

Full Length Paper

Effects of intratracheally instilled laser printer-emitted engineered nanoparticles in a mouse model: A case study of toxicological implications from nanomaterials released during consumer use



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ARTICLE INFO

Article history:

Received 1 October 2015

Received in revised form 4 December 2015

Accepted 18 December 2015

Available online 31 December 2015

Keywords:

Nanoparticles

Laser printers

Inflammation

Epigenetics

ABSTRACT

Incorporation of engineered nanomaterials (ENMs) into toners used in laser printers has led to countless quality and performance improvements. However, the release of ENMs during printing (consumer use) has raised concerns about their potential adverse health effects. The aim of this study was to use “real world” printer-emitted particles (PEPs), rather than raw toner powder, and assess the pulmonary responses following exposure by intratracheal instillation. Nine-week old male *Balb/c* mice were exposed to various doses of PEPs (0.5, 2.5 and 5 mg/kg body weight) by intratracheal instillation. These exposure doses are comparable to real world human inhalation exposures ranging from 13.7 to 141.9 h of printing. Toxicological parameters reflecting distinct mechanisms of action were evaluated, including lung membrane integrity, inflammation and regulation of DNA methylation patterns. Results from this *in vivo* toxicological analysis showed that while intratracheal instillation of PEPs caused no changes in the lung membrane integrity, there was a pulmonary immune response, indicated by an elevation in neutrophil and macrophage percentage over the vehicle control and low dose PEPs groups. Additionally, exposure to PEPs upregulated expression of the *Ccl5* (*Rantes*), *Nos1* and *Ucp2* genes in the murine lung tissue and modified components of the DNA methylation machinery (*Dnmt3a*) and expression of transposable element (TE) LINE-1 compared to the control group. These genes are involved in both the repair process from oxidative damage and the initiation of immune responses to foreign pathogens. The results are in agreement with findings from previous *in vitro* cellular studies and suggest that PEPs may cause immune responses in addition to modifications in gene expression in the murine lung at doses that can be comparable to real world exposure scenarios, thereby raising concerns of deleterious health effects.

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1. Introduction

The use of laser printers leads to exposure to various pollutants, including ozone, volatile organic compounds and particulate matter (PM), among other pollutants (He et al., 2007; Morawska et al., 2009; Wang et al., 2012). In particular, the release of a significant number of particles, the majority of which are nanoparticles, during the use of this growing technology has become a reason for concern. More recently, in order to

assess the complex chemistry of printer emitted particles (PEPs) and their potential health hazards, a Printer Exposure Generation System (PEGS) was recently developed to generate and sample airborne PEPs for subsequent physicochemical, morphological and toxicological analyses (Pirela et al., 2014a). The PM emission profiles from commonly used printers were evaluated and further characterization was performed on both raw toner powder and PEPs. The detailed analysis showed that laser printers emit up to 1.3 million particles/cm³ with modal diameters of <200 nm (Pirela et al., 2014a). More importantly, Pirela et al. (2014b) found nanoscale materials used in the toner formulation that become airborne during the use of a printer, thus, classifying toners as nano-enabled products (NEPs). Additionally, the authors found that toner powders and PEPs share a complex chemistry and contain elemental and organic carbon, as well as inorganic compounds such as nanoscale metals and metal oxides.

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While the physicochemical and morphological properties of PEPs have been studied in detail, their toxicological profiles remain largely unknown. In a series of recently published papers, several physiologically relevant cell lines (i.e., human small airway epithelial cells, microvascular endothelial cells, macrophages and lymphoblasts) were treated with various doses of PEPs using both mono- and co-culture exposure systems (Sisler et al., 2014; Pirela et al., 2015). In both studies, it was shown that PEPs triggered an unfavorable series of biological responses in macrophages, small airway epithelial cells and microvascular endothelial cells at doses comparable to approximately 8 h or more of consumer inhalation of PEPs. Specifically, cell treatment with PEPs led to significant changes in cell viability, hereditary genetic material changes, reactive oxygen species (ROS) generation and release of inflammatory mediators, among other adverse effects. Moreover, recent findings suggest that PEPs can also influence the cellular epigenome. Particularly, a 24-hour exposure to PEPs caused altered expression of DNA methylation machinery in small airway epithelial cells, in turn leading to changes in global DNA methylation and reactivation of transposable element (TE) LINE-1 and *Alu* (Pirela et al., 2015; Lu et al., 2015a).

Notably, the toxicity of PEPs remains poorly characterized *in vivo* with only a few published studies. Major discrepancy on those *in vivo* studies is the use of toner powders rather than the PM and gaseous pollutants emitted from laser printers. For example, Bai, Zhang (Bai et al., 2010) reported that mice exposed to printer toner particles showed significant pulmonary inflammation, damage to the epithelial-capillary barrier and enhanced cell permeability. Comparable inflammatory and fibrotic responses were also observed in rats exposed to toner powders (Morimoto et al., 2013). A historic rodent chronic inhalation exposure concluded that toner led to a substantial increase in lung weight, a chronic inflammatory response, pulmonary fibrosis and increased incidence of primary lung tumors in exposed rats (Muhle et al., 1991). However, as extensive as these studies were in identifying the biological response in the rodent lung following exposure to toner, they are limited by addressing only the toxicity of toner powder, which may be relevant to occupational settings and workers directly handling toner powders but is not applicable to consumers using laser printers.

In this study, we sought to further expand on the latest cellular toxicology studies performed by our group on PEPs (Sisler et al., 2014; Pirela et al., 2015; Lu et al., 2015b). Particularly, we present findings on the murine responses to intratracheal instillation exposures to various doses of PEPs. The endpoints evaluated included bronchoalveolar lavage (BAL) levels of lactate dehydrogenase, myeloperoxidase, cytokines and white blood cell differentials, as well as lung tissue expression of a number of genes involved in immune responses, cell survival and signaling, among other important biological processes.

2. Material and methods

2.1. Experimental design

Fig. 1 shows the experimental setup of the previously developed Printer Exposure Generation System (PEGS, (Pirela et al., 2014a)) used in this study. It consists of: a) a glovebox type environmental chamber to house the printer used in this study (Printer B1 in our previous publications: (Pirela et al., 2014a; Pirela et al., 2014b)) for uninterrupted operation; b) real time and time-integrated PM sampling and monitoring instrumentation to quantify particle size distribution and collect size-fractionated PEPs for analysis; and c) an animal inhalation exposure system for toxicological evaluation.

Groups of mice were exposed to various exposure doses of the smallest size fraction of PEPs (particles with an aerodynamic diameter smaller than 0.1 μm , $\text{PM}_{0.1}$) by intratracheal instillation. Following the exposure, animals were sacrificed and BAL was performed. The BAL fluid (BALF), blood and lung tissue were subsequently used to measure biochemical markers of inflammation, albumin and hemoglobin levels, white blood cell differentials and expression of a number of genes in addition to epigenetic analyses. In more detail:

2.2. Exposure characterization, sample preparation of size-fractionated airborne PM for intratracheal instillation exposures

2.2.1. Real time instrumentation for PM

A water-based condensation particle counter (WCPC Model 3785, TSI Inc., Shoreview, MN) was used to monitor the number concentration of particles sized from 5 to 1000 nm. A scanning mobility particle sizer (SMPS Model 3080, TSI Inc., Shoreview, MN) was also used in order to measure the particle size distribution (ranging from 2.5 to 210 nm) in the chamber. All the instruments were calibrated and background tests were performed at the beginning of each sampling experiment.

2.2.2. Size-selective integrated PM sampling and colloidal suspension preparation

The Harvard compact cascade impactor CCI, (Demokritou et al., 2004) was used to size fractionate and collect PM samples. The CCI operates with four stages and allows for collection of moderately large amounts of particles (mg level) for the following size fractions: $\text{PM}_{2.5-10}$, $\text{PM}_{0.1-2.5}$ and $\text{PM}_{0.1}$. The main advantage of CCI is the fact that size-fractionated PM is collected on pre-cleaned adhesive-free polyurethane foam (PUF) impaction substrates and Teflon filters from which the particles can be efficiently extracted using a water-based protocol. In summary, particles in the sampling substrates are extracted in deionized water

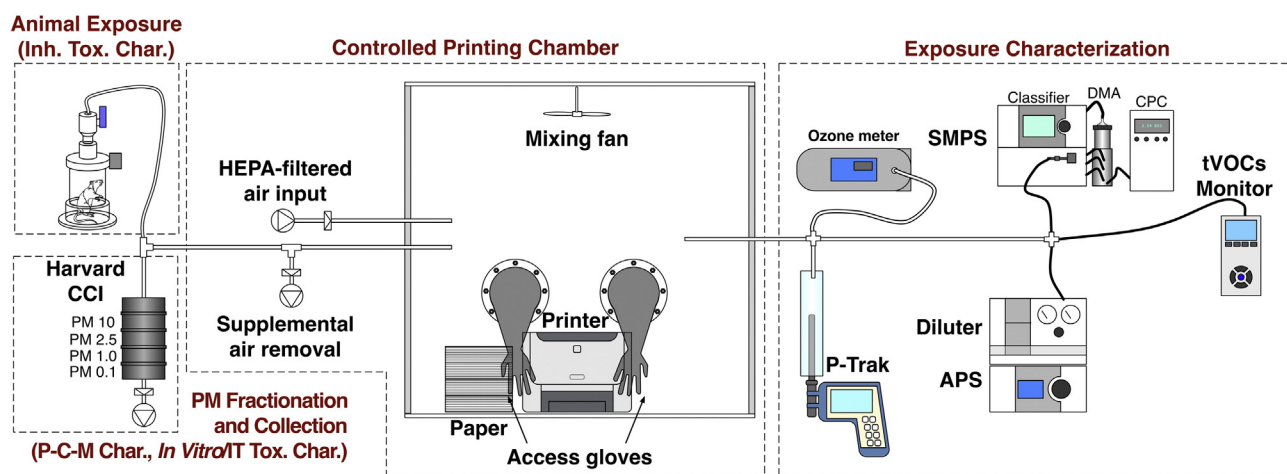


Fig. 1. Printer Exposure Generation System used to collect freshly generated PEPs for subsequent intratracheal instillations.

(DI H₂O) using a sonication protocol to allow for maximum extraction efficiency, no chemical alteration of the extracted particles and final particle suspension that is representative of the aerosol composition (Demokritou et al., 2004; Pirela et al., 2013; Chang et al., 2013; Pal et al., 2015; Khatri et al., 2013). In this study the PM_{0.1} size fraction was instilled. Thus, the extracted PEPs (PM_{0.1} size fraction) were dispersed in DI H₂O and the particle suspension characterized using a protocol developed by the authors (Cohen et al., 2012). In summary, the critical delivered sonication energy (DSE_{cr}), hydrodynamic diameter (d_H), polydispersity index (Pdl), zeta potential (ζ), and specific conductance (σ) were measured for all particle suspensions used in the study.

2.3. Intratracheal instillation exposures

2.3.1. Animals

Nine-week-old *Balb/c* male mice weighing an average of 24.25 ± 1.92 g were purchased from Taconic Farms Inc. (Hudson, NY). Mice were housed in groups of 4 in polypropylene cages and allowed to acclimate for 1 week before the studies were initiated. Mice were maintained on a 12-hour light/dark cycle. Food and water were provided ad libitum. All the animal protocols used in this study were approved by the Harvard Medical Area Institutional Animal Care and Use Committee (IACUC).

Once the particle suspensions were prepared for the intratracheal instillation exposure, as described above, each mouse was weighed and their respective exposure dose calculated at 2.5 mL/kg bw. The dosing solution was measured in a sterile syringe with an attached blunt-tipped 21-gauge gavage needle. The mice were anesthetized with vaporized isoflurane, quickly restrained on a slanted board and held upright by their upper incisor teeth resting on a rubber band. As the animals were under anesthesia, the tip of the needle was gently inserted into the trachea between the vocal cords, with the tip just above the tracheal bifurcation, and the dosing suspension was delivered in one bolus. The mice received an intratracheal instillation of PEPs (PM_{0.1}) at 0.5, 2.5 and 5.0 mg/kg bw or vehicle control (DI H₂O). After instillation, the animal was allowed to recover from anesthesia in a slanted position while the thorax was gently massaged to facilitate distribution of the instillate throughout the lungs. Each exposure group contained 3 or more mice.

It is worth noting that for this particular study, intratracheal instillation was chosen for exposure of mice to PEPs for various reasons primarily because intratracheal instillation allows for the delivery of a specific and accurate amount (dose) of PEPs and also to shorten exposure times and associated costs. The doses selected are specifically related to exposure durations (14, 71 and 142 h) occurring in the real world. While we recognized the pitfalls of using such a “bolus” method to expose animals (e.g., uniformity of delivery of the solution, dose rate issue), intratracheal instillation has been used routinely in the particle toxicology arena and it is widely accepted (Brain et al., 1976; Osier and Oberdorster, 1997; Driscoll et al., 2000). The 142-hour equivalent inhalation exposure dose was added in the study as a high dose in order to have a complete dose–response relationship assessment.

2.4. In vivo dosimetry considerations

Firstly, the mass of PEPs delivered to each mouse following intratracheal instillation of PEPs at the doses of 0.5, 2.5 and 5.0 mg/kg body weight was calculated using the average murine body weight of the mice used in the study (24.25 g). Secondly, the corresponding mass of PEPs instilled per lung surface area (mouse lung surface area = 82.2 cm²) to each mouse was calculated to be 1475, 7375 and 14,751 µg/m², respectively. This delivered mass per lung surface area was matched for the human lung. The human deposition mass flux (µg/min · m², mass per time and surface area) of the PEPs calculated by the Multiple Path Particle Deposition model (MPPD2) (Anjilvel and Asgharian, 1995) was used to determine the equivalent inhalation exposure time (min, hours) for each instilled mass of PEPs per surface area (µg/m²). Table 1 summarizes the parameters used for the MPPD2

simulation, which include both the human breathing parameters (tidal volume, breathing frequency, inspiratory fraction, pause fraction, functional residual capacity, head volume, breathing route) and the PEP airborne nanoparticle size distribution values (count median diameter, geometric standard deviation, particle mass concentration).

2.5. Bronchoalveolar lavage and analysis performed post-exposure to PEPs

Twenty-four hours after intratracheal instillations to PEPs, mice were given a fatal dose by intraperitoneal injection of FatalPlus (0.1–0.2 mL) and sacrificed by exsanguination, followed by bronchoalveolar lavage (BAL). The lungs were lavaged in situ with 12 washes of 0.75 mL of sterile 0.9% saline. The first two washes were pooled for biochemical assays. Cells were separated from the supernatant in all washes (400 ×g at 40 °C for 10 min). Total and differential cell counts, as well as hemoglobin measurements were made from the cell pellets. Total cell counts were performed manually using a hemocytometer. Cell smears were made with a cytocentrifuge (Shandon Southern Instruments, Inc., Sewickley, PA) and stained with Diff-Quick (American Scientific Products, McGaw Park, IL). Differential cell counts were performed by counting 200 cells per mouse. The supernatant fraction of the first two washes was clarified by sedimentation at 15,000 ×g for 30 min and used for measurement of enzyme activity, albumin and cytokine measurements. Standard spectrophotometric assays were used for lactate dehydrogenase (LDH), myeloperoxidase (MPO), albumin, and hemoglobin to identify damage to the lungs as described in Beck, Brain (Beck et al., 1982).

2.6. Multiplex cytokine analysis

Cytokine levels in BAL fluid were measured by Eve Technologies Corporation (Calgary, Alberta, Canada) using a MILLIPLEX Mouse Cytokine/Chemokine 32-plex kit (Millipore, St. Charles, MO) according to the manufacturer's protocol. The 32-plex consisted of eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-gamma, interleukin (IL)-1alpha, IL-1beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IFN gamma-induced protein (IP)-10, keratinocyte chemoattractant (KC), leukemia inhibitory factor (LIF), CXC motif ligand (LIX), monocyte chemoattractant protein (MCP)-1, macrophage (M)-CSF, monokine induced by gamma interferon (MIG), macrophage inflammatory protein (MIP)-1alpha, MIP-1beta, MIP-2, chemokine C-C motif ligand 5 (CCL5/RANTES), tumor necrosis factor (TNF)-alpha, and vascular endothelial growth factor (VEGF). The sensitivities of the assay to these markers ranged from 0.3 to 63.6 pg/mL.

2.7. Gene expression analysis

The RNA from the lungs of mice instilled with PEPs (2.5 mg/kg) or vehicle control (DI H₂O) was isolated. cDNA was amplified following the manufacturer protocol of the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The cDNA was used to analyze the following genes: epidermal growth factor receptor (*Egfr*), glutathione peroxidase 1 (*Gpx1*), peroxisome proliferator-activated receptor gamma (*Pparg*),

Table 1

Summary of parameters used in the human lung Multiple Path Particle Deposition model (MPPD2).

Human model	Breathing parameters	Airborne nanoparticle distribution
Functional residual capacity: 3300 mL	Tidal volume: 625 mL	Count mean diameter: 57.45 nm
Head volume: 50 mL	Breathing frequency: 12 breaths/min	Geometric standard deviation: 1.67
Breathing route: nasal	Inspiratory fraction: 0.5	Mass concentration: 23.86 µg/m ³
	Pause fraction: 0.0	

signal transducer and activator of transcription 3 (*Stat3*), vascular endothelial growth factor a (*Vegfa*), regulatory subunit of type II protein kinase a R-subunit domain containing 1 (*Riad1*), aldehyde oxidase 1 (*Aox1*), superoxide dismutase 1 (*Sod1*), transforming growth factor beta 1 (*Tgfb1*), nitric oxide synthase 1 (*Nos1*), *Ccl5*, B-cell lymphoma 2 (*Bcl2*), uncoupling protein 2 (*Ucp2*), serine–threonine protein kinase 1 (*Akt1*) and DNA (cytosine-5)-methyltransferase 3A (*Dnmt3a*) using TaqMan Universal polymerase chain reaction (PCR) Master Mix and TaqMan primers according to manufacturer guidelines. Relative gene expression was analyzed using the $2^{-\Delta\Delta CT}$ method with polymerase (RNA) II (DNA directed) polypeptide A (*POLR2a*) as the internal control.

2.8. Methylation and expression analysis of transposable element LINE-1

RNA and DNA were extracted simultaneously from flash-frozen cells and lung tissue of mice instilled with PEPs (2.5 mg/kg) or vehicle control (DI H₂O) using the AllPrep Mini Kit (Qiagen, Valencia, CA) for simultaneous RNA and DNA isolation according to the manufacturer's protocol. Analyses of methylation and expression of LINE-1 were performed by methylation-sensitive qRT-PCR as reported earlier in detail by the authors (Lu et al., 2015a). Briefly, 1 µg of genomic DNA was digested with 1 U of SmaI enzyme in 1X CutSmart buffer at 25 °C for 2 h. This was followed by a 16 h digestion at 37 °C in the presence of 1 U of the enzymes HpaII, HhaI, and AclI in 1X CutSmart buffer. The digestion was finalized by adding 0.5 U of BstUI enzyme in 1X CutSmart buffer for 4 h at 60 °C. All enzymes were purchased from New England Biolabs (Ipswich, MA, USA). Digested DNA was then analyzed by qRT-PCR on a ViiA 7 Real-Time PCR System (Applied Biosystems, Forrest City, CA, USA). DNA samples not digested with the restriction enzyme mix served as positive control, while samples 1) lacking the specific primers for DNA amplification and/or DNA template and 2) RAW264.7-derived DNA pre-treated with 5-azacytidine, a potent demethylating agent, served as negative controls. The threshold cycle (Ct) was defined as the fractional cycle number that passes the fixed threshold. The Ct values were converted into the absolute amount of input DNA using the absolute standard curve method and further normalized towards rDNA readings.

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0 (La Jolla, CA). Comparisons between all bronchoalveolar lavage fluid parameters after exposure to all doses of PEPs and vehicle control were evaluated using one-way analysis of variance (ANOVA) and Tukey correction for multiple comparison statistical significance. A p-value of 0.05 was considered significant. It is worth noting that the Kolmogorov–Smirnov normality test was used to assess the normality of the distribution of the dataset. The D'Agostino & Pearson omnibus and the Shapiro–Wilk normality tests were also run to assess normality of distribution. However, due to a small sample size, the D'Agostino & Pearson omnibus and the Shapiro–Wilk normality tests were unable to produce results regarding the normality of the data. However, it is expected that the data from animal studies to follow a Gaussian distribution and knowing that the control exposure group for this study is normally distributed, we assumed the rest of the data from the PEPs exposed groups also follow the same distribution.

3. Results

3.1. Characterization of PEP exposure

3.1.1. PEP size distribution

The complete description of the PEP size distribution of Printer B1 has been previously published (Pirela et al., 2014a). In summary, Printer B1 emitted close to 1.26 million particles/cm³ with a PM_{2.5} mass concentration of approximately 50 µg/m³. Furthermore, the PM emitted by Printer B1 had an average mobility diameter of 38.17 nm. It is also worth noting that detailed chemical analysis of the PEPs revealed a

complex mixture consisting of 62 and 97% organic, 10 and 0.5% elemental carbon, ~3% metal/metal oxides (e.g., aluminum, titanium) and ~25% other (e.g., phosphorus, sulfur) (Pirela et al., 2014b).

3.1.2. Colloidal properties of PEPs used in the IT study

Table 3 summarizes the particle behavior in suspension as described by diameter (d_H), zeta potential (ζ), polydispersity index (Pdl) and specific conductance (σ). Briefly, PEPs (PM_{0.1}) suspended in DI H₂O were generally monodispersed exhibiting a Pdl of 0.4 and had an average hydrodynamic diameter of approximately 180 nm.

3.2. In vivo dosimetry considerations

Fig. 2 shows the mass flux in humans following exposure to PEPs, estimated to be 1.732 µg/min·m² by the MPPD2 model, as a function of the human respiratory system. Table 2 shows the doses of intratracheal instillation exposure to PEPs performed in this study and the equivalent inhalation time a consumer would have to be exposed to PEPs to obtain the same deposition mass per lung surface area, which ranged from 13.7 to 141.9 h for the intratracheally instilled doses of 0.5, 2.5, 5.0 mg/kg.

3.3. Biological response in a mouse model following exposure to PEPs by intratracheal instillation

In order to assess the potential toxicity of PEP exposure by intratracheal instillation, mice treated with 0.5, 2.5 and 5.0 mg/kg of PEPs or DI H₂O (vehicle control) were sacrificed 24-hours post-exposure for the following analysis:

3.3.1. Pulmonary membrane integrity and neutrophil degranulation

The BALF from mice exposed to 0.5, 2.5 and 5.0 mg/kg of PEPs (PM_{0.1}) was evaluated and compared to that of the vehicle control group (DI H₂O). No significant differences in lactate dehydrogenase or myeloperoxidase were observed between the PEPs and the control treatment groups. Further, no differences were observed in the levels of hemoglobin or albumin across the different treatment groups (data not shown).

3.3.2. Inflammatory cellular response

Significant differences in white blood cell population were detected in the percent and number of neutrophils (Fig. 3A and B) as well as percent macrophages and lymphocytes (Fig. 3C and D) present in the BALF of mice exposed to PEPs at the highest dose (5 mg/kg) compared to both the vehicle control and the lowest dose of PEPs. Particularly, a dose-dependent elevation in the neutrophil percentage was visible across the three doses of instilled PEPs. Contrastingly, macrophage percentage in BALF was markedly lower in mice exposed to PEPs at 5 mg/kg when compared to both the vehicle control and the 0.5 mg/kg PEP exposure groups, suggesting enhanced adherence of macrophages to airway surfaces due to cell activation (Fig. 3C). The percent of lavageable lymphocytes also was noticeably lower at the highest PEP dose as opposed to the two lower PEP doses instilled (Fig. 3D).

Table 2

Comparison of doses of murine PEP exposures used in the study by intratracheal instillation with comparable human inhalation exposures to PEPs.

PEP exposure by intratracheal instillation (mg/kg bw)	Duration of consumer inhalation exposure of PEPs (h)
0.5	13.7
2.5	70.9
5.0	141.9

Table 3Properties of laser printer emitted particle dispersions in DI H₂O. d_H: hydrodynamic diameter, Pdl: polydispersity index, ζ: zeta potential, σ: specific conductance.

Material	Media	d _H (nm)	Pdl	ζ (mV)	σ (mS/cm)
PEPs (PM _{0.1})	DI H ₂ O	178.3 ± 3.459	0.403 ± 0.050	−20.6 ± 1.87	0.185 ± 5.8 × 10 ^{−4}

Notes: values represent the mean (±SD) of a triplicate reading.

3.3.3. Gene expression

Because our *in vitro* studies provided evidence of inflammatory response and oxidative damage due to exposure to PEPs, the expression of genes involved in these two important biological processes was evaluated *in vivo*. The lung tissue belonging to mice instilled with PEPs at 2.5 mg/kg was used to analyze the RNA and quantify the expression of a number of genes involved in inflammatory and oxidative damage responses. The genes evaluated included *Egfr*, *Gpx1*, *Pparg*, *Stat3*, *Vegfa*, *Riad1*, *Aox1*, *Sod1*, *Tgfb1*, *Nos1*, *Ccl5*, *Bcl2*, *Ucp2* and *Akt1*. In the PEP exposed group, there was an evident elevation in the fold induction of *Nos1*, *Ccl5* and *Ucp2* in comparison to the vehicle control group (Fig. 4).

3.3.4. Cytokine analysis

Out of 32 cytokines evaluated from the BALF of mice instilled with 2.5 mg/kg of PEPs, only leukemia inhibitory factor (LIF) was considerably upregulated by exposure to PEPs when compared to the vehicle control exposure group (Fig. 5).

3.3.5. Epigenetic alterations

Our previous *in vitro* studies clearly indicated that PEPs may affect the cellular epigenome within target cells. Here, exposure to 2.5 mg/kg of PEPs resulted in congruent epigenetic alterations. Fig. 6 shows the significant loss of DNA methyltransferase *Dnmt3a* and an elevated expression of TE LINE-1 observed in the whole lung tissue of mice instilled with PEPs.

4. Discussion

The aim of this study was to assess the effects of instilled PEPs emitted by laser printers using a mouse experimental model. This investigation is a part of a series of studies performed by our group to thoroughly evaluate the physicochemical, morphological and toxicological properties of PM emitted from laser printers (Pirela et al., 2014a, 2014b, 2015; Sisler et al., 2014; Lu et al., 2015b) and developed an integrated methodology that can be used to link exposures from particulate matter released across life cycle of nano-enabled products to toxicology and adverse health effects. In particular, in this research study we focused on the effect of consumer relevant PM exposure on the inherent rodent biological response.

Here, we present data on the outcome of exposure of mice to PEPs by intratracheal instillation as it pertains to various endpoints of interest: lung injury and inflammation as well as epigenetic response.

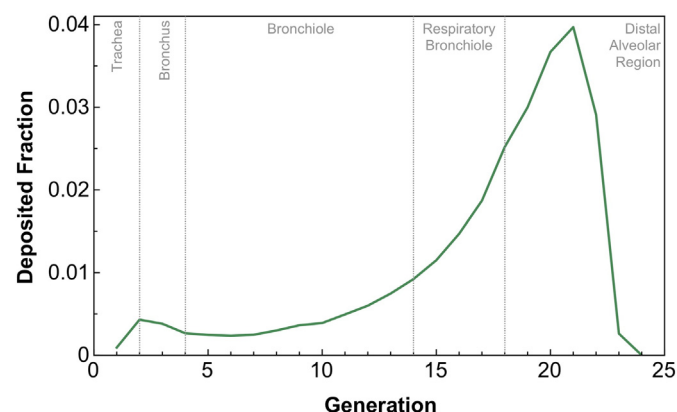


Fig. 2. Deposition fraction of the mass of PEPs inhaled as a function of human lung generation number.

The properties of the airborne PEPs were used to determine the range of doses of exposure used in the intratracheal instillation experiments. The material instilled in mice at 0.5, 2.5 and 5.0 mg/kg is equivalent to approximately 14, 71 and 142 h of consumer inhalation exposure to PEPs. It is worth noting that during printing, in addition to the PEPs there are also gaseous emissions (i.e., VOCs) that may also have deleterious effects (may act synergistically with emitted PM) when inhaled. This study focuses solely on possible effects from particulate phase (PEPs) and further studies are required to assess potential effects from gaseous phase pollutants.

Intratracheal instillation exposure to relatively low doses of PEPs did not compromise lung membrane integrity evidenced by insignificant variations in levels of LDH or MPO in the lavage fluid of exposed mice. However, in regards to the increase of both percentage and number of neutrophils obtained in the bronchoalveolar lavage fluid following exposure to PEPs, there seems to be an increase that certainly appears to be dose-dependent based on the three doses of PEPs instilled. Specifically, there is a significant difference between the control and 0.5 mg/kg PEP (PM_{0.1}) exposure groups to the 5.0 mg/kg bw PEP (PM_{0.1}) group, and while there is no statistical difference between the 2.5 and 5.0 mg/kg PEP (PM_{0.1}) exposure groups, there is a noticeable increase in the 5.0 mg/kg compared to the 2.5 mg/kg. It is worth noting that the inflammatory response reported after a high dose instillation of PEPs agrees with the study by Pirela, Molina (Pirela et al., 2013), in which the number of neutrophils was upregulated following instillation of PM_{0.1} and PM_{0.1–2.5} sampled in commercial photocopier center. The percentage of macrophages harvested by BAL decreased at the high dose of PEPs, suggesting that alveolar macrophages were more adherent to airway surfaces due to PEP-induced cell activation. Another plausible explanation of why there was a reduced number of macrophages lavaged, may be due to a toxic effect of PEPs on the murine alveolar macrophages. A clear dose–response relationship was observed in a previous study assessing the toxicity of PEPs on THP-1 macrophages (Pirela et al., 2015). Moreover, the reduction in lymphocytes at the highest PEP exposure compared to either the control group or the other two lower PEP doses, respectively, shows a regulation in the immune response to the exogenous PEPs. Lastly, in addition to no significant differences observed in the number of lymphocytes or macrophages between the PEPs exposed and the control groups, there are no discernable patterns in the macrophage and lymphocyte number population obtained in the bronchoalveolar lavage fluid. Therefore, no concluding statement can be made on this result.

After observing a substantial rise in expression of a variety of chemokines and cytokines in the *in vitro* toxicological assessments of PEPs (Sisler et al., 2014; Pirela et al., 2015), a similar inflammatory response could be expected following *in vivo* exposure to PEPs. However, out of 41 cytokines evaluated, only the expression of the LIF was significantly upregulated in mice instilled with PEPs (2.5 mg/kg) compared to the vehicle control. LIF is part of the IL-6 family of cytokines that is prominently elevated in pneumonia (Quinton et al., 2008). Principally, LIF has been associated with a protective role during pneumonia as well as having a central anti-inflammatory role during the early stages of an immune response (Quinton et al., 2012; Banner et al., 1998). Additionally, LIF was found to suppress cytokine production, cell death, airway hyperresponsiveness, alteration of epithelial membrane integrity and consequently, lung injury and inflammation. Particularly, the suppression of LIF signaling can lead to enhanced gene expression of *Ccl5* (RANTES) in small airway epithelial cells infected with the respiratory

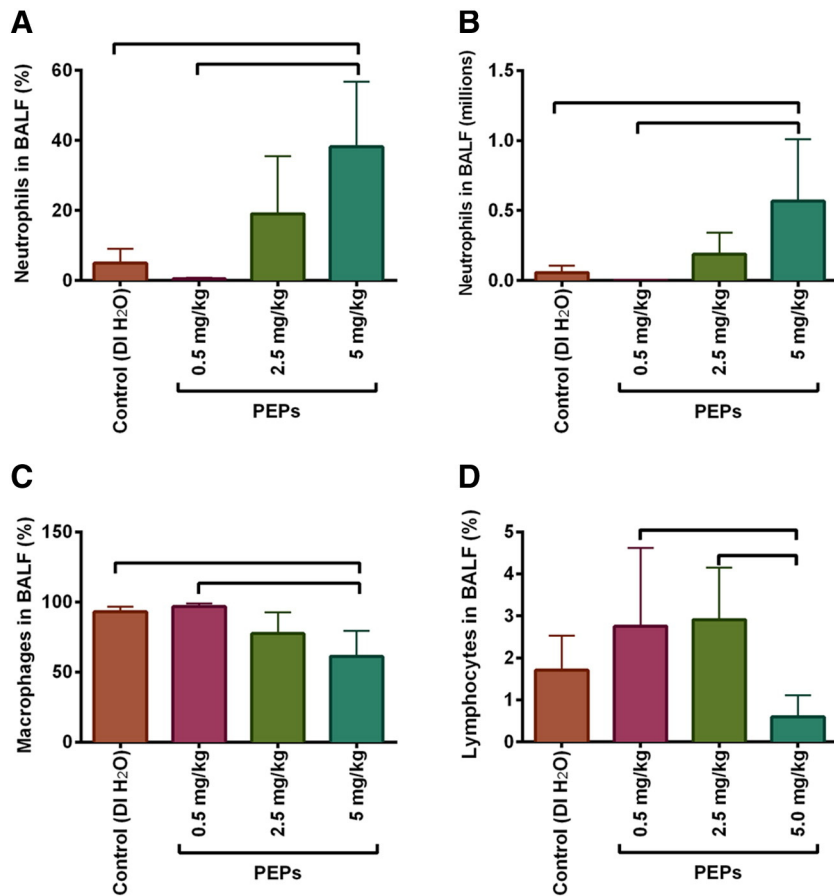


Fig. 3. BAL cell responses following exposure to PEPs by intratracheal instillation. Percentage (A) and values (B) of lavaged neutrophils harvested by BAL. Percentage of lavaged macrophages (C) and lymphocytes (D). Values are expressed as means (\pm SD). Bar represents a significant difference between the two groups ($p < 0.05$).

syncytial virus (Foronjy et al., 2014). Perhaps, the distinct increase in LIF post-instillation to PEPs provides protection against PEP-induced lung injury. In our study, no suppression of LIF was observed by enhanced expression of *Ccl5* and instead both LIF and *Ccl5* were upregulated following exposure to PEPs. Possibly, levels of microRNAs (i.e., miR-302) that negatively regulate *Ccl5* expression may be decreased due to PEP exposure, thus preventing the *Ccl5* mRNA degradation and leading to increased cellular levels of the latter. However, this is a speculation and more analyses have to be performed to make definitive conclusions and further evaluate the signaling pathway occurring after acute exposure to PEPs.

Additionally, instillation of PEPs (2.5 mg/kg) caused a substantial rise in the gene expression levels of three genes, namely *Ccl5* (RANTES), *Nos1* and *Ucp2*. Of interest, *Ccl5* (RANTES) is a pro-inflammatory chemokine that plays an important role in the trafficking of natural killer, dendritic cells, macrophages and the activation of leukocytes

(Aldinucci and Colombatti, 2014; Appay and Rowland-Jones, 2001). *Ccl5* (RANTES) was one of the cytokines whose expression was also up-regulated in both in vitro toxicology assessments of PEPs previously published by our group (Sisler et al., 2014; Pirela et al., 2015). More information is required to understand the association between gene expression changes and cytokine levels of LIF, involving further time course and dose response studies.

The uncoupling protein 2 (*Ucp2*), one of three UCPs, is an inner mitochondrial membrane protein acting as a natural regulator of ROS in the mitochondria by reducing the formation of a large proton gradient

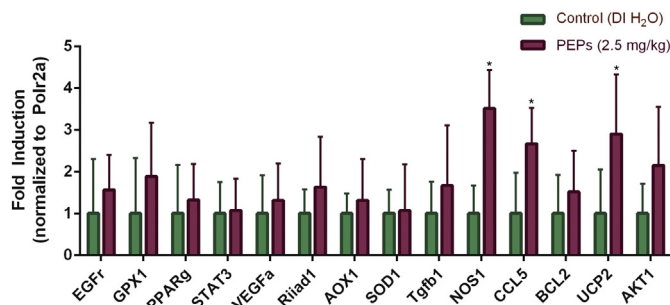


Fig. 4. Gene expression in lung tissue of mice instilled with PEPs (2.5 mg/kg). $n = 3$. * indicates significant difference when compared to the control exposure ($p < 0.05$).

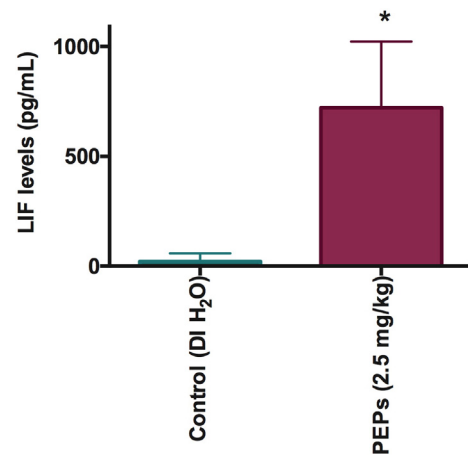


Fig. 5. Expression levels of the leukemia inhibitory factor (LIF) chemokine in the bronchoalveolar lavage fluid of mice exposed to PEPs (2.5 mg/kg). * indicates significant difference when compared to the control exposure ($p < 0.05$).

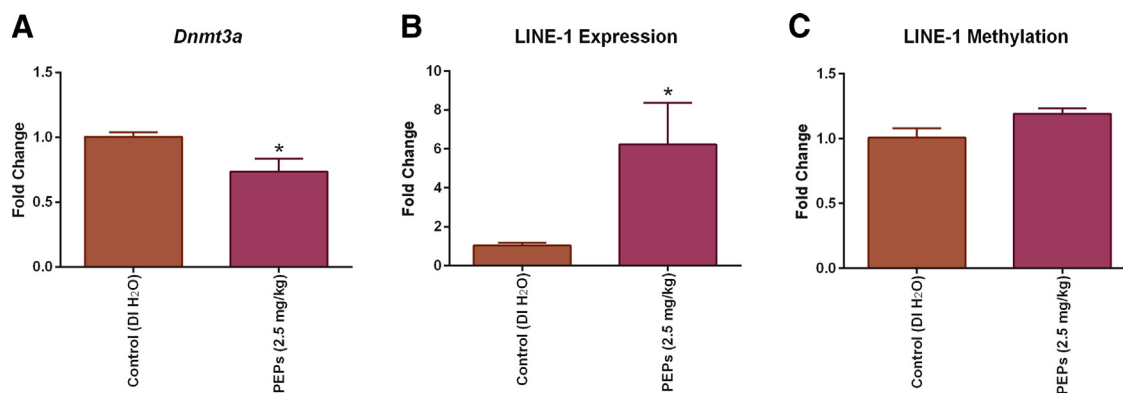


Fig. 6. Fold changes in methylation/expression of *Dnmt3a* and LINE-1 in murine lung tissue following exposure to PEPs (2.5 mg/kg). * indicates significant difference when compared to the control exposure ($p < 0.05$).

independent of a thermogenic pathway. Thus, this family of proteins protects against oxidative stress (Patti and Corvera, 2010; Echtay et al., 2002; Andrews et al., 2008; Jastroch et al., 2010; Krauss et al., 2005). A study by Steer, Mann (Steer et al., 2013) showed a significant induction of *Ucp2* in murine pulmonary alveolar macrophages following exposure to high concentrations of oxygen. Particularly, this increment in protein levels of *Ucp2* occurs in response to a rise in mitochondrial ROS production. Substantial gene modulations of *Ucp2* have been observed following exposures to other environmental stressors, such as arsenic, zinc oxide, octylphenol, nonylphenol, bisphenol A and tetrabromobisphenol A (Grasselli et al., 2014; Sarkar et al., 2014; Zhao et al., 2013; Song et al., 2012). Besides changes in expression of *Ucp2*, we observed a significant increase of *Nos1* following exposure to PEPs. *Nos1* is involved in the differentiation and function of immune cells in vitro and modulate immune responses and inflammatory process in vivo (Chakrabarti et al., 2012; Wahl et al., 2003; Martinelli et al., 2009; Sellers et al., 2013). *Nos1* is regulated by cytokines, microbial products, hormones and other intracellular factors (Forstermann et al., 1998; Iwase et al., 2000; Boissel et al., 2004; Dudzinski et al., 2006). Moreover, it has been found that there is an interaction between the *Nos1* gene and environmental factors, such as cigarette smoke, caffeine, and pesticides (Hancock et al., 2008). In a recent study (Levinsson et al., 2014), an association between polymorphisms in the *Nos1* gene and coronary heart disease and hypertension was discovered; thus, identifying the gene as an important biomarker for these diseases. The increased expression of *Nos1* and *Ucp2* would suggest that exposure to PEPs may lead to oxidative stress in the lung. Further mechanistic studies are required to reveal possible interdependencies and multiple pathways associated with possible cardiopulmonary effects associated with PEPs.

Another important outcome of this study is the congruence of epigenetic alterations observed in our previous in vitro studies with the alterations observed in the current in vivo model. Specifically, we identified that expression of DNA methyltransferase (*Dnmt3a*), the enzyme involved in regulation of DNA methylation, was significantly diminished in the lung tissue after intratracheal instillation exposure to PEPs. This finding is in a good agreement with the down-regulation of *Dnmt3a* in human small airway epithelial cells, observed in our previous in vitro study 24 h after exposure to PEPs (Lu et al., 2015a). Furthermore, loss of *Dnmt3a* expression was also reported in several other studies, devoted to exposure to particles of various sizes — from coarse ambient particles to nanoparticles (Miousse et al., 2014a; Gong et al., 2010). These findings suggest that DNA methyltransferase (*Dnmt3a*) may be ubiquitously targeted by particles, and further studies are warranted to determine if it may be useful as an epigenetic biomarker of exposure in nanoparticle toxicology.

DNA methyltransferases are critical for proper establishment of DNA methylation, and within the TEs, in particular. Expression of LINE-1, the most abundant mammalian retrotransposon that comprises nearly 20%

of their genome, is reported to be regulated by DNA methylation and directed by DNA methyltransferases (Bourc'his and Bestor, 2004; Miousse and Koturbash, 2015). Loss of DNA methyltransferases expression may lead to the loss of global and TEs-associated DNA methylation (Jones, 2012; Miousse et al., 2014b; Koturbash et al., 2011). Interestingly, in this study, similar to our previous investigations in vitro, (Lu et al., 2015a) we identified a non-significant trend towards LINE-1 DNA hypermethylation 24 h after exposure to PEPs. Further studies with larger numbers of mice are needed to evaluate the effects of PEP exposure and determine the time course of this phenomenon.

Particle exposure may also result in reactivation of TEs (Miousse et al., 2014a; Koturbash et al., 2011). In this study, we observed increased expression of LINE-1 24 h post-exposure to PEPs, similar to our findings from the previously published in vitro study (Lu et al., 2015a). Reactivation of LINE-1 may result in increased rates of its retrotransposition that may subsequently lead to genomic instability and development of disease (Miousse and Koturbash, 2015). Further, the early DNA hypermethylation effect observed in this study may be related to the inhibition of Tet-1 enzyme involved in active conversion of 5-methylcytosine into 5-hydroxymethylcytosine, which is a first step during the active DNA demethylation. This finding is in agreement with results published by our group recently (Lu et al., 2015a). LINE-1 mobilization, however, was not investigated in this study, since it has been shown that at least 120 h are needed in order to detect such event (Terasaki et al., 2013).

Altogether, our epigenetic findings confirm the ability of PEPs to target the cellular epigenome and suggest that in vitro studies may be used, although with caution, to investigate the epigenetic mechanisms of response to PEPs. Furthermore, it has been suggested that epigenetic parameters can be utilized in risk assessment (Koturbash et al., 2011; Goodman et al., 2010). Given congruent epigenetic responses observed in our in vitro and in vivo studies, further studies may support the use of epigenetic changes for the risk and safety assessment of nanomaterials. A large-scale study, investigating a platform of epigenetic alterations to a wide variety of nanomaterials both in vitro and in vivo, is clearly needed to identify specific parameters that can be further utilized for the assessment. Such study is currently underway in our laboratories and will be presented elsewhere in the future.

5. Conclusion

The study described here focuses on the assessment of toxicological potential of PEPs using an in vivo experimental animal model. Our data show that there may be an initiation of an immune response following the exposure to PEPs. Mice exposed to PEPs exhibited a variety of responses that translate into hallmarks of the initiation of an immune reaction due to the stress induced by PEPs. In toto, findings on lung injury, inflammation and changes in gene expression, point to possible adverse pulmonary effects. It is clear that these acute studies should be followed

by more detailed sub-acute and chronic studies in order to have more conclusive evidence on deleterious effects from such a widely used nano-enabled product. Finally, this experimental approach used here linking exposures to particulate matter released across life cycle (called LCPM) could be used to study other NEPs for a more realistic risk assessment of nanomaterials and nano-enabled products.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent views of the National Institute for Occupational Safety and Health or the Consumer Protection Safety Commission.

Funding information

This work was supported by the National Institute for Occupational Safety and Health and the Consumer Protection Safety Commission [212-2012-M-51174], National Institute of Health [HL007118, 1P20GM109005 and R01ES021764], University of Arkansas for Medical Sciences/National Institute of Health Clinical and Translational Science Award [UL1TR000039 and KL2TR000063], and the Arkansas Biosciences Institute, major research component of the Arkansas Tobacco Settlement Proceeds Act of 2000.

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