymes were then measured in the cell lysates.

The activity of SOD was measured using 15 μg protein of cell lysates. The cell lysates were incubated with xanthine oxidase enzyme and tetrazolium salt for 20 min at 37°C. The absorbance of the formazan salt resulting from the oxidation of tetrazolium salt was detected at 450 nm. The activity of SOD, expressed as percent inhibition of the formation of formazan, was then calculated.

The activity of catalase enzyme was measured as previously described (Aebi, 1984). An appropriate volume of cell lysate containing 50 μg protein was mixed with 1 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM H_2O_2 in 1 ml quartz cuvette. The decrease in absorbance of H_2O_2 was followed at 240 nm for 4 min. Catalase activity was calculated from the slope of the H_2O_2 absorbance curve and normalized to protein concentration.

The activity of glutathione reductase (GR) was directly measured as previously described (Guthenberg *et al.*, 1985) by mixing 50 μg protein of cell lysate with 1 ml of 0.1 M phosphate buffer supplied with 2 mM EDTA containing 20 mM NADPH and 20 mM GSSG. The decrease in NADPH absorbance was followed for 3 min at 340 nm. The activity of GR was calculated from the slope of NADPH absorbance curve and was normalized to protein content.

The activity of glutathione peroxidase (GPx) was measured using 50 μg protein of the cell lysates. The cell lysates were mixed with tert-butyl hydroperoxide (30 mM), reduced glutathione (2 mM), GR (0.5 unit/ml) and NADPH (0.25 mM) in 50 mM Tris HCl (pH 8) at 25°C. The decrease in NADPH absorbance was followed for 3 min at 340 nm. The activity of GPx was calculated from the slope of NADPH absorbance curve and was normalized to protein content.

Measurement of intracellular glutathione

HEp-2 cells grown to 85% confluence were exposed to nanoparticles (80 $\mu\text{g}/\text{cm}^2$) for 4 h. After the treatment, the amount of total and oxidized glutathione was measured by the recycling method (Rahman *et al.*, 2007). Particle-exposed cells were washed, scraped and lysed. Total glutathione was measured by reducing oxidized glutathione content using GR enzyme (3 units ml^{-1}) and NADPH (0.8 mM). The assay is based on the chemical reaction between GSH and DTNB to form TNB. The change in TNB absorbance was measured at 412 nm using a plate reader. To measure the amount of GSSG, 100 μl of cell lysate was incubated with 2-vinyl pyridine which covalently reacts with GSH but not GSSG, and the cell lysates were then treated

with GR, NADPH and DTNB. Data were compared to GSH and GSSG standard curves and were normalized to protein content. The ratio of GSSG to total glutathione was then calculated.

Detection of 8-isoprostane as a biomarker of lipid peroxidation

The supernatant of cells co-treated with CuO and resveratrol (100 μ M), deferoxamine (100 μ M) or D-penicillamine (100 μ M) for 4 h was collected, centrifuged at $12,000 \times g$ for 10 min to remove particles and cell debris and was used immediately to measure 8-isoprostane levels. 8-isoprostane, a biomarker of lipid peroxidation, was measured by a competitive enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical, Ann Arbor, MI) using 50 μ l of cell supernatant. The concentration of 8-isoprostane in samples were then calculated using an established standard curve.

Results

Cytotoxic effect of metal oxide nanoparticles on human laryngeal epithelial cells

To assess the toxicity of metal oxide nanoparticles, HEp-2 cells were treated with these particles at different doses (4, 8, 80, and 400 μ g/cm²) and viability after 5 h of exposure was determined. Cell viability decreased in a dose-dependent manner following exposure to CuO nanoparticles (Figure 1); however, SiO₂ and Fe₂O₃ nanoparticles were non-toxic (less than 10% non-viable) at all exposure doses tested as compared to cells treated with medium. Cell viability also decreased in a time-dependent manner with maximal cell death occurring within 5 h post-treatment. No morphological changes were observed in the epithelial cells during the exposure to CuO or Fe₂O₃ nanoparticles. CuO significantly decreased cell viability by 60% at the exposure dose of 1.9 log μ g/cm² (80 μ g/cm²). The cytotoxic effect of CuO nanoparticles was partially reversed when cells were co-treated with 100 μ M resveratrol (42% non-viable) suggesting that oxidative stress at least in part mediated the cytotoxic effect of CuO (Figure 2). In contrast, the copper chelators desferoxamine and D-penicillamine did not alter the cytotoxic effect of CuO on HEp-2 cells, indicating that CuO-induced cytotoxicity is mediated by the particles and not any dissolved metal fraction (Figure 2).

Metal oxide nanoparticles generate reactive oxygen species (ROS) *in vitro*

To compare the ability of metal oxide nanoparticles to induce oxidative stress in human epithelial cells, we first examined the ability of these particles to generate ROS in HEp-2 cells. Intracellular ROS was determined using 2,7 dichlorofluorescein diacetate (H₂DCFDA). H₂DCFDA diffuses into the cells and is hydrolyzed to H₂DCF, which reacts with ROS to form DCF. HEp-2 cells were exposed to silica, Fe₂O₃, and CuO (80 μ g/cm²) for 30 min; and DCF fluorescence was measured in cell lysates. Exposure of epithelial cells to SiO₂, Fe₂O₃ and CuO nanoparticles resulted in significant increases in DCF fluorescence (167%, 132% and 131%, respectively), when compared to cells not exposed to these particles (Figure 3).

Metal oxide nanoparticles alter the activity of antioxidant enzymes and the level of oxidized glutathione in cultured epithelial cells

When the production of ROS exceeds the ability of the cell to neutralize the effects of the radicals, accumulation of pro-oxidants occurs in the cell leading to a state of oxidative stress (Gilmour *et al.*, 2006). As oxidative stress in the cell increases, different biological outcomes such as change in the activity of antioxidant enzymes and the depletion of glutathione occur (Li *et al.*, 2003). To investigate the ability of metal oxide nanoparticles to alter the activity of cellular antioxidants, epithelial cells were exposed to each particle (80 μ g/cm²) and the activity of antioxidant enzymes was assessed. There was no difference in the activity of SOD in any of the exposed cell populations (Table 1). Epithelial cells exposed to Fe₂O₃ or CuO showed significant inhibition (21% and 25%, respectively) of catalase. However, no significant

differences were observed in SiO₂ exposed cells as compared to control cells, which were not exposed to particles. SiO₂ exposed cells exhibited a significant increase (15%) in the activity of GR, whereas CuO significantly inhibited (29%) the activity of GR in HEp-2 cells as compared to control cells not exposed to particles. The activity of GPx was significantly increased by CuO (150%); however, no significant differences were observed in SiO₂ exposed cells as compared to control (Table 1). Fe₂O₃ exposed cells demonstrated no differences in the activity of GR and GPx as compared to control. The ratio of oxidized to total glutathione increased (150%) after exposure to CuO, but not after exposure to SiO₂ or Fe₂O₃ (Figure 4). The oxidation of GSH indicates the inability of epithelial cells to scavenge ROS generated by CuO and the development of oxidative stress in these cells which may lead to oxidative damage.

Copper oxide nanoparticles increase 8-isoprostane production in HEp-2 cells

Isoprostanes are produced by the random non-enzymatic oxidation of cellular phospholipids by oxygen radicals. SiO₂ and Fe₂O₃ were excluded from this assay since they did not generate oxidative stress in HEp-2 cells as evidenced by normal ratio of GSSG to total GSH (similar to control cells exposed to medium) (Figure 4). In contrast, CuO induced oxidative stress, which can lead to oxidative damage and subsequently cell death. Analysis of culture supernatant from cells exposed to CuO nanoparticles indicated increased (1000%) levels of 8-isoprostane as compared to vehicle treated cells (Figure 5). Resveratrol, but not desferoxamine or D-penicillamine, significantly reduced (80%) the production of 8-isoprostane in CuO exposed cells.

Discussion

The manufacture and use of metal oxide nanoparticles is continuously expanding due to their wide applications and unique physicochemical properties. Since they have a very small size (<0.1 µm in diameter), they readily contaminate the environment and may pose a risk to humans. Thus, it becomes increasingly important to investigate and identify their possible toxicological effects and to identify which particles pose the greatest harm to human health. Since inhalation is a significant route of exposure to metal oxide nanoparticles, we have studied the impact of SiO₂, Fe₂O₃ and CuO nanoparticles on respiratory epithelial cells.

In our results there was significant variation in the ability of these particles to alter cell viability. CuO elicited a significant dose-dependent decrease in HEp-2 cell viability (Figure 1) as compared to cells treated with vehicle. In contrast, cell viability following exposure to SiO₂ and Fe₂O₃ remained unchanged; these particles were non-toxic to HEp-2 cells. Since all three particle types are fairly uniform in size, the number of particles per mg weight and the surface area was comparable between all of them. Thus, the chemical composition of the particles appears directly responsible for the decreased cell viability of HEp-2 cells. This is consistent with documented data demonstrating enhanced cytotoxicity following ingestion of CuO nanoparticles (Aruoja *et al.*, 2009). The mechanistic basis of this cytotoxicity; however was unknown.

Oxidative stress has been proposed as a common mechanism of cell damage induced by many types of nanoparticles (Stone *et al.*, 2007). We hypothesized that the cytotoxicity induced by CuO exposure in our studies was mediated by the generation of oxidative stress in these cells. Indeed, co-treatment with the antioxidant resveratrol mitigated the cytotoxic effect of CuO, suggesting that oxidative stress was responsible, at least in part, for the decreased viability. All metal oxide nanoparticles were able to generate ROS in HEp-2 cells; however, CuO was better able to inhibit the activity of catalase and GR enzymes and increase the activity of GPx as compared to cells exposed only to medium. This finding suggests that not only was CuO able to generate ROS (e.g. H₂O₂) in the exposed epithelial cells but also that it was more efficient

at blocking the antioxidant defenses of the cell as evidenced by an increase in the ratio of oxidized to total glutathione.

Usually cells respond to oxidative burden by fortifying their antioxidant defense mechanisms in order to protect themselves from any oxidative damage. However, if the defense mechanisms fail to neutralize the oxidative burden protein oxidation (Ramirez-Prieto *et al.*, 2006), lipid peroxidation (Gutteridge, 1995), DNA damage, mitochondrial perturbation and apoptosis occur (Li *et al.*, 2003). One of the eicosanoids produced by the oxidation of phospholipids is 8-isoprostane, a stable, water-soluble compound. Therefore, 8-isoprostane has been proposed as an ideal marker of oxidative stress and lipid peroxidation (Morrow *et al.*, 1995; Morrow and Roberts, 1997). The levels of 8-isoprostane in the supernatant of cells exposed to CuO was significantly elevated demonstrating that CuO induced oxidative damage in HEP-2 cells. Resveratrol completely protected HEP-2 cells from the oxidative damage associated with CuO exposure as evidence by the massive decrease in the level of 8-isoprostanes in the supernatant of these cells. However, it exhibited only partial protection from the cytotoxicity associated with CuO exposure in these cells. This suggests that oxidative stress was only partially responsible for the reduced viability induced by exposure to CuO and suggests that other mechanism(s) may be responsible for further reductions in cell viability.

Although SiO₂ and Fe₂O₃ were able to generate ROS in HEP-2 cells, the antioxidant defense system remained intact and no cytotoxicity was observed. Cell exposed to amorphous SiO₂ nanoparticles showed an increase in the activity of GR as compared to control. No differences in the activity of GPx, catalase, SOD or the ratio of oxidized to total glutathione were observed, suggesting that ROS generated in HEP-2 cells were scavenged by the antioxidant defense system and SiO₂ failed to generate oxidative stress. This was associated with the inability of SiO₂ particle to promote cell death even at the highest dose tested. Our data are consistent with previously published data demonstrating that amorphous silica nanoparticles do not significantly decrease the viability of respiratory epithelial cells and do not deplete GSH molecules (Cha and Myung, 2007). However, the exact reason why ROS was successfully scavenged in SiO₂ and Fe₂O₃, but not CuO, exposed cells is not clear. It is possible that different particles generated different radicals with different oxidative potency in HEP-2 cells.

Fe₂O₃ inhibited the activity of catalase enzyme in exposed cells, whereas, no differences in the activity of GR, GPx, SOD or the ratio of oxidized to total GSH as compared to cells exposed to vehicle were observed. Our data are consistent with recently published data demonstrating that Fe₂O₃ nanoparticles were not able to generate oxidative stress characterized by normal levels of oxidized DNA as compared to control in human alveolar epithelial cells (A549), even when these cells were incubated with Fe₂O₃ particles at the concentration of (40 µg/cm²) for 18 h (Karlsson *et al.*, 2008). Additionally, Fe₂O₃ nanoparticles (53 µg/cm²) were not able to decrease the viability of bronchial epithelial cells (BEAS-2B) cells after 24 h of exposure (Veranth *et al.*, 2007).

In our studies, CuO nanoparticles generated cytotoxicity at even the lowest doses. The cytotoxic effect of Cu²⁺ ion has been extensively studied. It has been proposed that Cu²⁺ decreases cell viability by binding to DNA resulting in DNA damage and cell death (Aruoma *et al.*, 1991). In addition, Cu²⁺ ions are able to induce apoptosis in neuronal cells by directly altering the expression of apoptotic genes (Chan *et al.*, 2008). Finally, it has been demonstrated that Cu ions can be released from the surface of CuO nanoparticles when they are suspended in Dulbecco's Modified Eagle's Medium and that the released component is responsible for some of the toxic effects (Midander *et al.*, 2009). Therefore, we tested whether Cu ions released from the particles to the cell media contribute in the cytotoxicity of CuO nanoparticles; however, both Cu-chelators utilized, desferoxamine and D-penicillamine, failed to mitigate the cytotoxic effect of CuO on HEP-2 cells. In addition, no differences in the level of 8-

isoprostane was observed in the supernatant of cells treated with Cu-chelators and CuO nanoparticles as compared to cells exposed to CuO only. Finally, desferoxamine and D-penicillamine are cell permeable and are capable of binding intracellular and extracellular free Cu ions should they become available. Our data suggest that even if Cu^{2+} ions were released in the cell or in the cell culture media, they do not significantly contribute to the cytotoxic effect or the oxidative damage associated with the exposure to CuO nanoparticles. This result is consistent with previous findings demonstrating that the dissolved portion of Cu from nanoparticles is insufficient to produce mortality in zebrafish exposed to CuO particles (Griffitt *et al.*, 2007).

Interestingly, different metal oxide nanoparticles have been shown to penetrate the cell membrane of respiratory epithelial cells with different efficacies (Park *et al.*, 2007). Once the particles are engulfed inside the cells, they are delivered to the lysosomes. Since the interior of the lysosomes is acidic (pH 4.5), many metal oxides, including CuO and Fe_2O_3 can be dissolved and released as Cu^{2+} and Fe^{3+} , respectively (Guo *et al.*, 2009). It remains possible that of all the particles studied here, CuO is the most efficient at penetrating the cell membrane and once inside the cell Cu^{2+} may be released and initiate the production of intracellular ROS or directly damage intracellular proteins prompting cell death. However, both Cu chelators, which can easily penetrate the cell membrane, did not mitigate the oxidative damage or cytotoxicity produced by CuO nanoparticles. This suggests that the biological effect seen in CuO exposed cells is least likely to be mediated through the release of Cu^{2+} ions either outside or inside the cells.

In our model, CuO but not Fe_2O_3 generated oxidative stress and induced cytotoxicity in HEp-2 cells. It has been proposed that transition metals such as Cu and Fe may contribute to the production of intracellular ROS via Fenton-type reaction (Stohs and Bagchi, 1995). Fe^{3+} and Cu^{2+} theoretically can be reduced by superoxide anion to Fe^{2+} and Cu^+ respectively, which then react with H_2O_2 to produce $(\text{OH}\cdot)$ (Stohs and Bagchi, 1995). However, the reduction capability of Fe^{3+} to Fe^{2+} in the absence of a reductant, which is crucial for initiation of the Fenton reaction, is not easily achievable under physiological conditions and occurs at an even slower rate when it is bound to chelators (Petrat *et al.*, 2003). Indeed, it was demonstrated that Fe_2O_3 nanoparticles are able to penetrate respiratory epithelial cell membrane and release Fe^{3+} in the acidic lysosomes, however, these Fe^{3+} ions were not reduced to Fe^{2+} under normal physiological conditions and hence did not cause oxidative stress (Guo *et al.*, 2009).

Collectively, it appears that the oxidative stress and the decrease in cell viability associated with exposure to CuO are generated by the particle itself rather than any released Cu^{2+} ions making the Fenton reaction an unlikely cause of oxidative stress in CuO exposed cells. On the other hand, Cu^{2+} rapidly reacts with superoxide making it difficult to scavenge and possibly allowing it to participate in redox cycling leading to sustained oxidative stress.

It has been proposed that the surface activity of metal oxide nanoparticles influence the biological effect of these particles. The manufacturing processes employed make the particles hydrophilic or lipophilic, catalytically active or passive and alter the electronic properties of the particle surface. It is possible that the surface of CuO has the ability to generate ROS such as H_2O_2 in cell medium which can then diffuse across the cell membrane of HEp-2 cells (Schubert and Wilmer, 1991). Alternatively, the extracellularly generated ROS may oxidize cell membrane lipids to produce 8-isoprostane which in turn can diffuse into the cell and initiate the production of intracellular or intramitochondrial ROS (Landar *et al.*, 2006). Interestingly, it was demonstrated that CuO nanoparticles were able to generate H_2O_2 in aqueous medium using O_2 in the presence of light (Bandara *et al.*, 2005). Additionally, CuO nanoparticles may directly oxidize membrane lipids and generate 8-isoprostane. Finally, CuO may penetrate the cell membrane and trigger an intracellular signaling network leading to the development of oxidative stress and apoptosis as previously documented with other metal oxide nanoparticles

(Park *et al.*, 2007). Understanding the relationship between the cellular response and the oxidative stress endpoints will be extremely useful in understanding the exact mechanism by which nanoparticles generate ROS in respiratory epithelial cells and should be further investigated.

Our data are to some extent consistent with a recently published report demonstrating the greater ability of CuO vs. Fe₂O₃ nanoparticles and CuO vs. Cu ions to induce cytotoxicity in a respiratory cell line (Karlsson *et al.*, 2008). In contrast to Karlsson's work, we observed the generation of significant levels of ROS in HEP-2 cells treated with CuO using the same assay (i.e. DCF fluorescence). The reasons for this are unclear and could simply be due to the use of different cell lines: A549 (human epithelial cells derived from a lung carcinoma) vs HEP-2 (human epithelial cells derived from an epidermoid carcinoma of the larynx) or different doses: 20 and 40 µg/cm² vs. 80 µg/cm² (the derived LD₅₀) of nanoparticles or different production mechanisms (Sigma vs. Alfa Aesar). Karlsson's data also clearly demonstrates that Cu ions at concentrations up to 20 µg/cm² have little effect on cell viability. To rule out the possibility that Cu ions were being released from CuO (either extracellularly or intracellular) and were themselves responsible for the adverse effects observed upon CuO exposure, we repeated many of our studies in the presence of two cell permeable Cu chelators, desferoxamine and D-penicillamine. Although our approach to address this same issue was different, we both arrived at a similar conclusion - Cu ions were not responsible for the observed adverse effects of CuO. Finally, our data extended Karlsson's work by demonstrating the following: 1) cell viability could be improved and oxidative damage reduced by alleviating the oxidative burden through the use of the antioxidant resveratrol, and 2) CuO diminished the activity of a variety of antioxidant enzymes.

In summary, our study demonstrated that there is significant variation among different metal oxide nanoparticles regarding their ability to generate oxidative stress and promote cell death. In fact, CuO nanoparticles were the only particles capable of inducing cell death. They were also the most potent at inducing oxidative stress. These effects were not due to the solubility of the transition metal and appear to involve sustained oxidative stress possibly due to redox cycling. This demonstrates that the chemical composition and possibly the reductive capacity of the particles have a great influence on the biological response of exposed cells. Exposure to CuO rather than Fe₂O₃ or SiO₂ nanoparticles may endanger human health and produce pulmonary diseases and/ or exacerbations of pre-existing respiratory diseases.

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Figure 1. Nanoparticles reduce human epithelial cell viability. Human epithelial (HEp-2) cells were incubated with increasing doses of SiO₂, Fe₂O₃ and CuO nanoparticles prior to assessment of cell viability. Data were normalized to cell viability of cells treated with medium only (100% viable) or cells treated with 0.1% saponin (0% viable). CuO significantly reduced cell viability as compared to vehicle only treated cells. Results are expressed as mean $\pm$ SE of 3 replicates (n = 4). Two-way ANOVA *p < 0.05 vs. vehicle.

Figure 2.

Resveratrol alters the cytotoxic effect of CuO in epithelial cells. Cell viability of HEp-2 cells exposed to CuO ($80 \mu\text{g}/\text{cm}^2$) with or without resveratrol (RV, $100 \mu\text{M}$), D-penicillamine (PA, $100 \mu\text{M}$) or desferoxamine (DF, $100 \mu\text{M}$) were assessed and were normalized to cell viability of medium-only, RV-only, PA-only and DF-only treated cells, respectively (100% viable) or 0.1% saponin treated cells (0% viable). Resveratrol but not desferoxamine or D-penicillamine alters the cytotoxic effect of CuO. Results are expressed as mean $\pm$ SE of 3 replicates, ($n = 4$). One-way ANOVA * $p < 0.05$ vs. CuO.

Figure 3. ROS generated in HEP-2 cells exposed to nanoparticles. Cells were pre-loaded with H₂DCFDA for 40 min and then treated with SiO₂, Fe₂O₃, CuO (80 µg/cm²) or vehicle. The intensity of DCF fluorescence was measured in cell lysates, and normalized to protein concentration. All particles generated ROS in HEP-2 cells. Data are expressed as mean ± SE of 2 replicates, (n = 4). One-way ANOVA, *p< 0.05 vs. vehicle, # p< 0.05 vs. SiO₂.

Figure 4. Ratio of oxidized to total glutathione in epithelial cells exposed to nanoparticles. The levels of GSSG and GSH were measured in cells exposed to SiO₂, Fe₂O₃, CuO (80 $\mu\text{g}/\text{cm}^2$) or vehicle and were normalized to protein concentration. CuO depletes GSH in exposed cells. Ratio is expressed as mean $\pm$ SE of 2 replicates, (n = 3). One-way ANOVA *p<0.05 vs. vehicle.

Figure 5.

Resveratrol reduces the level of 8-isoprostane induced by CuO. The level of 8-isoprostane was measured in the supernatant of cells co-treated with CuO ($80 \mu\text{g}/\text{cm}^2$) and desferoxamine (DF, $100 \mu\text{M}$), D-penicillamine (PA, $100 \mu\text{M}$) or Resveratrol (RV, $100 \mu\text{M}$). Resveratrol but not desferoxamine or D-penicillamine reduces the level of 8-isoprostane induced by CuO. Data are presented as mean $\pm$ SE ($n = 2$). One-way ANOVA * $p < 0.05$ vs. vehicle, # $p < 0.05$ vs. CuO.

Table 1

Cellular antioxidant enzyme activities following exposure to nanoparticles (80 $\mu\text{g}/\text{cm}^2$). The activity of superoxide dismutase (SOD), catalase, glutathione reductase, and glutathione peroxidase were measured and normalized to protein concentration. Data are expressed as mean $\pm$ SE of 2 replicates, (n =3). One-way ANOVA

Enzyme activity	vehicle	SiO ₂	Fe ₂ O ₃	CuO
total SOD (% inhibition rate)	66.28 $\pm$ 2.765	77.13 $\pm$ 3.609	78.30 $\pm$ 3.293	74.61 $\pm$ 2.949
catalase (unit/mg protein)	5.494 $\pm$ 0.0733	5.107 $\pm$ 0.1148	4.359 $\pm$ 0.2050*	4.105 $\pm$ 0.2176*
glutathione reductase (unit/mg protein)	0.0145 $\pm$ 0.0002	0.0163 $\pm$ 0.0005*	0.0140 $\pm$ 0.0003	0.0107 $\pm$ 0.0001*
glutathione peroxidase (unit/mg protein)	0.1684 $\pm$ 0.0070	0.1867 $\pm$ 0.0165	0.2039 $\pm$ 0.0163	0.3958 $\pm$ 0.0093*

*
p < 0.05 vs. vehicle.