Toxicology in Vitro 29 (2015) 389–397

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Toxicity assessment of aggregated/agglomerated cerium oxide nanoparticles in an *in vitro* 3D airway model: The influence of mucociliary clearance

C. Frieke Kuper [a,*], Mariska Gröllers-Mulderij [a], Thérèse Maarschalkerweerd [b], Nicole M.M. Meulendijks [c], Astrid Reus [d], Frédérique van Acker [d], Esther K. Zondervan-van den Beuken [a], Mariëlle E.L. Wouters [c], Sabina Bijlsma [a], Ingeborg M. Kooter [b]

[a] TNO, Utrechtseweg 48, 3704 HE Zeist, The Netherlands
[b] TNO, Princetonlaan 6, 3584 CB Utrecht, The Netherlands
[c] TNO, De Rondom 1, 5612 AP Eindhoven, The Netherlands
[d] TNO Triskelion bv, Utrechtseweg 48, 3704 HE Zeist, The Netherlands

ARTICLE INFO

Article history:
Received 8 June 2014
Accepted 21 October 2014
Available online 4 November 2014

Keywords:
Cerium oxide
In vitro 3D human bronchial epithelial model
Mucociliary apparatus
Mucociliary clearance

ABSTRACT

We investigated the toxicity of aggregated nanoparticles of cerium oxide (CeO_2) using an *in vitro* 3D human bronchial epithelial model that included a mucociliary apparatus (MucilAir™). CeO_2 was dispersed in saline and applied to the apical surface of the model. CeO_2 did not induce distinct effects in the model, whereas it did in BEAS-2B and A549 cell cultures. The absence of effects of CeO_2 was not because of the model's insensitivity. Nanoparticles of zinc oxide (ZnO) elicited positive responses in the toxicological assays. Respiratory mucus (0.1% and 1%) added to dispersions increased aggregation/agglomeration to such an extent that most CeO_2 sedimented within a few minutes. Also, the mucociliary apparatus of the model removed CeO_2 from the central part of the apical surface to the borders. This 'clearance' may have prevented the majority of CeO_2 from reaching the epithelial cells. Chemical analysis of cerium in the basal tissue culture medium showed only minimal translocation of cerium across the 3D barrier. In conclusion, mucociliary defence appeared to prevent CeO_2 reaching the respiratory epithelial cells in this 3D *in vitro* model. This model and approach can be used to study compounds of specific toxicological concern in airway defence mechanisms *in vitro*.

1. Introduction

Nanomaterials are included in a wide variety of industrial and commercial products as they can confer novel properties and functionalities. Nevertheless, concerns have been raised about their safety profiles. One particular area of concern is that of airborne nanomaterials and the potential harms that may result in the respiratory tract (Oberdörster, 2001). Examples of exposure to such airborne nanomaterials include additives in diesel post-combustion and occupational exposure during fabrication of nanomaterials.

In vivo inhalation studies in animals are representative ways of safety evaluation, but the costs, time required and political and societal pressures to replace, refine and reduce (3Rs) the amount of animals used in research mean that other approaches are needed. *In vitro* reconstructed respiratory tract human tissues cultured at air–liquid interfaces are increasingly being used for safety assessments of inhaled substances (Randell et al., 2011; Nichols et al., 2013; Huang et al., 2008, 2011, 2013). They consist of fully differentiated (human) cells that are morphologically and functionally similar to those found *in vivo*. These include characteristics such as ciliary beating, mucus production and representative metabolic activities. As a result they are considered to be more representative than monolayer cell cultures such as BEAS-2B, an

Abbreviations: CeO_2, cerium oxide; EDX, energy-dispersive X-ray spectrometry; LDH, Lactate dehydrogenase; TNFα, Tumour necrosis factor alpha; IL-1α, Interleukine 1alpha; IL-8, Interleukine 8; MCP-1, Monocyte chemotactic protein 1 (CCL2); sICAM-1, Soluble intercellular adhesion molecule 1 (CD54); SEM, scanning electron microscopy; TEER, Transepithelial electrical resistance; ZnO, zinc oxide; LPS, lipopolysaccharide; MMS, methyl methanesulfonate.

* Corresponding author. Tel.: +31 (0)88 866 17 36.
E-mail addresses: frieke.kuper@tno.nl (C. Frieke Kuper), mariska.grollers@tno.nl (M. Gröllers-Mulderij), therese.maarschalkerweerd@tno.nl (T. Maarschalkerweerd), nicole.meulendijks@tno.nl (N.M.M. Meulendijks), astrid.reus@tno.triskelion.nl (A. Reus), frederique.vanacker@tno.triskelion.nl (F. van Acker), esther.zondervan-vandenbeuken@tno.nl (E.K. Zondervan-van den Beuken), marielle.wouters@tno.nl (M.E.L. Wouters), sabina.bijlsma@tno.nl (S. Bijlsma), ingeborg.kooter@tno.nl (I.M. Kooter).

http://dx.doi.org/10.1016/j.tiv.2014.10.017
0887-2333/

immortalized human bronchial epithelial cell line, and A549, an adenocarcinomatous human alveolar epithelial cell line.

The A549 cell line is the most widely applied *in vitro* system to test (nano) particle inhalation toxicity. This makes sense because the alveolar epithelial lining covers a far greater surface area than the respiratory lining. Moreover, respiratory tract toxicity evaluation in general has long been focused on lungs, because this was the site of most concern in humans. In addition, particles are reported to exert effects especially in the alveolar region (reviewed by Gradon and Sosnowski (2014)). However, particles may deposit predominantly on the respiratory epithelium (Lippmann et al., 1980; Oberdörster et al., 2005) that covers nasal passages, larynx, trachea and extra-pulmonary bronchi. These tissues are not routinely examined in inhalation studies, which may explain why knowledge surrounding particle deposition in these anatomical regions is relatively poor. The new OECD412 guideline for inhalation toxicity highlights the significance of these organs (OECD guideline on subacute inhalation toxicity, 2009).

Cerium oxide (CeO_2) nanomaterials have widespread use as (automotive) catalysts, in solar cells, gas sensors, and in metallurgic and glass and ceramic applications (Murray et al., 1999; Zheng et al., 2005). Many of these applications are dispersive in nature and are associated with increased risk of exposure with unknown health and safety implications. CeO_2 has been studied *in vivo* in various sub-acute inhalation studies. This includes an examination of the entire respiratory tract and the potential toxicity of CeO_2 nanoparticles (Cassee et al., 2011; Gosens et al., 2014; Landsiedel et al., 2014). These studies confirmed that the main target for cerium oxide is the alveolar region and not the respiratory epithelium despite high deposition rates. Moreover, numerous data are available on *in vitro* toxicity of CeO_2, with these data typically having been obtained via immersion exposure to cell lines (reviewed by US EPA, 2009). Despite this wealth of data, such cell lines lack key features (such as mucociliary apparatus) meaning that there is a risk that *in vitro* cellular assessments of toxicity may overestimate potential effects of compounds. We investigated the behaviour of CeO_2 nanoparticles in respiratory mucus and assessed toxicity in an *in vitro* 3D human airway model (MucilAir™; Epithelix Sàrl) that included a mucociliary apparatus. Nano particles of zinc oxide (with its toxicity being related to its solubility (Cho et al., 2013)) were used as a positive control particle. ZnO exerts *in vivo* toxicity in the upper respiratory tract as well as in the alveolar region (Vandebriel and De Jong, 2012; Landsiedel et al., 2014). We also assessed CeO_2 toxicity in A549 and BEAS-2B cell lines to benchmark our data.

2. Materials and methods

Commercially available CeO_2 nanoparticles (product number 544841) and ZnO nanoparticles (purity 99.99%, CAS #1314-13-2) were obtained from Sigma–Aldrich Chemie BV (Zwijndrecht, the Netherlands). Solutions of methyl methanesulfonate (MMS, 3 μg/cm^2; Sigma Aldrich), lipopolysaccharide (LPS (30 μg/cm^2; Sigma Aldrich) and Triton X100 (0.3 μg/cm^2; Sigma Aldrich) were also used as positive controls in the toxicity assays (see Table 1). Respiratory mucus was obtained from Epithelix Sàrl (Geneva, Switzerland).

2.1. Dispersions and dextran tablet exposure methods

Particle dispersions were prepared in saline (containing 10 mM HEPES and 1.25 mM $CaCl_2$), or Dulbecco's modified eagle medium (DMEM) and DMEM/fetal calf serum (FCS) at concentrations of 1 or 2 mg/ml, and sonicated on ice twice for 150 s with an amplitude of 210 μm on ice (Bransonic Sonifier S-250 D/S-250 A, 1/8″ tapered tip). The dispersions were vortexed for 5 s and put in a 37 °C water bath. Instead of via dispersions, particles can also be embedded into an inert and neutral substance as a carrier to avoid the problem of unstable dispersions. Dextran has been used as carrier to deliver particles onto the apical surfaces of MucilAir™. It is a bacterial by-product with the dextran macromolecule consisting of glycan groups linked end to end. It was tested by the supplier and found to be inert to MucilAir™ (Huang et al., 2013). The preparation of dextran tablets with CeO_2 and ZnO was done by Epithelix Sàrl (Geneva, Switzerland). In this experiment ZnO nanoparticles were used as a control.

2.2. Characterisation of CeO_2

2.2.1. Surface free energy, dynamic light scattering and zeta potential

CeO_2 was characterised under the exposure conditions, namely the dispersion of particles in the BEAS-2B and A549 media (DMEM and DMEM/FCS) and saline for the 3D model, at a temperature of 37 °C and at a representative concentration range. A Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) was used to determine zeta potential and hydrodynamic size distribution (via dynamic light scattering (DLS)). Average size and polydispersity index (PDI) were determined according to ISO22412. The PDI scale was 0–1 with 0 representing a monodisperse state and 1 representing a polydisperse state. Due to high conductivity of the dispersions (because of the high salt concentration in the media; measured with Cyberscan PCD 6500, Eutech®), and the influence of proteins in the media (which have their own zeta potential), measurement settings of the Zetasizer were adjusted to use an applied voltage of 50 V and a relaxation time between measurements of 45 s.

2.2.2. Scanning electron microscopy and element analysis

CeO_2 particle shape and size were determined with a Field Emission SEM (FEG-SEM/EDX; Tescan MIRA-LMH FEG-SEM). Dry powders of the particles were prepared by placing them onto conducting carbon tape for examination by SEM. CeO_2 particle dispersions (3D saline, with or without respiratory mucus) were filtered and dried on SEM stubs, and were analysed in Quantomix capsules, with the backscattered electron technique (BEI). Quantomix capsules are made from stainless steel coated with a thin polymer film, and can contain about 15 μl of dispersions. Element analysis was performed with energy-dispersive X-ray spectrometry (EDX) (Bruker AXS spectrometer with a Quantax 400 workstation and an XFlash 5030 detector with an active area of 30 mm^2 and super light element window (SLEW)), which allows X-ray detection of elements higher than boron ($Z > 5$).

2.3. 3D bronchial cell culture model (MucilAir™) and BEAS-2B and A549 cell cultures

2.3.1. 3D model

Fully differentiated bronchial epithelial MucilAir™ models (Epithelix Sárl, Geneva, Switzerland), reconstituted from primary human cells, were used for the 3D bronchial cell culture experiments. The models were maintained on 24-well Transwell® culture supports at an air–liquid interface using MucilAir™ culture medium (Epithelix Sàrl, Geneva, Switzerland; supplemented with 1% amphotericin, 1% penicillin/streptomycin and 0.5% gentamicin). The models were maintained in a humidified incubator at 37 °C and 5% CO_2. Upon receipt, the MucilAir™ models were maintained in culture for at least one week prior to performing the experiments. Culture medium was refreshed every 2–3 days. The basolateral culture medium was refreshed approximately one hour before exposure to the test compounds. The MucilAir™ models were also rinsed with saline containing 10 mM HEPES and 1.25 mM $CaCl_2$ to