Cellular Effects in an *In Vitro* Human 3D Cellular Airway Model and A549/BEAS-2B *In Vitro* Cell Cultures Following Air Exposure to Cerium Oxide Particles at an Air–Liquid Interface

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**Abstract**

There is a need for representative *in vitro* models to assess the effects of airborne particles on lung health. The objective of this study was to assess the cellular effects of cerium oxide (CeO2) particles exposed via an air–liquid interface in three relevant cell models in parallel. BEAS-2B, A549, and MucilAir™ cells were exposed for 1 hour to CeO2 particles comprising a range of doses (0.04–3.0 µg/cm²) and sizes (0.75 µm and 13.8 nm primary particle size). MucilAir cells are complex 3D cell systems with intact mucociliary system and are cultured at air–liquid interface, in contrast to the simple cell lines A549 and BEAS 2B, which are essentially immersion models. Cell responses varied according to cell type used. BEAS-2B and A549 cells (normally cultured in liquid media) were sensitive to air exposure alone and exposure to CeO2 particles in terms of cytotoxicity, inflammation, and genotoxicity. MucilAir cells normally cultured at an air–liquid interface were insensitive to the air stream alone and only exhibited oxidative stress response after exposure to CeO2 particles. Minimal or no effects on gene expression were detected in all cell types. Variations according to different sizes and doses of CeO2 were apparent but did not result in any definitive patterns. The minimal effects, except for the oxidative stress response, observed in the MucilAir cells are likely because of the presence of extracellular defensive mechanisms, such as intact mucociliary system, that were not present in the other cell types. Further studies with MucilAir cells and other compounds are warranted to understand wider applicability in inhalation toxicology research.

**Key words:** A549, air–liquid interface, BEAS-2B, cerium oxide, human epithelial airway model, nanoparticles

**Introduction**

Both epidemiological and animal studies are widely used to investigate potential adverse effects of airborne particles. However, the approaches have limitations in terms of interpreting outcomes and reproducibility. For animal studies, a key issue is that they are not always a good model to predict toxicity in humans because they are not fully representative of the human lung1 and respiratory tract, plus deriving mechanistic understanding from such studies is challenging on many levels. On top of this there is a general trend toward reducing the use of animals in such research. Therefore, there is a clear desire to move toward more widespread usage of *in vitro* models that are predictive of *in vivo* toxicity. For the assessment of airborne particles, there is a particular interest in lung cell-derived models that resemble human anatomical and cellular features as closely as possible.

Most *in vitro* studies that model respiratory processes use human cell lines derived from the epithelium of the airways and alveolar (gas exchange) regions with most of these studies focusing on the use of monocultures. However, coculture studies that attempt to model human exposure in a more meaningful way show that cellular responses can differ substantially from those seen in monocultures.2–7 Furthermore, missing morphological features and cellular mechanisms and the fact that many cell lines are used with a liquid exposure route (rather than a more relevant air exposure) mean that estimations of toxicological effects of particles are likely to deviate, sometimes substantially, from reality in humans.

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Interest in systems that more fully mimic cellular processes in humans is therefore warranted from the perspective of better predictability. One such approach is in vitro 3D cell models that include various cellular apparatus and metabolic pathways that more fully represent airway cells found in humans. We have previously shown that aggregated/agglomerated nanosized cerium oxide (CeO$_2$) does not induce distinct toxicological responses in one such 3D model (MuclAir™) but does in widely used BEAS-2B and A549 cell cultures. The 3D model was not insensitive to particles as positive responses were elicited in the presence of particles of zinc oxide. Rather, mucociliary defense mechanisms “cleared” CeO$_2$ before it came into contact with the cells.

The exposure route in the above experiments was via addition of particles in liquid, which, as alluded to earlier, is not ideal when compared to actual exposure in humans. We therefore wanted to investigate how this model performed when such CeO$_2$ particles are applied via an air–liquid interface (ALI). In line with our previous studies, toxicological outcomes in the MuclAir 3D cell model and BEAS-2B and A549 cell lines (which are normally cultured in liquid media) were investigated in parallel and under similar ALI exposure conditions to CeO$_2$ particles. We hypothesized, based on the previous studies, that (toxicological) responses in the MuclAir 3D model would be less pronounced than those in the cell lines because of the presence of cellular characteristics such as a mucociliary clearance system, real tight junctions, and intact repair/homeostasis mechanisms. Additionally, stress caused by exposure to air was investigated as a potential factor that might hamper meaningful toxicological assessments.

Methods

An overall schematic of the aerosol generation and exposure system and experimental setup can be found in Figure 1.

Aerosol generation and characterization of particles

The test atmosphere was generated by aerosolizing two different sizes of CeO$_2$ (termed micro- and nano-CeO$_2$; primary particle size 0.75 μm and 13.8 nm, respectively; according to Sigma-Aldrich MSDS data) using a turntable dust feeder, an eductor (Fux Valve Development Corp., and method of Cheng et al.) supplied with humidified compressed air at 0.8 kg/cm$^2$. The total air flow was 69.7 L/min for the generation of the micro-CeO$_2$ test atmosphere and 60.8 L/min for the nano-CeO$_2$ test atmosphere. The test atmosphere was introduced at the top of a buffer chamber and directed downward.

The test atmosphere for the high-dose group in both the 3D model and cell lines was extracted from the buffer chamber using a mass flow controller (Bronkhorst Hi Tec B.V.) connected to a vacuum source. For the mid- and low-dose groups, the test atmospheres were diluted using an AirVac educator, with 50% relative humidity from four separate Vitrocell™ modules, each module supporting three inserts (n = 3). In addition, two incubator controls were run in parallel (n = 2). The test atmosphere for the high-dose group was extracted from the buffer chamber using a mass flow controller connected to a vacuum source. For the mid- and low-dose groups, the test atmosphere was diluted using an AirVac educator, with 50% relative humidity among all dosed groups.

Air–liquid interface exposure system

A Vitrocell™ system (Vitrocell Systems GmbH) was used to expose cells at an ALI to CeO$_2$ aerosols. The setup consisted of four modules, each supporting three inserts (with adaptable well size) (Fig. 1). Each module had two parts, a turntable dust feeder and venturi that aerosolize the particles, a bellows to dilute the particles, and a buffer chamber to supply the test atmosphere. The test atmosphere was generated by aerosolizing micro-sized and nanosized CeO$_2$ via a turntable dust feeder and an eductor supplied with humidified compressed air. The test atmosphere was introduced at the top of a buffer chamber and directed downward. An air control, and high-, mid-, and low-dose groups were tested simultaneously for each particle size in four separate Vitrocell™ modules, each module supporting three inserts (n = 3). In addition, two incubator controls were run in parallel (n = 2). The test atmosphere for the high-dose group was extracted from the buffer chamber using a mass flow controller connected to a vacuum source. For the mid- and low-dose groups, the test atmosphere was diluted using an AirVac educator, with 50% relative humidity among all dosed groups.
base and a lid, and both were heated with circulating water to 37°C. The base contained three stainless steel wells filled with medium in which the inserts with the cells were placed. The lid contained three individual trumpet-shaped tubes through which the aerosol (or medical air as a control) was drawn into the three individual exposure inserts. To ensure equal dose per number of cells for each model, flow rates were adjusted to the well sizes. Flow rates were 5 mL/min (for A549 or BEAS-2B cells, 6 well size), or 1.5 mL/min (for MucilAir cells, 24 well size). The calculated final doses were 0.04 (low), 0.17 (mid), and 0.71 (high) μg/cm² cell culture area for the A549 and BEAS-2B cells, and, respectively, 0.15, 0.67, and 3.00 μg/cm² for the MucilAir cells (see Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/aivt). To ensure equal distribution of the aerosol among the three wells in each module, isokinetic sampled aerosol was directed to the wells by in-house-developed distribution tubes.

**Deposited dose**

To determine the actual deposited dose, a separate experiment was performed using MucilAir inserts using the same exposure conditions. After exposure the presence of Ce was analyzed using an Element XR High Resolution Inductively Coupled Plasma Mass Spectrometer (Thermo) in the basal medium during the exposure and the basal medium during the postexposure period and in the cell lysate (see section MucilAir, 3D human bronchial epithelia model, for cell treatment during and after the exposure).

**Experimental setup**

Two independent but similar exposures were performed for each cell type and each test substance, respectively termed exposures 1 and 2 (see Supplementary Table S2 for an overview). Cell culture medium was used for LDH and cytokine analyses (see section Cellular effects) after both exposures. Cells from exposure 1 were used for gene expression analyses, whereas cells from exposure 2 were used for HO-1 analyses (2/3 of the cells) and Comet assay (1/3 of the cells; see section Cellular effects). During each exposure, two untreated inserts were placed in an incubator (1 hour, 37°C, 5% CO₂). These incubator control cultures were used to compare air-exposed cells with cells that were not air-exposed in the Vitrocell system. Furthermore, during exposure 2, additional inserts were placed in an incubator to serve as positive controls for the Comet assay (cells treated with 25 μg/mL MMS, n = 2 inserts), the LDH assay (cells exposed to 0.1% Triton-X100, n = 2 inserts), and the cytokine analyses (cells exposed to a cytokine mix containing TNF-α, IFN-γ, and IL-1β in a concentration of 10 ng/mL, n = 2 inserts). Except for the placement in the incubator, the nonexposed and the positive control inserts were treated similarly to the air and CeO₂ exposed cells.

**MucilAir, 3D human bronchial epithelial model**

MucilAir are fully differentiated bronchial epithelial cell models (Epithelix Sàrl) and are reconstituted from primary human cells from healthy donors. According to the manufacturer of the model, the cells mimic in vivo tissue of the human respiratory epithelium through inclusion of a variety of cellular characteristics that are likely to provide some protection toward exposure to potentially toxic compounds. These features include the presence of goblet, basal, and ciliated cells; a mucus layer; ciliary beating; tight junctions; active ion transport; and various metabolic activities such as cytochrome p450 activity and the release of cytokines, chemokine, and metalloproteinase. We have previously shown that interactions between particles and biomatrices such as mucus present in the model are likely to mediate toxicological responses and potentially provide protection after exposure to particles.

Upon receipt, the MucilAir models were maintained in culture (on 24-well Transwell® culture supports at an ALI using MucilAir culture medium [supplemented with 1% amphotericin and 0.5% gentamicin] in a humidified incubator at 37°C with 5% CO₂) for at least 1 week before performing the experiments. Culture medium was refreshed every 2–3 days. The basolateral culture medium was refreshed approximately 24 hours before exposure. The MucilAir models were rinsed with saline (0.9% NaCl, 1.25 mM CaCl₂ and 10 mM HEPES buffer) approximately 24 hours before and again just before exposure to ensure that each individual model contained a mucus layer of comparable thickness. Just before exposure, the inserts were transferred to the exposure device (Fig. 1). The glass wells of the exposure device contained MucilAir culture medium to feed the cell monolayer from the basal side during the 1-hour exposure. Cells were exposed at a flow rate of 1.5 mL/min. Two independent but duplicate exposures were performed for each particle dosage. After exposure, the MucilAir inserts were cultured for another 24 hours under ALI conditions with 0.7 mL MucilAir culture medium at the basal site in a humidified incubator (∼37°C, 5% CO₂).

**Cell lines and culture conditions**

The human lung epithelial carcinoma cell line A549 (CCL-185) and the human bronchial epithelial cell line BEAS-2B (CRL-9609) were obtained from ATCC. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with Glutamax-I® supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator (∼37°C, ∼5% CO₂).

For air–liquid exposures, cells were seeded at a density of 40,000 cells onto track-etched polyethylene terephthalate (PET) membrane inserts (Falcon; BD Biosciences) in 6-well cell culture plates (4.2 cm² cell culture area/insert). After 72 hours, the inserts were transferred to the air–liquid phase with 3 mL of DMEM/HEPES cell culture medium and 1% FCS below the membrane.

Cells were cultured in air–liquid conditions for 16–24 hours before exposure. Just before exposure, the inserts were transferred to the exposure device. The glass wells of the exposure device contain DMEM/HEPES culture medium with 1% FCS, to feed the cell monolayer from the basal side during the 1-hour exposure. Cells were exposed using a flow of 5 mL/min. Two independent but similar exposures were performed for each particle type and size. After exposure, the cell inserts were cultured for another 24 hours under submerged conditions with 2 mL regular cell culture medium (DMEM Glutamax, 10%
FCS) on top of the cell insert (apical site), in a humidified incubator (≈37°C, 5% CO₂) as previously described.12,13

Cellular effects: cytotoxicity, cytokines, oxidative stress, Comet assay, and RNA isolation for microarray analysis

After the 24-hour postexposure period, the cell cultures were analyzed for markers of oxidative stress, inflammation, and cytotoxicity. For both cell lines, the apical cell culture media were collected. For MucilAir cells, the basal cell culture media were collected. Media were analyzed for the release of lactate dehydrogenase (LDH-assy kit; Roche), and the release of cytokines (IFN, MCP-1, IL-8, ICAM-1, IL-6, IL-13, IP-10, IL-1β) using multiplex analyses (Human Inflammation 20plex FlowCytomix Multiplex; Ebiosciences). From the first exposures (exposure 1), RNA was isolated using Nucleospin RNA II spin-columns, according to manufacturer’s instructions. RNA expression was analyzed using Illumina beadchip (humanHT-12v4). From the second exposures (exposure 2), 2/3 of the cells were used to determine heme oxygenase (HO-1) protein expression using an ELISA according to the manufacturer’s instructions (Enzo Life Sciences BVBA). The other 1/3 of the cells was used to measure genotoxicity using the comet assay. The comet assay procedure was based on the method described by Singh et al.14 and others.15,16 In short, two slides were prepared for the Comet assay from each culture. Cell suspension was mixed with 0.5% low-melting agarose (37°C) and spread onto each of the slides. The slides were then immersed in chilled lysis buffer (2.5 M NaCl, 0.1 M Na₂EDTA, 0.01 M Tris, and 1% Triton X-100 in distilled water, pH 10) and incubated at 2–10°C overnight. Thereafter, slides were incubated for 30 min in ice-cold electrophoresis buffer (0.3 M NaOH and 0.001 M Na₂EDTA in distilled water, pH >13) for DNA unwinding, followed by 30 min electrophoresis at 25 V and 300 mA in a Roth electrophoresis chamber (catalog number N610.1; Carl Roth GmbH & Co. KG) filled with ice-cold electrophoresis buffer and a three-times 5 min incubation in neutralization buffer (0.4 M Tris in distilled water, pH 7.5). The slides were immersed in ethanol for at least 5 min and air-dried. For scoring, each slide was stained with ethidium bromide. Fifty randomly chosen cells were analyzed per slide (Comet Assay IV; Perceptive Instruments Ltd.). In total 300 cells per treatment concentration were evaluated. Average median for each treatment concentration was calculated from the median score calculated from each slide. The presence of ghost cells was also recorded, as an indication of cytotoxicity (necrosis or apoptosis).

Microarray analysis

To further investigate how A549, BEAS-2B, and MucilAir cells were affected by the different CeO₂ particles (nano- and microforms), we performed microarray studies using Illumina beadchip (humanHT-12v4) to investigate any changes in gene expression after exposure. The microarray data were generated at Services XS, Leiden, The Netherlands. For the RNA expression analysis, website arrayanalysis.org17 was used to perform quantile normalization and quality control (QC). First, all samples were normalized together and a principal component analysis (PCA) plot was generated from all normalized samples as part of QC. Four samples (1 for A549, 2 for BEAS-2B, and 1 for MucilAir) were found to be outliers and were therefore removed from further analysis. Subsequently, normalizations, PCAs, and QC were performed for the samples of each model separately (no further outliers were detected).

Statistical analysis

We performed six independent experiments, that is, both nano-CeO₂ particles and micro-CeO₂ particles for each of the three cell systems used. Each experiment consisted of duplicate exposures, where the first was used for RNA analysis and the second was used for HO-1 protein analyses and Comet assay. LDH and cytokine analyses were conducted for both exposures. Each exposure had three wells for each dose group (control, low, mid, or high concentration). For each study parameter, we took the natural logarithm of the value measured in each well. The values were averaged over the triplicate wells and the difference between the average of the particle-exposed wells and the average of air control wells was calculated. Two-sided t-tests were conducted to judge if particle exposure resulted in different responses compared to air controls. The difference between particle exposure and air exposure was considered statistically significant if the p-value was less than 0.05; cases for which 0.01 < p < 0.05 are indicated with a single asterisk and cases for which p < 0.01 are indicated with a double asterisk in respective figures. The t-tests were based on averages of three wells under one and the same exposure.

LDH and IL-8 responses were studied in the exposures to air and to a particle concentration of 50 mg/m³, which had repeated exposures under the same conditions, to assess the random variation among the three wells relative to the random variation among similarly exposed units and relative to the systematic variation between different exposures. For example, the LDH–air exposure measurements involved 3 cell types, 6 independent exposures per cell type (including 2 pilot exposures not further mentioned), 3 wells per exposure, and a total of 54 measurements. Using a nested analysis of variance,18 we split the variation of the LDH measurements around the overall mean value into the variation among cell types, the variation among the six exposures within one cell type, and the variation among the three wells in one and the same exposure. A graphical representation is given of the nested analysis of variance in Supplementary Figure S1a (see ref.19 for the rationale behind the graphical analysis). Similar analyses were performed for the LDH–50 mg/m³ exposure measurements, as well as for the IL-8–air and IL-8–50 mg/m³ exposure measurements (Supplementary Fig. S1b, S1c, and S1d, respectively). We conclude that there are sufficient independent exposures to make the systematic differences clearly visible.

For the gene expression analysis, the normalized microarray data per model were processed via the R package limma20 for statistical analysis. CeO₂-exposed cells of a certain dose and particle size were compared with their coexposed air controls. The resulting p-values from all limma analyses were adjusted for multiple testing using the procedure of Benjamini and Hochberg21 to obtain false discovery rates (FDRs). Genes with an FDR <5% were considered statistically significantly regulated. Genes with an absolute fold change above 2 were considered to be regulated genes.
Table 1. Concentration of Cerium Oxide Particles (mg/m³)

<table>
<thead>
<tr>
<th>Model</th>
<th>Exposure</th>
<th>Dose group</th>
<th>Buffer chamber</th>
<th>Low</th>
<th>Mid</th>
<th>High</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A549</td>
<td>1</td>
<td></td>
<td>1083</td>
<td>47.9</td>
<td>183</td>
<td>969</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>953</td>
<td>45.6</td>
<td>165</td>
<td>881</td>
</tr>
<tr>
<td>BEAS-2B</td>
<td>1</td>
<td></td>
<td>964</td>
<td>44.5</td>
<td>171</td>
<td>874</td>
</tr>
<tr>
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<td>2</td>
<td></td>
<td>697</td>
<td>43.0</td>
<td>159</td>
<td>802</td>
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<tr>
<td>MucilAir</td>
<td>1</td>
<td></td>
<td>891</td>
<td>39.2</td>
<td>145</td>
<td>778</td>
</tr>
<tr>
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<td>2</td>
<td></td>
<td>986</td>
<td>44.3</td>
<td>174</td>
<td>878</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>929</td>
<td>44</td>
<td>166</td>
<td>864</td>
</tr>
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</table>

Actual concentrations of nanosized and microsized cerium oxide (CeO₂) particles (mg/m³) in the buffer chamber as well as in the low-, medium-, and high-dose groups (Vitrocell modules) for all three cell models measured by gravimetric analysis in two independent replicated experiments. The last row depicts the average of the values in that corresponding column.

(although not necessarily significantly regulated) and counted to look for trends in the number of regulated genes.

Results

Exposure

The concentration of the test atmosphere in the high-dose group nano-CeO₂ was close to the concentration in the buffer chamber (average exposure concentration was 864 mg/m³ compared to 929 mg/m³ in the buffer chamber; see Table 1). The mid- and low-dose nano-CeO₂ had an average concentration of 166 and 44 mg/m³, respectively. For the micro-CeO₂ exposure the efficiency of the generated test atmosphere was lower, resulting in a lower cell concentration (average exposure concentration was 428 mg/m³ compared to 1066 mg/m³ in the buffer chamber, see Table 1). The mid and low dose had an average concentration of 66 and 14 mg/m³, respectively. Aerodynamic particle size (APS) data indicated an average particle size of 1.6 (geometric standard deviation [gsd] 2.0) and 5.2 (gsd 1.8) μm for nano- and micro-CeO₂, respectively (Table 2). The final calculated doses of CeO₂ particles received by the A549 and BEAS-2B cells were 0.0357 (low dose), 0.1600 (mid dose), and 0.07143 (high dose) μg/cm². MucilAir models received 0.1500 (low dose), 0.6720 (mid dose), and 3.000 (high dose) μg/cm². Full elaboration of these calculations is given in Supplementary Table S1.

The mean deposition rate of Ce in all three cell types and basal media was 1% or less of the initial exposure concentration. SEM analyses indicated that nano-CeO₂ particles were regular round-shaped agglomerates, whereas micro-CeO₂ particles were irregular flake-shaped (Fig. 2). SEM analyses also indicated that particle concentrations were 5.1 × 10⁶ and 8.4 × 10⁵ particles/cm² for nano- and micro-CeO₂, respectively, and 14% of the nano-CeO₂ particles were <100 nm, whereas 11% of the micro-CeO₂ particles were <100 nm. The mean diameter of the particles was 300 nm for nano-CeO₂ and 370 nm for micro-CeO₂ (Table 3).

Cellular responses to air exposure in MucilAir and cell lines A549 and BEAS-2B

Figure 3 illustrates the cellular response of the three models to the control air exposure. The responses of LDH leakage and IL-8 release upon air exposure (without CeO₂) are expressed relatively to the incubator controls (no air) for the different cell types used. According to expectations, responses after exposure to air alone in the MucilAir cells did not differ from incubator controls. Air exposure of BEAS-2B cells, however, resulted in a significantly increased LDH response (132%, p < 0.05) and IL-8 release (173%, p < 0.001) compared to its incubator controls. Similarly, exposure of A549 cells to air alone resulted in significant increases of both LDH and IL-8 responses (233% with p < 0.001 and 125% with p < 0.05, respectively) compared to its incubator controls.

Cellular responses to CeO₂ particle exposure in MucilAir and cell lines A549 and BEAS-2B

The data on oxidative stress, inflammatory, cytotoxic, and genotoxic responses are expressed as the percentage change in the particle-exposed cells when compared to air control cells (Fig. 4).

Oxidative stress response. HO-1 expression in MucilAir cells was significantly increased at all doses in comparison to control (air alone) (p < 0.05 in all cases). Increases in expression of 158%, 151%, and 119% were observed for the low,
mid, and high dose, respectively (Fig. 4A). Upon exposure to micro-CeO₂, HO-1 expression in MucilAir cells also significantly increased at all doses ($p < 0.05$ in all cases). Increases in expression of 126%, 118%, and 108% were observed at low-, mid-, and high-dose exposures in comparison to air alone (Fig. 4A). No significant effects on HO-1 expression were seen in either A549 or BEAS-2B cell lines compared to their corresponding air controls upon exposure to any of the different doses or sizes of CeO₂.

Inflammatory cytokines and cytotoxicity (LDH). Exposure to CeO₂ resulted in detectable amounts of MCP-1, IL-13, and IL-1β in all cell systems and at (almost) all concentrations, but these amounts did not differ significantly from those in air control-exposed cells (data not shown). There was a significant increase in IL-8 release in BEAS-2B after nano-CeO₂ exposure at the highest dose (147%, $p < 0.05$; see Fig. 4B), but not in MucilAir cells or A549 cells. Microsized CeO₂ exposure did not result in any changes in IL-8 release in any of the cell types. The only significant increase in LDH leakage was seen for the highest dose of nano-CeO₂ in BEAS-2B cells (153%, $p < 0.05$; Fig. 4C). LDH leakage did not differ from air controls in all other cell types or exposure conditions.

Genotoxicity response. A dose-related increase in the tail intensity was observed for nano-CeO₂-exposed cell lines A549 and BEAS-2B but not for MucilAir cells (Fig. 4D). Exposure to micro-CeO₂ did not result in any significant increase in the tail intensity in comparison to control in all cell types and for all exposure conditions.

Global gene expression response. Only two genes in the micro-CeO₂-exposed MucilAir cells showed statistically significant changes in gene expression after a correction for FDR of ≤5% (Supplementary Table S3). This occurred at the low dose and high dose. No other statistically significant changes in gene expression were observed in any of the other cell types or when any cell type was exposed to nanosized CeO₂. Numbers of genes with a fold change ≥2 but not significantly up- or downregulated are also given in Supplementary Table S3.

A PCA of gene expression profiles (comprising 47,323 normalized gene expression levels in each sample) indicated that the MucilAir model differed from the cell lines in terms of gene expression profiles and that the cell lines clustered (Fig. 5). The analysis indicated that the first two principal components only accounted for approximately 30% of the variance in the data. PCAs based on the three cell types alone did not reveal any specific groupings based on whether samples were controls or had been exposed to CeO₂. The first two principal components in these analyses only accounted for <25% of variance in the respective datasets (data not shown). Taken together, these analyses suggest that the biggest factor influencing gene expression is cell type and that the effects of CeO₂ are very limited in terms of gene expression.

**Discussion**

We investigated a panel of cellular responses in MucilAir cells (a 3D bronchial epithelial model) after exposure via air to two different sizes of CeO₂ (termed nano- and micro-CeO₂). In parallel we examined A549 and BEAS-2B cells under similar conditions of exposure, with the assumption that responses would be different (the cell types differed in terms of respiratory tract anatomical source, morphology, and tumorigenic nature). Exposure did not result in significant effects in terms of inflammatory or cytotoxicity parameters and genotoxicity (Comet assay) in MucilAir cells, whereas responses were observed in the cell lines. Conversely, HO-1 responses were

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**Table 3. SEM Analyses Derived Nano-CeO₂ and Micro-CeO₂ Particle Concentrations and Mean Diameters**

<table>
<thead>
<tr>
<th>Particle characteristics</th>
<th>Nano-CeO₂</th>
<th>Micro-CeO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>PN ($n$/cm$^3$)</td>
<td>5.1E+06</td>
<td>8.4E+03</td>
</tr>
<tr>
<td>PN &lt;100 nm ($n$/cm$^3$)</td>
<td>7.4E+05</td>
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<tr>
<td>PN &lt;100 nm (%)</td>
<td>14</td>
<td>11</td>
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<tr>
<td>Mean diameter particles (nm)</td>
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</tr>
<tr>
<td>Mass mean diameter particles (µm)</td>
<td>1.5</td>
<td>4.0</td>
</tr>
<tr>
<td>Smallest particle detected (nm)</td>
<td>10</td>
<td>50</td>
</tr>
</tbody>
</table>

PN, particle number.
**FIG. 3.** LDH (A) and IL-8 (B) response of MucilAir, BEAS-2B, and A549 to air exposure (without any CeO\(_2\)). The biological LDH (A) and IL-8 (B) response (y axis) of the cells tested is expressed as %LDH and %IL-8 of air-exposed samples (n = 3) divided by the average expression of incubator control samples (n = 2). Indicated are the average and standard deviation of the resulting three ratios multiplied by 100 to obtain percentage values. The difference between air-exposed and incubator controls was considered statistically significant if the p-value was less than 0.05; cases for which 0.01 < p < 0.05 are indicated with a single asterisk (*), and cases for which p < 0.001 are indicated with a triple asterisk (***)

**FIG. 4.** HO-1 (A), IL-8 (B), LDH response (C), and Comet assay (D) of MucilAir (▲), BEAS-2B (■), and A549 (◆) for 1-hour exposure to nanosized (left column) and microsized CeO\(_2\) (right column). Assays were measured as described in Materials and Methods. The points represent the percentage of change in the CeO\(_2\)-exposed wells when compared to the air exposure group. The response to air is taken as 100% for each cell type. n = 3 for each point, except for IL-8 and LDH, for which n = 6. The difference between particle exposed and air exposures was considered statistically significant if the p-value was less than 0.05; cases for which 0.01 < p < 0.05 are indicated with a single asterisk (*), and cases for which p < 0.01 are indicated with a double asterisk (**)
observed in MucilAir cells but not in the cell lines. Gene expression analysis indicated that there were very limited effects of exposure to CeO₂ after correction for a 5% FDR. In short, cell responses in the MucilAir model exposed via air to CeO₂ were minimal compared to those of the cell lines.

**Dose**

The experimental setup used a simultaneous exposure mechanism to deliver air alone (control) and the low, mid, and high doses of each particle size to each cell type. Only very low levels of particles were deposited on the exposed cells after a 1-hour exposure. This is in line with previous studies with comparable exposure duration of 1 hour.13,22 Because of differences in well sizes between the MucilAir model and cell lines, flow rates were adapted to ensure that exposure doses were as close as possible between each cell type. We hypothesized that the protective characteristics associated with the MucilAir model would result in low or no effects at the low dose that was given to the cell lines and therefore adjusted upward the doses received by this model. In effect, the final low dose received by the MucilAir model matched the final mid-dose received by the cell lines. Even with this overlap and subsequent higher dose given to the MucilAir model, no LDH (cytotoxicity) or cytokine releases were observed. In line with our previous studies, these observations help support the notion that the protective physiology of the MucilAir model is likely preventing CeO₂ from reaching the underlying epithelial cells in sufficient amounts and this results in a lack of cellular responses.

**Exposure to particles**

Aerosolizing CeO₂ results in agglomeration or aggregation of particles. Such agglomerates or aggregates are likely to be the predominant form of particle that interacted with all types of cells in our experiments. Only a very small proportion of particles were below 100 nm in both the nano-CeO₂ and micro-CeO₂ (14% or 11%, respectively). Mean particle size was either 300 nm (nano-CeO₂) or 370 nm (micro-CeO₂), suggesting that there was only a minor size difference between the two sizes of particles. Such agglomeration or aggregation has been reported before.23,24 Given that agglomeration has such an effect on final particle size, we restricted our conclusions to view the two sizes of particles as two different exposures, but not draw conclusions on biological outcomes in relation to size of CeO₂.
Cellular responses upon exposure to air alone in MucilAir cells and cell lines BEAS-2B and A549

Exposure of both BEAS-2B and A549 cell lines to air alone in the exposure system resulted in increased LDH leakage and IL-8 release. This did not occur in the MucilAir cells. Such effects have been reported before for A549 cells in which increases in IL-8 and LDH leakage and reductions in cell viability were observed after exposure to air in a similar system. Although it is important to account for such effects in subsequent experiments, it highlights that detecting effects of particles in such cell lines when used at an ALI is likely to be difficult. This is simply because such cells, which are normally cultured under a liquid medium, can be stressed by exposure to air (the control we selected was air alone rather than no exposure to air at all). MucilAir cells did not express such stress to air, which we suggest may be because of the presence of a continuous mucus layer and distinct ciliary movement.

Cellular responses to CeO₂ particle exposure in MucilAir cells and cell lines BEAS-2B and A549

A consistent effect of exposure to CeO₂ particles in MucilAir cells was that HO-1 was elevated in comparison to air alone. This was independent of dose level or size of particle used (nano- or microsized). We have previously seen this effect after exposure to CeO₂ via droplets, and this supports the observation that some level of oxidative stress was induced by exposure to particles. Increases in HO-1 were not observed in the other cell lines. This merely suggests that there was no change at the time point measured and does not discount the possibility that these cells had already passed tier 1 in the hierarchical oxidative stress model, in which antioxidant enzymes are induced to restore cellular redox homeostasis.

IL-8 release and LDH leakage were observed only in BEAS-2B cells exposed to the highest dose of nanosized CeO₂. No similar effects were observed in A549 or MucilAir cells. As IL-8 release and LDH leakage are markers of inflammatory and cytotoxicity response, respectively, this suggests that BEAS-2B cells are sensitive to such a dose. A549 cells showed considerable LDH leakage after exposure to air alone (see Fig. 3), and so cytotoxic effects of CeO₂ may be masked by the effect of exposure to air or absent. IL-8 release was only limited upon exposure to air alone, and therefore we can only assume that CeO₂ has no effect on this parameter in A549 cells.

For MucilAir cells there was no evidence of increases in IL-8 release or LDH leakage after exposure to any dose or size of particle and air alone. Thus, CeO₂ most probably had no effect on cytotoxicity or inflammation in this experiment at the doses used. Whether this is a result of the protective physiological characteristics of MucilAir cells or lack of effects at the doses used is unclear.

We note that our previous study with CeO₂ via droplets used a dose of 30 µg/cm² and a 1.5-fold increase in IL-8.
was observed after a 3-hour exposure. Thus, the dose used in the current experiment (3.0 μg/cm²) might be too low to induce any significant inflammatory effects in the cell model.

Genotoxicity, according to Comet assay data, was observed in both A549 and BEAS-2B cells at certain doses and particle size but not in MucilAir cells. As alluded to earlier, this may be because of the protective physiological characteristics of MucilAir cells or that the dose used was simply too low to induce significant effects.

We investigated gene expression levels in the three different cell types after exposure to CeO₂ or controls, with the assumption that expression levels would be different. Only two genes in the MucilAir cells appeared to be significantly up- or downregulated after exposure to microsized particles of CeO₂ in comparison to control. No other changes in gene expression could be detected in the experiments. While some clustering was observed in a principal components analysis, the only plausible explanation was that cell type was the biggest factor influencing gene expression. The effects of CeO₂ were limited in terms of gene expression levels and this was probably because of the dose used in the study.

\textbf{Comparison of results derived from the use of different sizes of CeO₂}

It is clear from our data that exposure to nanosized CeO₂ resulted in the most evident effects, whereas exposure to microsized CeO₂ largely did not. We are cautious about concluding much from these observations in terms of linking the size of particles to cellular responses. As already discussed, agglomeration meant that the actual sizes of particles interacting with the cells were likely not that different.

Taken together, our data suggest that when CeO₂ is applied via an ALI to the widely used BEAS-2B and A549 cell lines, cytotoxicity and genotoxicity effects can be expected. Conversely, such effects cannot be expected in MucilAir cells. This is likely because of a combination of cellular characteristics offering protection from CeO₂, the low final exposure that is achievable in the apparatus used, and indeed that CeO₂ may simply be less reactive than originally expected. Although we have previously reported\(^a\) cellular effects in MucilAir cells upon exposure to zinc oxide and much reduced effects caused by CeO₂ exposure, further experiments are still required to delineate between these different confounding factors.

\textbf{Conclusions}

Cell responses to air exposure to different sizes (0.75 μm and 13.8 nm primary particle size) and doses (0.04–3.0 μg/cm²) of CeO₂ for 1 hour varied according to cell type tested. The bronchial epithelial cell line BEAS-2B and the tumor cell line A549 resembling the alveolar cell pneumocyte II cells were sensitive to air itself (based on an IL-8 and LDH response), and exposure to CeO₂ particles resulted in cytotoxicity (LDH), the release of (pro)inflammatory cytokines (IL-8), and genotoxicity (a positive Comet assay). The 3D bronchial epithelium model MucilAir, which harbors a variety of physiologically relevant cellular mechanisms, such as intact mucociliary system, was less sensitive to air exposure. MucilAir cells also responded to particles with an oxidative stress response only. Minimal or no effects on gene expression were detected in all cells. The combination of air exposure and the features with the 3D airway cell model made our experiments as realistic as is currently possible in terms of \textit{in vitro} testing of toxicological outcomes. Further studies with such 3D models and with other compounds are warranted to understand its applicability in risk assessments and wider research into mechanisms of action of particles that interact with the respiratory tract.

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\textbf{Author Disclosure Statement}

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