In Vitro Assessment of Nano-Cerium Oxide ($nCeO_2$) and Nano-Ferric Oxide (nFe_2O_3) on Fibrogenic and Carcinogenic Potential

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ABSTRACT

The present study investigated the specific cellular responses to $nCeO_2$ and nFe_2O_3 in primary human lung cells and developed an *in vitro* sub-chronic exposure model to predict the potential fibrogenic and carcinogenic effects. Primary human lung fibroblasts were treated with $nCeO_2$, which induced a dose-dependent increase in collagen production (a hallmark of fibrosis). In separate experiments, primary human small airway epithelial cells were exposed to a sub-lethal concentration of $nCeO_2$ and nFe_2O_3 for 6 weeks to evaluate their effects on cell transformation and invasion. Our results showed that $nCeO_2$ induced proliferation of lung epithelial cells and nFe_2O_3 induced neoplastic-like transformation of epithelial cells, suggesting their potential carcinogenicity. The *in vitro* model and new data described in this study, demonstrate the impact and significance of a simple high-throughput platform to screen nanomaterial fibrogenicity and/or carcinogenicity, and address the critical need for evaluating nanomaterials for risk assessment.

Keywords: nanomaterial, human cell, *in vitro* model, fibrogenicity, carcinogenicity

Disclaimer: The findings and conclusions in this abstract are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

1 INTRODUCTION

Engineered nanomaterials (ENMs) including metal oxides and carbon nanotubes (CNTs) are currently being manufactured for wide applications, such as composite materials, process catalysts, electronic components, medical products, and cosmetics [1, 2]. Inhalation of these respirable particles has raised potential adverse human health concerns. Accumulating data have suggested potential lung disorders induced by pulmonary exposure to these EMNs in animal models. Recent *in vivo* studies indicated cancer promotion by multi-walled CNT (MWCNT) [3] and our previous *in vitro* work showed sub-chronic CNT exposure can cause cultured human lung epithelial cell transformation, and induce tumor formation when these cells were injected into nude mice [4]. These findings raise critical ENM-induced carcinogenesis concerns, especially with rapid growth of ENM applications.

Existing data are mainly on short-term toxic effects of ENMs, while nanoparticle-induced lung diseases are long-term/multi-step processes. Animal studies are limited due to the countless and rapidly expanding number of ENMs, since it is time consuming and cost prohibitive. The long-term exposure studies necessary for mechanistic investigations are lacking. The present study addressed current knowledge gaps on relevant low dose/long term cellular response of ENMs, and developed *in vitro* tools for evaluating fibrogenic or carcinogenic potentials of ENMs, especially $nCeO_2$ and nFe_2O_3 compared to MWCNT.

Based on previous studies showing the deposition of inhaled metal oxides in the deep lung tissues such as alveoli, interstitium and pleura, the present study investigated the specific cellular responses to metal oxides in target lung cell types, including fibroblasts and epithelial cells.

2 METHODS

2.1 Nanoparticles

Nano cerium oxide ($nCeO_2$, size dXRD = 17 nm, SSA = 61 m^2/g) and ferric oxide (nFe_2O_3, size dXRD = 20 nm, SSA = 50 m^2/g) were manufactured via the Versatile Engineered Nanomaterial Generation System (VEGNES) flame spray ionization, and subsequently sterile filtered by the Demokritou Laboratory at Harvard University, MA [5]. Particle stock solutions were prepared and sonicated according to previously

published protocols [6,7]. MWCNT (Mitsui, Inc.) suspensions were prepared by adding 1.5% bovine serum albumin (BSA) and sonicated for dispersion. All materials were characterized for x-ray diffraction diameter, zeta potential (NanoSeries ZetaSizer, Malvern Instruments), BET surface area, density, dynamic light scattering and Field Emission Scanning Electron Microscopy (FESEM).

2.2 Fibrogenesis Assay

For *in vitro* fibrogenic effect test, primary human lung fibroblasts (Lonza, Walkersville, MD) were cultured in 6 well plate and treated with $nCeO_2$ in triplicate at doses (0-2.5 μg/cm^2) which are relevant to *in vivo* exposure doses. Direct stimulation of collagen production (a hallmark of fibrosis) by $nCeO_2$ was evaluated by Western blotting.

2.3 *In Vitro* Sub-chronic Exposure of Nanoparticles in Cultured Human Lung Cells

Primary human small airway epithelial cells (pSAEC) (Lonza, Walkersville, MD) were exposed to a sub-lethal concentration (0.0625 μg/cm^2) of $nCeO_2$ and nFe_2O_3 in triplicate for 6 weeks and their effects on cell prolifiration was evaluated and compared to MWCNT (0.06 μg/cm^2), a known inducer of cell transformation *in vitro* [8, 9]. Saline and dispersant exposed cells were passaged throughout exposure as negative controls.

2.4 Cell Transformation Assays

Carcinogenesis hallmark assessments of the nanoparticle-treated pSAEC cells were performed by a set of cell transformation assays: 1) *Cell proliferation:* the nanoparticle-treated or control pSAEC cells (5x10^3/well) in 96-well plates were incubated in a no-particle culture medium for 24 h or 48 h. Cell proliferation was determined by WST-1 spectrophotometric assay. 2) *Cell migration and invasion:* the nanoparticle-treated or control pSAEC cells were suspended in media and held for 24 or 48 h, respectively, on Transwell migration or Matrigel® invasion inserts (BD Biosciences) in normal SAGM medium with 5% FBS containing SAGM in basal chamber as a chemoattractant. Inserts were DifCo stained, dried and photographed using bright field microscopy. 3) *Colony formation:* the nanoparticle-treated or control pSAEC cells (4.3x10^4) were mixed 1:2 in agar/media containing Difco agar, 15% FBS, 2x minimal MEM medium, Clonetics® growth supplements and 1% gentamicin. Suspended cells were slowly layered onto cooled agar/media in triplicate 6-well plates and allowed to solidify at a final cell density of 1x10^4. Colonies were photographed using dark field inverted microscopy at 14 and 21 days and were then counted.

2.5 Statistical Analysis

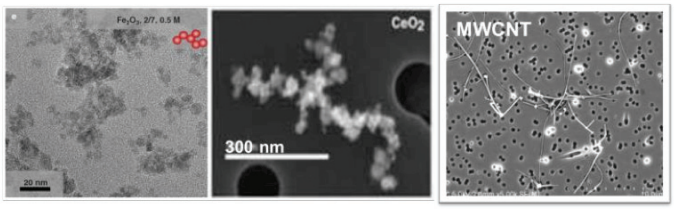
Significance of the treatments was determined by comparing values to the control using one-way ANOVA analyses followed by Dunnett's or Tukey-Kramer HSD post-hoc tests (α = 0.05).

3 RESULTS

3.1 Nanoparticle Characteristics

Nanoparticles used in the present study were dispersed as described in the "Materials and Methods" section. Characteristics of these nanoparticles are shown in Figure 1.

A



B

ENM	Manufacturer	Process	d_{XRD} (nm)	d_{BET} (nm)	Length (μm)	SSA (m^2/g)	d_H (nm)	ζ medium (mV)	ζ water (mV)
$nCeO_2$	Harvard University	VEGNES FSP	23.7	33.6	n/a	59	241 ±9.53	-10.8	ND
nFe_2O_3	Harvard University	VEGNES FSP	12.2	11.9	n/a	96	218	ND	20.7
MWCNT	Mitsui Inc.	CVD	8.2	n/a	81 ±5	26	78	-8.41	-40.6

Figure 1. Engineered nanomaterial characterization. A) TEM or SEM of nFe_2O_3 (left, Ref. 4), $nCeO_2$ (middle, Ref. 3) and MWCNT (right). B) resources of ENM, size, BET surface area and suspension characterization for each ENM. dXRD = x-ray diffraction, dBET = diameter based on surface area/density, SSA = specific surface area, dH = mean mode diameter in culture medium, z = zeta potential.

3.2 $nCeO_2$ induced collagen I *in vitro*, an indication of fibrogenic potential

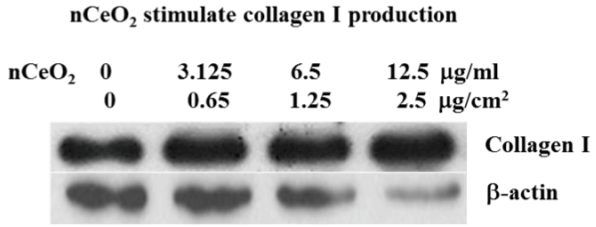


Figure 2. Effects of $nCeO_2$ on collagen I *in vitro*. Human lung fibroblast cells were untreated (0) or treated with the indicated concentrations of $nCeO_2$ for 1 (data not shown) and 3 days. Collagen I expression were measured using Western blotting and β-actin serves as a load-standard. Data showed that $nCeO_2$ induced a dose-dependent increase in collagen I production when compared to β-actin loading control by lung fibroblasts which suggested a direct fibrogenic effect of the $nCeO_2$. This data is consistent with our *in vivo* observation [Ref. 10] that pulmonary exposed $nCeO_2$ induced rat lung fibrosis in weeks.

3.3 $nCeO_2$ or nFe_2O_3 induced cell proliferation or cytotoxicity

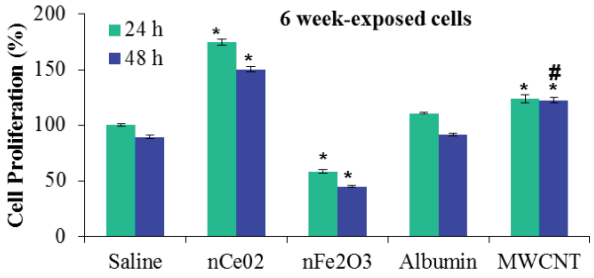
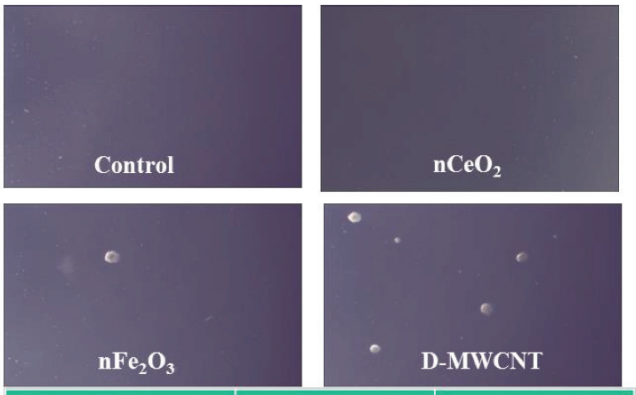


Figure 3. Primary human small airway epithelial cells were treated with $nCeO_2$ or nFe_2O_3 and un-treated cell served as passage control, while albumin dispersed multi-walled carbon nanotubes (MWCNT) which had been well-studied were used as a positive control with albumin only-exposed cell as a dispersion agent background control. After a continually sub-chronic exposure (6 weeks), the treated and control cells were tested for their cellular stimulation or cytotoxic effect using WST-1 assay for 24 or 48 hours. * and # indicate treatments different from Saline control and Albumin control, respectively ($P \leq 0.05$). Data showed cell growth stimulating effect of $nCeO_2$ while cytotoxic effect of nFe_2O_3 after 6 weeks in vitro exposure.

3.4 Induction of pSAEC attachment-independent growth by nFe_2O_3 *vs*. Dispersed-MWCNT



Nanomaterial	Population 1 Colonies	Population 2 Colonies
Saline	0	0
DISP (Albumin)	0	0
$nCeO_2$	0	0
nFe_2O_3	12	0
SWCNT	0	0
D-MWCNT	17	0

Figure 4. Counts of colonies displaying attachment-independent growth in soft agar assay for primary human SAECs exposed to different engineered nanometal oxides and carbon nanotubes. Primary SAECs were exposed for 6 weeks, passaged and then plated onto solidified agar supplemented with culture growth factors and 15% FBS. At Day 21, triplicate plates per population were counted and pooled.

3.5 Summary of sub-chronic expsoure bio effects of $nCeO_2$ and nFe_2O_3

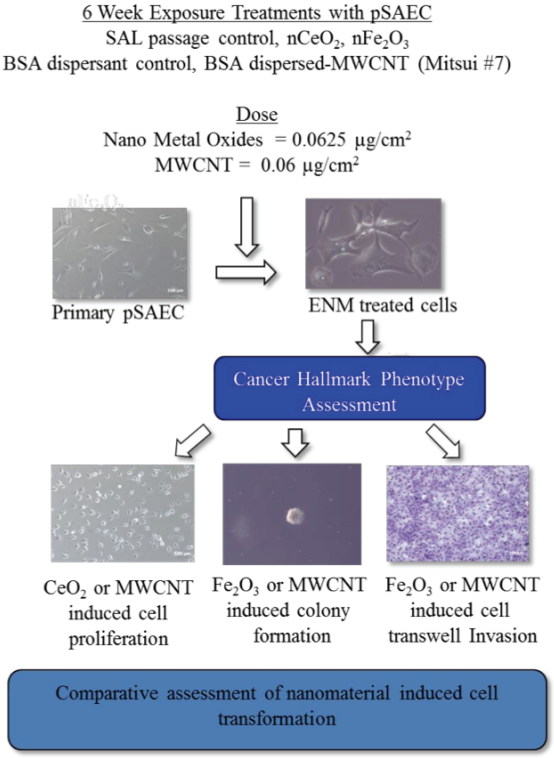


Figure 4. *In vitro* exposure and cancer hallmark screening model to assess subchronic nanomaterial exposure to primary human lung epithelium. $nCeO_2$, nFe_2O_3 and bovine serum albumin (1.5% w/v)-dispersed Mitsui #7 MWCNT stock suspensions were prepared from non-dispersed ENMs by sonication in ultrapure water. Neoplastic transformation assessment model of ENM-exposed primary small airway epithelial cells.

4 DISCUSSION

This study utilized primary human lung fibroblast cells to assess ENM exposure-associated bio-effects of $nCeO_2$, which resulted in direct collagen induction *in vitro*. This result is consistent with *in vivo* data showing $nCeO_2$-induced lung fibrosis in animals [10], thus validating the *in vitro* assay as a predictive tool to screen the fibrogenic potential of nanoparticles. Sub-chronic exposure studies showed that nFe_2O_3 induced neoplastic-like behaviors of pSAEC cells at an occupationally relevant dose, while MWCNT showed a modest induction compared to albumin dispersant control. Further isolation and characterization of nFe_2O_3 induced pSAEC colonies will be needed to determine the tumor-forming potential. These results will guide animal studies to confirm these observations and to examine the relevancy of our sub-chronic *in vitro* exposure model. The present data demonstrate the impact and significance of an *in vitro* high-throughput platform to screen fibrogenic and carcinogenic potential of nanomaterials and support *in vivo* studies, which collectively help to address the critical need for evaluating nanomaterials for risk assessment.

5. ACKNOWLEDGEMENTS

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