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***In vivo* epigenetic effects induced by engineered nanomaterials: A case study of copper oxide and laser printer-emitted engineered nanoparticles**

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Abstract

Evidence continues to grow on potential environmental health hazards associated with engineered nanomaterials (ENMs). While the geno- and cytotoxic effects of ENMs have been investigated, their potential to target the epigenome remains largely unknown. The aim of this study is twofold: 1) determining whether or not industry relevant ENMs can affect the epigenome *in vivo*; and 2) validating a recently developed *in vitro* epigenetic screening platform for inhaled ENMs. Laser printer-emitted engineered nanoparticles (PEPs) released from nano-enabled toners during consumer use and copper oxide (CuO) were chosen since these particles induced significant epigenetic changes in a recent *in vitro* companion study. In this study, the epigenetic alterations in lung tissue, alveolar macrophages, and peripheral blood from intratracheally instilled mice were evaluated. The methylation of global DNA and transposable elements (TEs), the expression of the DNA methylation machinery and TEs, in addition to general toxicological effects in the lung were assessed. CuO exhibited higher cell-damaging potential to the lung, while PEPs showed a greater ability to target the epigenome. Alterations in the methylation status of global DNA and TEs, and expression of TEs and DNA machinery in mouse lung were observed after exposure to CuO and PEPs. Additionally, epigenetic changes were detected in the peripheral blood after PEPs exposure. Altogether, CuO and PEPs can induce epigenetic alterations in a mouse experimental model,

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Declaration of interest

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which in turn confirms that the recently developed *in vitro* epigenetic platform using macrophage and epithelial cell lines can be successfully utilized in the epigenetic screening of ENMs.

Keywords

Printer-emitted engineered nanoparticles; copper oxide; epigenetics; DNA methylation; transposable elements

Introduction

The unique physicochemical properties of ENMs are being exploited for use in a variety of commercial nano-enabled products, including electronics, cosmetics, printer toners, and building materials, as well as a wide variety of products for medical applications (Bello et al., 2013; Pirela et al., 2014a; Pyrgiotakis et al., 2014, 2015; Setyawati et al., 2015a; Tay et al., 2015).

Evidence continues to grow and recent studies clearly demonstrate that exposure to ENMs may trigger epigenetic alterations in the target tissues and cells even at non-cytotoxic, low doses, in addition to cytotoxicity, induction of inflammatory response and oxidative stress, DNA damage and penetration of biological barriers (Chia et al., 2015; Choi et al., 2008; Cohen et al. 2014a; Demokritou et al. 2013; Gong et al., 2010; Halappanavar et al., 2011; Lu et al., 2015; Sharifi et al., 2012; Setyawati et al., 2013, 2015b; Sisler et al. 2014; Watson et al. 2014; Zhou et al. 2014). Epigenetics is defined as somatically heritable changes in gene expression without alterations in DNA sequence and includes methylation of DNA, histone modifications, regulation by non-coding RNAs and nucleosome positioning. Such epigenetic alterations are associated with the development and progression of numerous pathological states and diseases (Kelly et al., 2010), therefore, including epigenetic effects as part of risk assessment screening of exposures to nanoparticles is of paramount importance.

The ability of environmental stressors to target the cellular epigenome is becoming recognized. Of particular interest are the effects associated with the alterations in DNA methylation, an epigenetic mechanism responsible for the proper expression of genetic information in a tissue-, cell-, and sex-specific manner, as well as silencing of TEs (Jones, 2012). Only limited cellular studies exist indicating that short-term exposure to ENMs (*e.g.*, silica nanoparticles and CuO) may affect global and TEs-associated DNA methylation, as well as the expression of DNA methylation machinery (Gong et al., 2010; Lu et al., 2015). These influences may lead to reactivation and retrotransposition of TEs as well as genomic instability (Hedges et al, 2007). Particularly, the retrotransposon LINE-1 is the most abundant repetitive element in mammalian genomes comprising 17% and 19% of mouse and human genomes, and the changes in methylation and the associated aberrant expression of LINE-1 are reported in both human and experimental cancers, which has been recognized as one of various driving forces in the pathogenesis of numerous diseases (reviewed in Miousse and Koturbash, 2015).

Recently, a study by the authors describing a novel *in vitro* screening platform aimed to assess cellular epigenetic effects of inhaled industry relevant ENMs including PEPs (Pirela

et al., 2014a,b, 2015a,b), CuO, and titanium oxide (TiO₂), as well as mild-steel welding fumes (MS-WF) served as control particles (Lu et al., 2015). It was reported that 24 h *in vitro* exposure to these particles at low or non-cytotoxic dose (0.5 and 30 µg/mL), led to epigenetic alterations in both human and mouse macrophages as well as human small airway epithelial cells. The most pronounced changes were observed following exposure to PEPs and CuO, which led to affect the methylation and expression of the most abundant TEs in mammalian genomes – LINE-1 and SINE/*Alu*, and the DNA methylation machinery.

The main goal of this companion *in vivo* study was to validate the *in vitro* findings of the study described above, using a mouse experimental model. Particularly, the epigenetic assessment was performed on the murine lung tissue and alveolar macrophages – target cells that constitute the first line of the pulmonary defense against inhaled particles (Hiraiwa and van Eeden, 2013). Furthermore, it was of interest to identify possible noninvasive epigenetic markers in the peripheral blood of the exposed animals which served as potential biomarkers of exposure. Figure 1 summarizes the overall research strategy and experimental design for both the *in vivo* and the previously published *in vitro* studies (Lu et al., 2015).

Methods

Sources and characterization of ENMs

Two types of ENMs – a widely used in commercial applications CuO and PEPs were used in this *in vivo* validation study, which were part of the materials tested in the *in vitro* companion study (Lu et al., 2015). The properties for both CuO and PEPs nanoparticles are described in great detail in the previous studies (Lu et al., 2015; Pirela et al., 2014a). In summary: 1) the primary particle size of CuO was 58.7 nm determined by the nitrogen adsorption/Brunauer–Emmett–Teller (BET) method (Lu et al., 2015); 2) PEPs generated from Printer B1 (as previously referred to the recent publication) possess complex chemistries and contain large amounts of organic carbon (96.51%), elemental carbon (0.48%), and metals (2.67%). The primary particle size of PEPs was below 100 nm detected by both transmission electron microscopy and real time aerosol instrumentation (Pirela et al., 2014a).

In vitro and *in vivo* dosimetric considerations

It is of paramount importance to bring *in vitro* and *in vivo* doses to same scale for the validation purposes (Cohen et al. 2014b; Pal et al., 2015; Pirela et al., 2015a). Based on previous studies (Kroll et al., 2011; Landsiedel et al., 2014), alveolar surface area of rodent lung and the surface area of *in vitro* configuration were used to convert the *in vivo* instillation dose to the equivalent cell delivered *in vitro* dose. The integrated dosimetric platform previously developed by the authors was used to the conversion of cell administered dose and cell delivered dose (Cohen et al. 2014b; DeLoid et al. 2014; Pal et al., 2015). In more detail:

The average mouse weight used in this study was 25 g and the alveolar surface area of adult mouse is 82.2 cm² (Knust et al., 2009). The *in vitro* configuration used for epigenetic studies of our *in vitro* companion paper was 100 mm diameter dish with surface area of 78.5 cm²

and the exposure volume was 8 mL for each dish (Lu et al., 2015). According to the mouse body weight instillation dose, the total particle mass instilled to each mouse and mass per alveolar surface area were calculated. Moreover, based on the 78.5 cm² surface area of the *in vitro* dish, the equivalent cell delivered *in vitro* mass dose was counted. Because of the particokinetics of the ENMs in the media suspension that define their settling rate and *in vitro* dosimetry (Cohen et al., 2014b), the mass delivered to the cell is not necessarily equal to the mass administered *in vitro*. Therefore, the fraction of administered particle mass that is deposited on the cells as a function of *in vitro* exposure time (f_D) needs to be calculated in order to match the *in vivo* lung deposited dose. The f_D as a function of *in vitro* exposure time is calculated using the hybrid experimental/numerical Volumetric Centrifugation Method-*In vivo* Sedimentation, Diffusion and Dosimetry (VCM-ISDD) methodology recently developed by the authors (Cohen et al. 2014b; DeLoid et al. 2014; Pal et al. 2014). The mean media-formed agglomerate hydrodynamic diameter (d_H) and the VCM-measured effective density of formed agglomerates (DeLoid et al. 2014) were used as input to the VCM-ISDD fate and transport numerical model in order to estimate the f_D as a function of time. Based on our companion *in vitro* study (Lu et al., 2015), the f_D was detected for CuO and PEPs suspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 100 U/mL penicillin–streptomycin (DMEM/10%FBS, Life Technologies) or small airway epithelial cell growth medium with the SAGM bullet kit (SAGM, LONZA, Allendale, NJ) for a 24 h *in vitro* exposure using 100 mm diameter dishes. Thus, the cell administrated *in vitro* mass dose was calculated by the equivalent cell delivered *in vitro* mass dose divided by specific f_D .

Animal protocol

Eight-week-old BALB/c male mice weighing approximately 25 grams were purchased from Taconic Farms Inc. (Hudson, NY). Mice were housed in an environmentally controlled room at $25 \pm 1^\circ\text{C}$ with a relative humidity of $50 \pm 10\%$ under a 12-h light/dark cycle and allowed to acclimate for 1 week before the studies were initiated. Food and water were provided *ad libitum*. The mice were randomly assigned to two groups (group 1 and group 2). Group 1 is used for immediate sorting of alveolar macrophages from the lung tissue of exposed mice, and the lungs from group 2 animals were directly stored at -80°C for epigenetic analysis. In each group, there were three exposures (4 mice per exposure): vehicle control (deionized water at 2.5 mL/kg), CuO at 2.5 mg/kg body weight, and PEPs at 2.5 mg/kg body weight. Mice were intratracheally instilled with these treatments and sacrificed 24 h after administration with an intraperitoneal injection of Fatal-Plus (0.2 mL of 78 mg/mL, Vortech Pharmaceuticals, Ltd, Dearborn, MI). Subsequently, blood samples were collected from the inferior vena cava with anticoagulant tubes, and centrifuged at 200 g for 15 min at 4°C . The resulting pellets were collected and stored at -80°C for epigenetic analysis. All the protocols and studies involving the animals were conducted in accordance with the guiding principles in the Use of Animals in Toxicology and the Animal Care and Use Committee of Harvard University.

Sorting of alveolar macrophages from bronchoalveolar lavage fluid (BALF) using flow cytometry

After 24 h, mice from group 1 were anaesthetized with an intraperitoneal injection of Fatal-Plus (0.2 mL of 78 mg/mL), and sacrificed by exsanguination. Bronchoalveolar lavage (BAL) was performed by lavaging the lung with 12 washes of sterile 0.9% saline using 1 mL of saline for the first lavage, then 0.75 mL for subsequent washes. The pellet from all the BALF was collected. The first two washes of the BAL were analyzed for the levels of lactate dehydrogenase (LDH) and myeloperoxidase (MPO) following a published protocol (Beck et al., 1982).

Viable cells in BALF pellets were counted, and resuspended in Cell Staining Buffer (Biolegend, San Diego, CA) at $5-10 \times 10^6$ cells/mL and distributed 100 μ L/tube of cell suspension ($5-10 \times 10^5$ cells/tube) into 2 mL plastic tubes. Then, anti-mouse CD16/CD32 antibody (Biolegend) was used to block Fc receptors by pre-incubating cells with 10 μ g/mL purified antibody on ice for 10 min. Next, appropriately conjugated fluorescent antibodies (Anti-Mouse CD45 PerCP-Cyanine 5.5 (eBioscience, San Diego, CA) and Brilliant Violet 605TM anti-mouse CD11c Antibody (Biolegend) were added at predetermined optimum concentrations based on each antibody protocol and incubated on ice for 15 min in the dark. After washing twice with at least 2 mL of Cell Staining Buffer by centrifugation at 350 g for 5 min at 4°C, the cell pellets were resuspended in 0.4 mL of Cell Staining Buffer and 5 μ L 4',6-diamidino-2-phenylindole (DAPI, 1 μ g/mL, Biolegend) was added to exclude the dead cells. After incubation on ice for 3 min in the dark, the cells were sorted by a BD FACSARIAIIu (488 nm 25mW Sapphire laser, 405 nm 50mW Cube laser, and 594 nm 200mW MPB laser) at 20 psi through a 100 μ m nozzle using a gating strategy that identified viable, singlet CD45⁺CD11c⁺ cells. Fluorescence Minus One (FMO) controls were used to delineate negative boundaries for fluorescent markers. The alveolar macrophages were identified as CD45⁺CD11c⁺ based on previous studies (Mircescu et al., 2009, Zaynagetdinov et al., 2013) and stored at -80°C for epigenetic analysis.

Nucleic acids extraction

RNA and DNA were extracted simultaneously from the flash-frozen alveolar macrophages (group 1) and lung tissue (group 2) using the AllPrep Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. RNA and DNA from blood pellets were extracted using the PLASMA KIT (QIAGEN). DNA concentrations were analyzed by the Nanodrop 2000 (Thermo Scientific, Waltham, MA), and DNA integrity was evaluated on 1% agarose gel.

Analysis of 5-Methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) levels

RNaseA (Sigma, St. Louis, MO) was added to 1 μ g of genomic DNA to a final concentration of 0.02 mg/mL and incubated at 37°C for 15 min. Purified DNA was digested into component nucleotides using Nuclease P1, snake venom phosphodiesterase, and alkaline phosphatase as previously described (James et al., 2010). The detailed methodology can be found in the Supplementary Materials.

Analysis of methylation status of TEs

Methylation at the LINE-1 and SINE B1 elements was assessed by methylation-sensitive quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). The detailed method has been described in Supplementary Materials, with primers listed in Supplementary Table 1.

Gene and transposable elements expression analysis

cDNA was synthesized from 1 µg RNA using the High-Capacity Reverse Transcription Kit (Life Technologies, Carlsbad, CA). qRT-PCR was performed using 10 ng of cDNA per reaction and the TaqMan Universal PCR Master Mix, no AmpErase® UNG (Life Technologies) on a ViiA 7 instrument (Life Technologies). Assay IDs used in the study have been provided in Supplementary Table 2. Primers for determination of mRNA abundance of LINE-1 and SINE B1 elements are provided in Supplementary Table 3. The Ct values for all genes were determined relative to the control gene *GAPDH* or *Rps29* (Supplementary Tables 2 and 3). The Ct were calculated using each exposed group means relative to control group as described previously (Schmittgen and Livak, 2008). All qRT-PCR reactions were conducted in triplicate and repeated twice.

Analysis of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-G) levels

In addition to the *in vivo* epigenetic effects listed above, oxidative damage to DNA was also measured for both *in vitro* and *in vivo* systems used in this study. 8-oxo-G is an indicator of oxidative damage to DNA, and the methodology of detecting the 8-oxo-G level in genomic DNA was the same as "Analysis of 5-mC and 5-hmC levels" mentioned above. For the *in vivo* experiments, the 8-oxo-G levels in the lung tissue were detected of the exposed mice from group 2.

For *in vitro* experiments, the 8-oxo-G levels in murine macrophages (RAW264.7 cells) and human small airway epithelial cells (SAEC cells) after CuO and PEPs exposures were assessed. These cell lines were also employed in the *in vitro* companion paper (Lu et al., 2015). The RAW264.7 cells were purchased from ATCC (Manassas, VA) and cultured in DMEM/10%FBS. The SAEC cells were a gift from Jennifer Sisler (NIOSH, Morgantown, WV) and cultured in SAGM. The preparation and characterization of particle suspensions, and *in vitro* cell cultures and exposures were performed as described in great details in the *in vitro* companion paper (Lu et al., 2015). In summary: 1) the d_H of CuO was 1367 ± 73.12 nm in SAGM and 828.3 ± 95.49 nm in DMEM/10%FBS, and the colloidal properties of CuO suspension were stable both in these two media after 24 h post-sonication; 2) the d_H of PEPs was 381.7 ± 40.2 nm in SAGM and 298.0 ± 5.73 nm in DMEM/10%FBS, and the colloidal properties of PEPs suspension were also stable in these two media after 24 h post-sonication; 3) the exposure dose and time to cells were 0.5 and 30 µg/mL for 24 h.

Statistical analysis

Significance was determined by one-way analysis of variance (ANOVA), followed by Dunnett's test using GraphPad Prism 5.0 software. A *p* value < 0.05 was considered to be significant. Data are presented as mean ± standard error.

Results

In vitro and *in vivo* dosimetric considerations

The Supplementary Table 4 shows the *in vitro* and *in vivo* dosimetric considerations. The 2.5 mg/kg body weight instillation dose used in this study was equal to a total particle mass of 62.5 µg instilled to each mouse, resulting to a 0.76 µg/cm² instilled mass per alveolar surface area. For the 78.5 cm² surface area of the *in vitro* dish, the equivalent cell delivered *in vitro* mass dose was calculated to be 59.7 µg. Moreover, based on our companion *in vitro* study (Lu et al., 2015), the f_D was found to be 1 for CuO suspended both in DMEM/10%FBS and SAGM for a 24 h *in vitro* exposure using 100 mm diameter dishes. For PEPs, the f_D was 1 in SAGM and 0.93 in DMEM/10%FBS. Thus, the equivalent cell administered *in vitro* mass dose was calculated to be 59.7 µg for CuO suspended either in DMEM/10%FBS or SAGM. For PEPs, the equivalent cell administered mass dose was calculated to be 59.7 µg for SAGM and 64.2 µg for DMEM/10%FBS. The equivalent administered *in vitro* volumetric doses were approximately 8.0 µg/mL for CuO and PEPs in these two media, which is within the range of the administered doses (0.5 and 30 µg/mL) used in the companion *in vitro* study (Lu et al., 2015).

Effects of CuO and PEPs on the integrity of the murine lung

First, we sought to determine whether exposure to the tested ENMs at an instillation dose of 2.5 mg/kg body weight would lead to extensive pulmonary membrane damage as measured by the LDH assay. As shown in Figure 2A, CuO exposure led to a significant 3.6-fold increase in LDH release in comparison to the control group. On the contrary, levels of LDH remained unchanged after exposure to PEPs. This increase in cellular membrane damage after instillation to CuO was also accompanied with an increase, although insignificant, in MPO level, an indicator of neutrophil degranulation (Figure 2B). No changes in MPO activity were noted in BALF from mice exposed to PEPs.

In vitro and *in vivo* effects of CuO and PEPs on the activation of oxidative stress responses

Exposure to CuO and PEPs also induced an increase in DNA oxidative lesions, which was evident by a substantial elevation in the levels of 8-oxo-G in genomic DNA (Figure 2C–E). Exposure to CuO and PEPs led to a significant increase in the 8-oxo-G levels in RAW264.7 cells and an insignificant increase in SAEC cells after 24 h of exposure (Figure 2C and D).

It is worth noting that intratracheal exposure to these two ENMs was also characterized by a marked increase in the levels of 8-oxo-G in the murine lung tissue (Figure 2E).

Moreover, effects on the expression of the oxidative-stress-inducible gene heme oxygenase 1 (*Hmox1*) were detected. A significant increase in *Hmox1* expression was observed in the alveolar macrophages (1.9-fold) and lung tissue (2.8-fold) after exposure to CuO (Figure 2F and G). Exposure to PEPs did not significantly impact the *Hmox1* mRNA levels *in vivo*.

Effects of exposure to CuO and PEPs on the methylation status of global DNA and TEs *in vivo*

Mice instilled with either to CuO or PEPs exhibited increases in 5-mC levels in lung tissue (17%, $p=0.7$ in CuO and 25%, $p=0.04$ in PEPs, Figure 3A). Meanwhile, both CuO and PEPs were able to cause statistically significant increases in the 5-hmC levels in the lung tissue 24 h post intratracheal instillation (Figure 3B). Limited number of alveolar macrophages from the BALF did not allow for analysis of 5-mC and 5-hmC.

To further investigate whether the overall increase in 5-mC in response to ENM exposure *in vivo* is associated with hypermethylation of TEs, we addressed the methylation status of LINE-1 and SINE B1 in the mouse lung. Hypermethylation of the LINE-1 element at the 5'-untranslated region (5'UTR), although of small magnitude and insignificant, was observed in the mouse lung tissue after exposure to PEPs (Figure 3D). Changes in the methylation of other LINE-1 regions were also detected, with the significant hypermethylation of 3'UTR in the alveolar macrophages observed in response to PEPs exposure (1.5-fold) (Figure 3E-J). Exposure to CuO did not lead to significant alterations in the methylation of LINE-1 *in vivo*.

SINE B1 elements were hypermethylated in the alveolar macrophages after intratracheal exposure to both CuO and PEPs, while hypomethylation was observed in the lung tissue of mice exposed to PEPs, although all of them were insignificant (Figure 3K and L).

Effects of exposure to CuO and PEPs on the expression levels of DNA methylation machinery *in vivo*

Proper DNA methylation is sustained through the function of DNA methyltransferases, which are responsible for copying the methylation patterns to the newly synthesized DNA strand during replication, as well as the methylation of newly created sites. Expression of all three DNA methyltransferases – *Dnmt1*, *Dnmt3a*, and *Dnmt3b* was affected by CuO/PEPs *in vivo* (Figure 4A and B). Particularly, intratracheal exposure to PEPs significantly increased the expression of *Dnmt1* in the alveolar macrophages, while CuO treatment markedly reduced the expressions of *Dnmt1* and *Dnmt3b* in the lung tissue. PEPs also down-regulated *Dnmt3a* expression in the alveolar macrophages and lung tissue, however, exposure to CuO only significantly reduced the expression of *Dnmt3a* in the murine lung tissue.

The expression of *Tet1* methylcytosine-deoxygenase, an enzyme involved in the conversion of 5-mC into 5-hmC, was significantly decreased in both alveolar macrophages and lung tissue after exposure to either CuO or PEPs (Figure 4C and D).

Effects of exposure to CuO and PEPs on the reactivation of LINE-1 and SINE B1 *in vivo*

Altered methylation and loss of DNA methyltransferases function may lead to reactivation of TEs. Insignificant loss of expression of LINE-1 was observed in the alveolar macrophages after exposure to both CuO and PEPs (Figure 5A). Figure 5B shows the significant reactivation of LINE-1 retrotransposon in the lung tissue, where a 6.0-fold increase in LINE-1 expression was detected in response to PEPs exposure.

Expression of another TE – SINE B1 was also affected (Figure 5C and D). The level of SINE B1 transcript was markedly increased 3.7-fold in the lung tissue from the animals

exposed to PEPs. No significant effects were detected in the lung tissue after CuO treatment and in the alveolar macrophages of mice exposure to CuO and PEPs.

Epigenetic alterations in the peripheral leukocytes in response to CuO and PEPs exposures *in vivo*

Epigenetic parameters that were mostly affected in *in vivo* systems were also assessed in the peripheral leukocytes of mice exposed to either CuO or PEPs. Expression of maintenance DNA methyltransferase *Dnmt1* was diminished in the leukocytes in response to CuO (2.2-fold, $p=0.06$, Figure 6A), congruent with the effects observed in the lung tissue of CuO-exposed mice (Figure 4). At the same time, exposure to PEPs has led to an increase in *Dnmt1* mRNA level, although not significant (1.6-fold, $p=0.07$), and a marked 3.1-fold increase in *Dnmt3a* was also detected after PEPs exposure (Figure 6A–B). These changes correlated well with the unchanged transcription status of LINE-1 elements after CuO exposure, and the 2.3-fold increase in its expression after exposure to PEPs (although insignificant, Figure 6D), while the methylation status of LINE-1 was not affected by either ENMs (Figure 6C).

Discussion

This investigation is part of a series of studies using both *in vitro* and *in vivo* experimental platforms to evaluate the potential of ENMs to target the epigenome. In our previous *in vitro* companion study, it was reported that short-term exposure to some ENMs results in alterations in global and TEs-associated DNA methylation and hydroxymethylation as well as the expression of DNA methylation machinery, and leads to reactivation of LINE-1 and SINE B1 *in vitro* (Lu et al., 2015).

The aim of this study was to validate the findings of our previous *in vitro* companion study using an *in vivo* murine model. Two industry-relevant ENMs (CuO and PEPs) from the *in vitro* study were used for this *in vivo* validation. An intratracheal instillation dose of 2.5 mg/kg body weight corresponds to a cell administered *in vitro* volumetric dose of approximate 8.0 $\mu\text{g/mL}$ for both ENMs and the culture media used for RAW264.7 and SAEC cells. This dose is within the range of the administered doses (0.5 and 30 $\mu\text{g/mL}$) used in the companion *in vitro* study (Lu et al., 2015) and corresponds to 70.9 h consumer inhalation exposure duration (hours) to PEPs (Pirela et al., 2015b).

The data presented in this study showed that intratracheal instillation of CuO nanoparticles, but not of PEPs, led to a dramatic increase in LDH release, a reliable marker of pulmonary injury. Similarly, the injury was accompanied by an elevation in the MPO activity, which indicates neutrophil degranulation in the BALF of mice exposed to CuO, but not to PEPs. These findings are in agreement with those from our published *in vitro* companion study where CuO induced cytotoxicity at 30 $\mu\text{g/mL}$ dose in RAW264.7 cells while PEPs did not show any cytotoxicity at both 0.5 and 30 $\mu\text{g/mL}$ doses (Lu et al., 2015). Similar findings were found for CuO in other published *in vivo* and *in vitro* toxicological assessment studies (Jeong et al., 2015). The extensive damage to the integrity of the murine lung by CuO may be due to the oxidative stress caused by Cu via the Fenton reaction, since it is a transition metal (Karlsson et al., 2013). The dissolution of Cu ions from CuO may also be a

contributing factor to the lung injury observed in the mice after instillation of CuO (Ahamed et al. 2015). It is worth noting that for the PEPs, in addition to LDH and MPO, levels of hemoglobin and albumin, inflammatory cellular response, gene expression, and chemo-/cytokine secretion were measured in the BALF of BALB/c mice 24 h after intratracheal instillation of 0.5, 2.5 and 5 mg/kg of PEPs (Pirela et al., 2015b). A pulmonary immune response was induced by exposure to PEPs, which was indicated by an elevation in neutrophil and macrophage percentage and the leukemia inhibitory factor (LIF) cytokine. Additionally, exposure to PEPs upregulated expression of the *Ccl5 (Rantes)*, *Nos1* and *Ucp2* genes in the murine lung tissue, which are involved in both the repair process from oxidative damage and the initiation of immune responses to foreign pathogens. For CuO nanoparticles, there is evidence on their ability to cause lung injury, severe neutrophilic inflammation, and neoplastic lesions in lung as summarized in *in vivo* toxicological assessment studies reviewed in Ahamed et al. (2015).

Further *in vitro* and *in vivo* analysis of the oxidative potential of these two ENMs using mouse macrophages, human small airway epithelial cells and lung tissue from exposed mice showed that both CuO and PEPs are capable of up-regulating 8-oxo-G, suggesting an increase in oxidative DNA lesions in both the *in vitro* and *in vivo* testing platform. Additionally, elevated levels of another indicator of oxidative stress, *Hmox1*, were observed in both systems. A marked elevation in *Hmox1* was found in RAW264.7 cells treated with CuO, while exposure to PEPs had higher impact in SAEC cells (6.3-fold, $p < 0.01$, Lu et al., 2015). Similar trends were observed in the alveolar macrophages from mice instilled with CuO, which was revealed higher increase in *Hmox1* mRNA level in comparison to that from the treatment with PEPs. These findings suggest that both of the investigated ENMs may cause oxidative damage to DNA in a cell-dependent manner, which was detected in the *in vitro* and *in vivo* systems.

Evidence continues to grow indicating the ability of ENMs (e.g., silica nanoparticles and CuO) lead to alterations in the methylation status of global DNA and TEs (Gong et al., 2010; Lu et al., 2015). In this study, the lungs of mice intratracheally instilled with CuO and PEPs exhibited a unidirectional increase in 5-mC and 5-hmC levels. However, it is worth noting that no significant effects were identified in the levels of 5-mC and 5-hmC in RAW264.7 and SAEC cells in response to CuO and PEPs exposures in the *in vitro* companion paper (Lu et al., 2015).

The methylation status of LINE-1 and SINE B1/*Alu* TEs may also be affected even in the absence of gross alterations in gene-specific methylation (Koturbash et al., 2011a,b). Methylation of these abundant TEs is thought to be the primary mechanism that prevents them from unwanted reactivation (Bourc'his and Bestor 2004; Miousse and Koturbash 2015). In our companion *in vitro* paper (Lu et al., 2015), exposure to CuO and PEPs significantly affected the methylation status of LINE-1 element, particularly in the 5' UTR region where there were: 1) hypomethylation in RAW264.7 cells due to CuO exposure and, 2) hypermethylation due to both ENMs in SAEC cells. In contrast, effects in the animal model lacked significance in the methylation of LINE-1 5'UTR in both alveolar macrophages and lung tissue. However, a marked hypermethylation of LINE-1 3'UTR was exhibited in the alveolar macrophages from mice instilled with PEPs. The hypermethylation

of SINE B1/*Alu* was detected after CuO exposure both in RAW264.7 and SAEC cells *in vitro*, but not for PEPs (Lu et al., 2015). The similar effect was found *in vivo* in the alveolar macrophages after both CuO and PEPs treatments.

In addition to presenting epigenetic modifications caused by CuO and PEPs in a mouse experimental model, this study aimed at validating the ability of the recently developed *in vitro* screening platform reported in our published companion paper (Lu et al., 2015). However, some discrepancies were identified when comparing the *in vitro* and *in vivo* data as presented above (*i.e.*, the methylation status of global DNA and TEs). One possible explanation for these differences in results may be the cell populations assayed. For example, the various cells (*e.g.*, non-small airway epithelial cells, endothelial cells, smooth muscle cells, nerve cells, fibroblasts) obtained from the lung tissue of treated mice, were not represented in the *in vitro* companion study (Lu et al., 2015). Consequently, this mismatch in the cell lines evaluated in both studies could contribute to discrepancies in the methylation status of global and TEs observed, as shown by Meissner and colleagues (Meissner et al., 2008).

On the other hand, congruent results were observed in the analysis of DNA methyltransferases expression between the *in vitro* and *in vivo* models and of the tested ENMs. In general, the down-regulation of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* was observed in RAW264.7 and SAEC cells (Lu et al., 2015) as well as the murine alveolar macrophages and lung tissue from the *in vivo* model. It is worth noting that according to recently published data, particles (*i.e.*, nanoparticles, coarse ambient particulate matter) cause decreases in DNA methyltransferases expression (Miousse et al., 2014, 2015b; Lu et al., 2015; Pirela et al., 2015a) and increases in their enzymatic activities (Miousse et al., 2015b), explaining the foci of hypermethylation within the genome. Given the limited amount of tissue material, its extraordinary high cost, including generation of sufficient amount of real-world nanoparticles such as PEPs, the analysis of DNA methyltransferases enzymatic activity could not be performed in this study but needs to be further investigated. Furthermore, significant losses in expression of major methylcytosine-deoxygenase, *Tet1*, were detected due to CuO or PEPs exposure in the lung tissue and alveolar macrophages of instilled mice and in SAEC cells (Lu et al., 2015). The alterations in *Tet1* expression suggest that increments in 5-mC and 5-hmC levels of exposed mice may be underlined by the diminished ability of *Tet1* to further convert 5-mC into 5-hmC and further into 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) during the demethylation process. However, studies providing a clear link between the decreased expression of *Tet1* and conversion of 5-mC into 5-hmC and 5-hmC further into 5-fC and 5-caC are warranted.

The mechanisms by which ENMs exert their epigenetic effects are unclear, given that the understanding of epigenetic effects in nanotoxicology is in its infancy. In our both studies, *in vitro* and *in vivo*, we observed congruent decreases in the expression of DNA methyltransferases, the enzymes needed for establishment of proper DNA methylation and silencing of transposable elements. It is plausible to consider that one of the possible mechanisms of epigenetic alterations may be associated with the metal content in CuO and PEPs. While the majority of metals are weak mutagens, they can negatively affect the DNA methyltransferases enzymatic activity (Fragou et al., 2011), leading to alterations in DNA

methylation. Generation of reactive oxygen species, associated with metals, may compromise the normal redox status, alter glutathione content and affect one-carbon metabolism pathway, resulting in aberrant DNA methylation (Koturbash et al., 2012). The exact mechanisms of CuO and PEPs-associated epigenotoxicity, however, still need to be determined. DNA methyltransferases are among the major regulators of TE's expression, and loss of *Dnmts* expression may lead to TE's reactivation (Jones, 2012; Miousse et al., 2015a). Increases in the expression of the LINE-1 and SINE B1/*Alu* retrotransposons were observed in response to short-term CuO and PEPs exposures both *in vivo* and *in vitro* (Lu et al., 2015). More significant effects were observed in response to PEPs exposure in the SAEC cells and lung tissue than with CuO. Since reactivation of these two TEs may lead to their retrotransposition and subsequently lead to development of genomic instability, these end-points should be addressed in the future studies by investigating the long-term consequences of exposure to ENMs. Taken together, the *in vivo* results are mostly in agreement with those from the *in vitro* companion study (Lu et al., 2015) with the exception of data referred to methylation status of global DNA and TEs.

The observed epigenetic effects caused by single exposure to ENMs would, most probably, be reversible once the stressor is eliminated. However, the long-term/chronic low-dose exposures to ENMs may lead to permanently altered cellular epigenome and development of diseases. Therefore, chronic studies investigating the long-term consequences of low-dose exposure to ENMs are needed to further understand the observed effects.

Finally, analyses of epigenetic parameters suggest that peripheral leukocytes may be targeted by PEPs but not by CuO, as evidenced by the increased mRNA levels of *Dnmt1* and *Dnmt3a* and the reactivation of LINE-1 retrotransposon post PEPs exposure. These findings, together with others from this study, suggest that while CuO exhibits higher cell-damaging potential, PEPs exhibits greater potency in targeting the epigenome. However, it is unclear whether the changes in peripheral leukocytes are the result of the interaction of PEPs with the cells or the indirect cascade responses induced by exposure to PEPs.

Conclusions

In this *in vivo* study, it was shown that exposure to two ubiquitous industry-relevant ENMs – CuO and PEPs – affects the epigenome. These results are mostly in agreement with our *in vitro* companion study. While some differences were observed (*e.g.*, methylation status of global DNA and TEs), the general patterns of *in vitro* and *in vivo* effects showed a high degree of consistency. These findings suggest that both *in vitro* and *in vivo* systems can be successfully utilized in future toxicological and epigenetic investigations on ENMs.

Another important finding of this study is the translational applicability of the data obtained from the mouse model and the human cells used in the *in vitro* system. In particular, we observed very congruent responses in the expression of TEs and DNA methylation machinery between primary small airway cells and mouse lung tissue. Thus, both of our *in vitro* and *in vivo* studies confirm the ability of some ENMs to target the epigenome, while suggesting *in vitro* platforms may be useful to assess epigenetic changes due to ENMs

exposure. Furthermore, epigenetic parameters show potential to be included into the risk and safety assessment as they provide information on the biological properties of ENMs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Overall research strategy and experimental design for the *in vitro* (Lu et al., 2015) and *in vivo* epigenetic studies.

Figure 2.

Lung injury and oxidative stress induced by ENMs. (A–B) Lactate dehydrogenase release (A) and myeloperoxidase activity (B) in BALF after exposure to CuO and PEPs *in vivo*. (C–E) 8-oxo-G levels in genomic DNA after exposure to CuO and PEPs *in vitro* and *in vivo*; (C) in RAW264.7 cells, (D) in SAEC cells, and (E) in lung tissue isolated from exposed animals. (F–G) The *Hmox1* gene expression was measured in the alveolar macrophages (F) and lung tissue (G) isolated from exposed animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ANOVA with Dunnett's test. 8-oxo-G values are not available for alveolar macrophages.

Figure 3.

Levels of global and TEs-associated DNA methylation in response to ENMs exposure. (A–B) Global DNA methylation was assessed by measuring 5-mC (A) and 5-hmC (B) levels in whole genome DNA by HPLC. (C–J) The methylation status of the four regions of LINE-1 after exposure to CuO and PEPs; (C–D) The methylation status of the 5' UTR region of LINE-1 in the alveolar macrophages (C) and lung tissue (D) isolated from exposed animals, (E–F) The methylation status of the open reading frames 1 (ORF1) region of LINE-1 in the alveolar macrophages (E) and lung tissue (F), (G–H) The methylation status of the ORF2 region of LINE-1 in the alveolar macrophages (G) and lung tissue (H) isolated from exposed animals, (I–J) The methylation status of the 3' UTR region of LINE-1 in the alveolar macrophages (I) and lung tissue (J) isolated from exposed animals. (K–L) The methylation status of SINE B1 in the alveolar macrophages (K) and lung tissue (L) isolated from exposed animals. * $p < 0.05$, *** $p < 0.001$, ANOVA with Dunnett's test. Whole genome values are not available for alveolar macrophages. AM: alveolar macrophages.

Figure 4.

Analysis the expression of DNA methylation machinery. (A–B) The expression levels of DNA methyltransferases *Dnmt1*, *Dnmt3a* and *Dnmt3b* in the alveolar macrophages (A) and lung tissue (B) isolated from the exposed animals of CuO and PEPs treatments. (C–D) The expression levels of methylcytosine-deoxygenase *Tet1* in the alveolar macrophages (C) and lung tissue (D) isolated from the exposed animals of CuO and PEPs treatments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ANOVA with Dunnett's test.

Figure 5.

Retrotransposon transcript levels in response to ENMs exposure. (A–B) The expression levels of LINE-1 5' UTR in the alveolar macrophages (A) and lung tissue (B) isolated from the exposed animals of CuO and PEPs treatments. (C–D) The expression levels of SINE B1 in the alveolar macrophages (C) and lung tissue (D) isolated from the mice of CuO and PEPs exposures. * $p < 0.05$, ANOVA with Dunnett's test.

Figure 6.

Epigenetic parameters in peripheral blood of ENMs-exposed mice. (A–B) The levels of *Dnmt1* (A) and *Dnmt3a* (B). (C–D) The methylation (C) and expression (D) of the 5' UTR region of LINE-1 retrotransposon in the peripheral leukocytes of exposed mice. * $p < 0.05$, ANOVA with Dunnett's test.