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RESEARCH ARTICLE

REVISED Diurnal variation in the proinflammatory activity of urban fine particulate matter (PM$_{2.5}$) by *in vitro* assays [version 3; referees: 2 approved]

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Abstract
Background: Ambient particulate matter (PM) smaller than 2.5 μm in diameter (PM$_{2.5}$) undergoes diurnal changes in chemical composition due to photochemical oxidation. In this study we examine the relationships between oxidative activity and inflammatory responses associated with these diurnal chemical changes. Because secondary PM contains a higher fraction of oxidized PM species, we hypothesized that PM$_{2.5}$ collected during afternoon hours would induce a greater inflammatory response than primary, morning PM$_{2.5}$.
Methods: Time-integrated aqueous slurry samples of ambient PM$_{2.5}$ were collected using a direct aerosol-into-liquid collection system during defined morning and afternoon time periods. PM$_{2.5}$ samples were collected for 5 weeks in the late summer (August-September) of 2016 at a central Los Angeles site. Morning samples, largely consisting of fresh primary traffic emissions (primary PM), were collected from 6-9am (am-PM$_{2.5}$), and afternoon samples were collected from 12-4pm (pm-PM$_{2.5}$), when PM composition is dominated by products of photochemical oxidation (secondary PM). The two diurnally phased PM$_{2.5}$ slurries (am- and pm-PM$_{2.5}$) were characterized for chemical composition and BV-2 microglia were assayed *in vitro* for oxidative and inflammatory gene responses.
Results: Contrary to expectations, the am-PM$_{2.5}$ slurry had more proinflammatory activity than the pm-PM$_{2.5}$ slurry as revealed by nitric oxide (NO) induction, as well as the upregulation of proinflammatory cytokines IL-1β, IL-6, and CCL2 (MCP-1), as assessed by messenger RNA production.
Conclusions: The diurnal differences observed in this study may be in part attributed to the greater content of transition metals and water-insoluble organic carbon (WIOC) of am-PM$_{2.5}$ (primary PM) vs. pm-PM$_{2.5}$ (secondary PM), as these two classes of compounds can increase PM$_{2.5}$ toxicity.

Keywords
Photochemistry, Los Angeles, PM2.5, Oxidative stress, Traffic, Primary PM, Secondary PM, Neuroinflammation

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Introduction

Particulate matter (PM) with an aerodynamic diameter less than 2.5 μm (fine PM or PM$_{2.5}$), is associated with diverse health problems and chronic diseases, including asthma, chronic obstructive pulmonary disease (COPD), lung cancer, and coronary heart disease (Delfino *et al.*, 2005; Delfino *et al.*, 2011; Dockery *et al.*, 1993; Dominici *et al.*, 2006; Kaufman *et al.*, 2016; Kim *et al.*, 2013; Landrigan *et al.*, 2018; Shah *et al.*, 2013). Findings of recent epidemiological studies extend chronic PM$_{2.5}$ exposure risk to Alzheimer's disease and accelerated cognitive decline (Cacciottolo *et al.*, 2017; Chen *et al.*, 2015; Chen *et al.*, 2017). Corresponding rodent models show robust indicators of inflammatory and oxidative stress to PM$_{2.5}$ fractions in pathological responses of aorta (Li *et al.*, 2003), brain (Cheng *et al.*, 2016b; Levesque *et al.*, 2011; MohanKumar *et al.*, 2008; Morgan *et al.*, 2011), and lung (Zhang *et al.*, 2012).

In addition to the epidemiological associations with chronic disease, we must also consider diurnal variations in airborne particulate matter chemistry that are not included in most long-term epidemiological studies. Diurnal variation in air pollution toxicity is suggested by diurnal variations in emergency department admissions for dementia (Linares *et al.*, 2017), ischemic stroke (Han *et al.*, 2016), and respiratory conditions (Darrow *et al.*, 2011). Although these admissions were more strongly associated with ozone than with PM$_{2.5}$ in all three of these studies, diurnal changes in PM$_{2.5}$ chemistry must also be considered as an influencing factor. Freshly emitted primary PM undergoes photochemical oxidation reactions over the course of the day, catalyzed by ultraviolet (UV) sunlight, which results in diverse oxidized organic and inorganic products (secondary PM) (Forstner *et al.*, 1997; Grosjean & Seinfeld, 1989), along with concomitant changes in PM toxicity. These diurnal changes in PM$_{2.5}$ composition and associated toxicity are relevant to and may inform future long-term epidemiological studies of primary and secondary particulate matter. While prior studies in the

Los Angeles air basin have shown extensive diurnal variations in PM composition and size, the findings of PM oxidative activity have been inconsistent and differ between various assays of oxidative potential (Saffari *et al.*, 2015; Verma *et al.*, 2009; Wang *et al.*, 2013b).

The current study further examined diurnal variations in composition and oxidative potential of PM samples collected at the central Los Angeles site used in the three studies mentioned above. However, unlike these earlier studies, PM samples were collected by a direct aerosol-into-liquid collection method to provide time-integrated aqueous PM$_{2.5}$ slurries for both morning and afternoon periods. This technology allows for a more comprehensive analysis than the filterable (i.e. water extracted) particulate samples examined in our prior studies (Morgan *et al.*, 2011; Saffari *et al.*, 2015; Verma *et al.*, 2009; Woodward *et al.*, 2017a).

Microglia were used for *in vitro* assays of oxidative and inflammatory responses to PM$_{2.5}$ exposures because of their increasingly recognized role in environmental neurotoxicology (Krafft, 2015). Air pollution can induce premature microglial activation, as documented in rodent models (Cheng *et al.*, 2016a; Hanamsagar & Bilbo, 2017; Morgan *et al.*, 2011) and as indicated for young adults living in the highly polluted Mexico City (Calderón-Garcidueñas *et al.*, 2008; Calderón-Garcidueñas *et al.*, 2018). Microglia (BV-2) cell cultures were assayed for induction of nitric oxide (NO) and for proinflammatory gene mRNA responses of interleukins 6 and 1β (IL-6 & IL-1β), and monocyte chemoattractant protein 1 (MCP-1), also known as chemokine (C-C motif) ligand 2 (CCL2). These markers were chosen because of their *in vivo* and *in vitro* responses to ultrafine PM shown in prior studies (Cheng *et al.*, 2016b; Morgan *et al.*, 2011; Woodward *et al.*, 2017b).

We hypothesized that afternoon PM$_{2.5}$ (pm-PM$_{2.5}$), with its high proportion of secondary photochemical oxidation products, would have greater oxidative and proinflammatory activity than freshly emitted, primary PM collected during morning hours (am-PM$_{2.5}$).

Methods

Particulate sample collection

All sampling was done at the University of Southern California Particle Instrumentation Unit (PIU), located approximately 150 meters downwind (east) of the Los Angeles I-110 freeway (34°1'9" N, 118°16'38" W). PM$_{2.5}$ samples were collected weekdays during the morning rush hour period of 6am–9am, as well as during the afternoon hours of 12pm–4pm, when photochemical products of primary PM oxidation are dominant in the atmosphere. The 5-week sampling campaign was conducted during late summer (August and September) of 2016, ensuring maximum UV sunlight exposure to enhance photochemical oxidation reactions.

Particle collection employed a novel high-volume aerosol-into-liquid collector developed and built at USC's Sioutas Aerosol Laboratory, which provides concentrated slurries of fine and/or ultrafine PM (Wang *et al.*, 2013a). A 2.5 μm cut-point

slit impactor at the inlet to the online sampling system removed PM larger than 2.5 μm in diameter and ensured that only $PM_{2.5}$ was captured in the aerosol-into-liquid collector. This sampler operates at 200 liters per minute (lpm) flow; two inlet aerosol streams, each at 100 lpm flow, are merged and passed through a steam bath where ultrapure water vapor condenses on the surfaces of airborne particles, growing the droplets to 2–3 μm in diameter. Downstream of the hot water bath, particles enter an electronic chiller, where they are cooled and condensed, passing through an impactor and accumulating in the aerosol-into-liquid collector as an aqueous $PM_{2.5}$ slurry.

For each sampling condition, morning and afternoon, one time-integrated slurry sample was collected for chemical speciation and biological assays. At the end of each morning and afternoon daily sampling period, each aqueous slurry sample was added to its corresponding total sample collection bottle that was kept refrigerated. At the end of the 5-week sampling period, these continuously refrigerated, cumulative aqueous slurry samples were then used in the *in vitro* assays. While it is possible that changes in PM composition might occur during sampling, the advantage of using the direct aerosol-into-liquid system is that PM is collected directly into an aqueous suspension and does not undergo an aqueous extraction and re-suspension process, thereby significantly reducing the possibility of any artifact formation. The benefits of this collection method compared to conventional filter sampling systems have been discussed extensively in the literature (e.g. Saarikoski *et al.,* 2014; Wang *et al.,* 2013b; Zhao *et al.,* 2005).

PM gravimetric analysis

To determine mass loadings of the $PM_{2.5}$ slurry samples, 47 mm Zefluor filters (Pall Life Sciences, Ann Arbor, MI, USA) were used to capture $PM_{2.5}$ passing through a parallel airstream at a flow rate of 9 lpm. Mass of the $PM_{2.5}$ filter samples was determined gravimetrically by pre- and post-weighing the Zefluor filters, equilibrated at controlled temperature (22–24 °C) and relative humidity (of 40–50%) conditions. Slurry PM concentrations were calculated from the filter mass loadings and air volume sampled per time period.

PM chemical species analysis

Aqueous $PM_{2.5}$ slurry samples were analyzed for metals and trace elements, total carbon (TC), and inorganic ions. Analyses were performed in triplicate on one aliquot of each slurry, morning (am-$PM_{2.5}$) and afternoon (pm-$PM_{2.5}$). Total metals and trace elements were quantified using magnetic-sectored Inductively Coupled Plasma Mass Spectroscopy (SF-ICPMS) following acid extraction, while analysis of the samples for inorganic anions was achieved by ion chromatography (IC) (Zhang *et al.,* 2008). Total carbon was determined using a Sievers 900 Total Carbon Analyzer (Sullivan *et al.,* 2004). Uncertainty values for all analyses are reported in the results as analytical error. Each uncertainty value is calculated as the square root of the sum of squares of the instrument and blank uncertainty components (S.D. of triplicate analyses, S.D. of triplicate blank measurements).

Microglial *in vitro* assays

BV-2 Cell Culture. $PM_{2.5}$ slurry samples were assayed with immortalized BV-2 microglia (RRID: CVCL_0182)

(Eun *et al.*, 2017; Gresa-Arribas *et al.*, 2012). BV-2 cells were cultured in Dulbecco's Modified Eagle's Medium/Ham's F12 50/50 Mix (DMEM F12 50/50; # 11320033, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; #45000–734, VWR, Radnor, PA), 1% penicillin/streptomycin (#P4333–100ML, Sigma-Aldrich, St. Louis, MO), and 1% L-glutamine (Glutamax; #35050061, Life Technologies, Carlsbad, CA) in a humidified incubator (37 °C/5% CO_2). For cell treatments, $PM_{2.5}$ slurries were diluted in the same isotonic and pH-balanced culture media and applied to cells for up to 24 hours. Cell culture experiments were done in triplicate per endpoint.

Nitrite Assay. Nitric oxide (NO) was assayed in BV-2 cell media by the Griess reagent (Cheng *et al.*, 2016b; Ignarro *et al.*, 1993). BV-2 cells at 60–70% confluence in 96-well plates (2×10^6 cells/plate) were treated with both am-$PM_{2.5}$ and pm-$PM_{2.5}$ at doses of 1, 5 and 20 μg/mL, 200 μL/well. At 30-minute, 60-minute and 24-hour timepoints, duplicate 50 μL aliquots of cell media were removed from each treatment well and transferred to a new 96-well plate. Within this same 96-well plate, a series of nitrite standards (50 μL/well) ranging from 0.10 to 10 μM prepared from a $NaNO_2$ stock solution were added, thus allowing a standardization curve to be generated for use in determining the NO concentration in each treatment well from measured absorbance data. After transferring all aliquots, 50 μL of Griess reagent was added to each well and the plate was allowed to incubate at room temperature (21–23 °C) for 10 minutes, followed by spectrophotometric analysis at 548 nm absorbance using a SpectraMax M2 microplate reader (Molecular Devices, San Jose, CA, USA). The nitrite assay was performed in triplicate, with six data points collected at each $PM_{2.5}$ concentration per condition.

Quantitative Polymerase Chain Reaction (qPCR). The quantitative polymerase chain reaction (qPCR) assay was used to quantify upregulation of cytokines and chemokines associated with the microglial neuroinflammatory response, including IL-6, CCL2 (MCP-1), and IL-1β. BV-2 microglia were seeded in 6-well plates at 10^6 cells/well and grown overnight at 37 °C/5% CO_2, followed by treatment with aqueous am-$PM_{2.5}$ and pm-$PM_{2.5}$ slurries diluted to 10 μg/mL in isotonic and pH-balanced cell culture media. A control condition, consisting of pure media diluted with ultrapure water, was also prepared. After 24 hours of incubation, treated cells were trypsinized and harvested for RNA extraction. Total cell RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was prepared from 1 μg of RNA (RT Master Mix, BioPioneer, San Diego, CA). Specific primers for each gene were used in conjunction with the qPCR Master Mix (BioPioneer) to run real time qPCR reactions.

Genes examined by qPCR included IL-1β (forward: 5' CTAAAG-TATGGGCTGGACTG 3'; reverse: 5' GGCTCTCTTTGAACA-GAATG 3'), IL-6 (forward: 5' TGCCTTCTTGGGACTGATGCT 3'; reverse: 5' GCATCCATCATTTCTTTGTAT 3'), MCP-1 (forward: 5' CCCAATGAGTAGGCTGGAGA 3'; reverse: 5' TCTGGAC-CCATTCCTTCTTG 3'), and GAPDH (forward: 5' AGACAGCCG-CATCTTCTTGT 3'; reverse: 5' CTTGCCGTGGGTAGAGT-CAT 3') (Integrated DNA Technologies, Skokie, IL). Data were

normalized to GAPDH and quantified as ΔΔCt. qPCR was repeated, with 12 data points collected per treatment (am-PM$_{2.5}$ and pm-PM$_{2.5}$; 10 µg/mL).

Statistical analysis. Results were evaluated by 2-way repeated measures ANOVA statistical analysis and Bonferroni post hoc tests using GraphPad Prism (v. 6.04) statistical software.

Results
Nitric Oxide (NO)
A dose-dependent NO response to PM$_{2.5}$ treatments relative to control was observed at all timepoints (30 min., 60 min., 24 hr.), which was greater for am-PM$_{2.5}$ than pm-PM$_{2.5}$ exposures (Figure 1). am-PM$_{2.5}$ samples induced consistently higher levels of NO for all concentrations and post-exposure timepoints, with a peak effect, 7-fold greater than control (p = 0.0077), observed at 60 minutes in response to the highest am-PM$_{2.5}$ dose of 20 µg/mL (Figure 1A). At 30 minutes post-treatment, there was also a significant 5.3-fold increase of am-PM$_{2.5}$ relative to control (p = 0.0020), and a significant difference between the responses to am-PM$_{2.5}$ and pm-PM$_{2.5}$, with am-PM$_{2.5}$ eliciting a 3.1-fold greater NO response than pm-PM$_{2.5}$ (p = 0.0094). There was also a significant a significant 2.9-fold increase of am-PM$_{2.5}$ relative to control (p = 0.0007) at 24 hours post-treatment. The NO responses to pm-PM$_{2.5}$ paralleled the effects of am-PM$_{2.5}$ exposures, but were at least 50% smaller (Figure 1B): the 20 µg/mL pm-PM$_{2.5}$ treatment induced 1.7-, 3.5-, and 2.0-fold increases in NO concentration relative to control at 30 min., 60 min. and 24 hrs., respectively, but these effects were not significant. The acute effects of PM exposure seen within the first hour of exposure, at 30 and 60 minutes post-treatment, are due to direct NO induction, while the sustained effect still measurable

after 24 hours indicates that there has been upregulation of the iNOS enzyme that produces NO. Thus, this overall effect is two-fold, with the increase in NO secretion due to PM$_{2.5}$ exposure mediated by two distinct mechanisms, acute and delayed.

Inflammatory gene responses
BV-2 cells were treated with 10 µg/mL of am-PM$_{2.5}$ and pm-PM$_{2.5}$ and analyzed for mRNA responses by qPCR after 24 hours incubation. The 10 µg/mL dose was chosen as below threshold for metabolic impairment based on prior studies from our group (e.g. Cheng *et al.*, 2016b; Morgan *et al.*, 2011; Woodward *et al.*, 2017b). Induction of all three cytokines was increased by both morning and afternoon PM$_{2.5}$ samples, with more modest responses to pm-PM$_{2.5}$ (Figure 2). As shown in Figure 2A, treatment with am-PM$_{2.5}$ induced a significant 4.8-fold increase in IL-1β expression relative to control (p = 0.0070). Both am-PM$_{2.5}$ and pm-PM$_{2.5}$ induced significant increases in IL-6 mRNA production relative to control, with am-PM$_{2.5}$ exposure resulting in a 5.1-fold increase (p < 0.0001) and pm-PM$_{2.5}$ resulting in a 3.5-fold increase (p = 0.0046) (Figure 2B). Treatment with am-PM$_{2.5}$ also induced a significant 2.0-fold increase in MCP-1 mRNA production (p = 0.0022), while pm-PM$_{2.5}$ had a 33% smaller effect (Figure 2C). This difference in MCP-1 mRNA production induced by am-PM$_{2.5}$ as compared to pm-PM$_{2.5}$ was marginally significant (p = 0.0527).

Chemical composition of PM$_{2.5}$ slurry samples
The am-PM$_{2.5}$ and pm-PM$_{2.5}$ time-integrated aqueous slurry samples were analyzed for chemical composition, including total carbon (TC), inorganic ions, and total metals and trace elements, and are presented as PM$_{2.5}$ mass fractions in Figures 3A, 3B, and 3C, respectively. PM$_{2.5}$ TC content
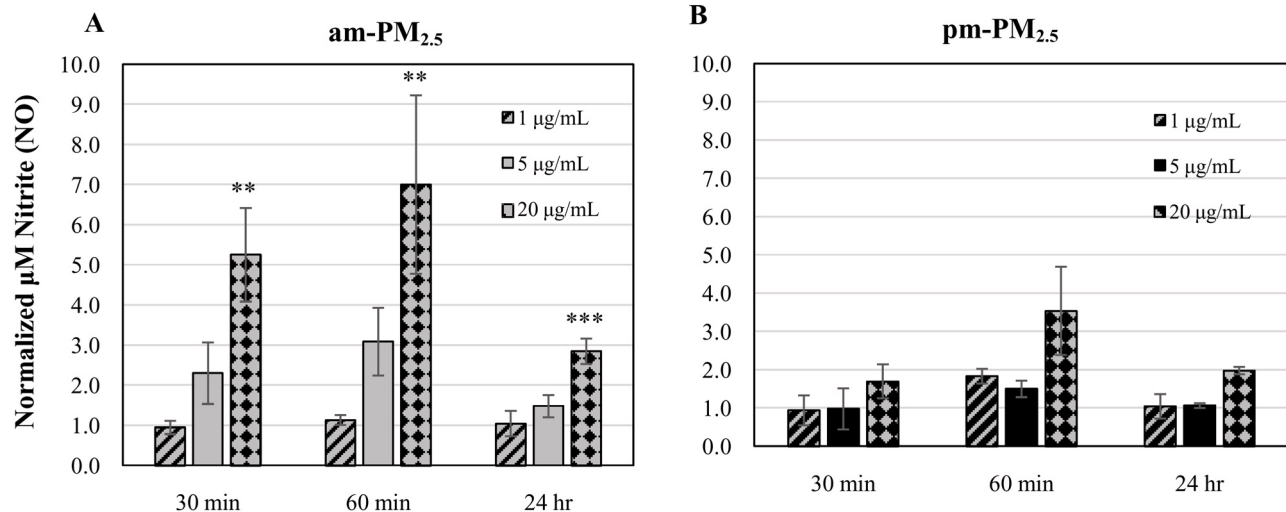
Figure 1. Nitric oxide (NO) induction by microglia. BV-2 microglial responses to PM$_{2.5}$ slurries *in vitro*, assayed in culture media by the Griess reaction (control = 1.0 µM nitrite). **A.** Morning samples (am-PM$_{2.5}$); **B.** Afternoon samples (pm-PM$_{2.5}$). am-PM$_{2.5}$ samples induced consistently higher NO responses for all concentrations and post-exposure timepoints. At 30 minutes post-treatment, there was a significant effect of am-PM$_{2.5}$, as well as a significant difference between the responses to am-PM$_{2.5}$ and pm-PM$_{2.5}$ (overall ANOVA: p = 0.0017; am-PM$_{2.5}$ 20 µg/mL vs. control: 5.3-fold increase, p = 0.0020; am-PM$_{2.5}$ 20 µg/mL vs. pm-PM$_{2.5}$ 20 µg/mL: 3.1-fold increase, p = 0.0094). There was also a significant effect of am PM$_{2.5}$ at 60 minutes post-treatment (overall ANOVA: p = 0.010; am-PM$_{2.5}$ 20 µg/mL vs. control: 7.0-fold increase, p = 0.0077). At 24 hours a significant effect of am-PM$_{2.5}$ treatment was also observed (overall ANOVA: p = 0.0005; am-PM$_{2.5}$ 20 µg/mL vs. control: 2.9-fold increase, p = 0.0007). Mean ± SE (n = 3 experiments). 2-way repeated measures ANOVA statistical analysis with Bonferroni post hoc tests: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.
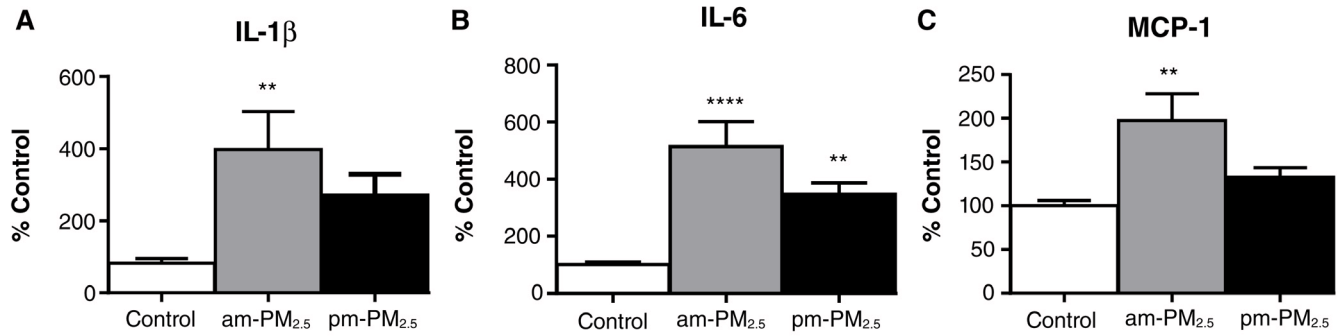
Figure 2. Inflammatory gene mRNA induction in microglia. After exposing BV-2 cells to 10µg/mL of morning (am-PM$_{2.5}$) and afternoon (pm-PM$_{2.5}$) slurries, cellular mRNA production was assessed by qPCR. Relative to control, both am-PM$_{2.5}$ and pm-PM$_{2.5}$ exposures increased mRNA levels of **A.** Interleukin 1β (IL-1β), **B.** Interleukin 6 (IL-6), and **C.** monocyte chemoattractant protein 1 (MCP-1). Treatment with am-PM$_{2.5}$ induced a significant 4.8-fold increase in IL-1β expression relative to control (overall ANOVA: p = 0.0090; am-PM$_{2.5}$: 4.8-fold increase, p = 0.0070). Both am-PM$_{2.5}$ and pm-PM$_{2.5}$ induced significant increases in IL-6 mRNA production (overall ANOVA: p < 0.0001; am-PM$_{2.5}$: 5.1-fold increase, p < 0.0001; pm-PM$_{2.5}$: 3.5-fold increase, p = 0.0046). Treatment with am-PM$_{2.5}$ also induced a significant increase in MCP-1 mRNA production, while pm-PM$_{2.5}$ had an effect 33% smaller than am-PM$_{2.5}$ (overall ANOVA: p = 0.0028; am-PM$_{2.5}$: 2.0-fold increase, p = 0.0022; am-PM$_{2.5}$ vs. pm-PM$_{2.5}$: p = 0.0527). Mean ± SE (n = 12). 2-way repeated measures ANOVA statistical analysis with Bonferroni post hoc tests: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.
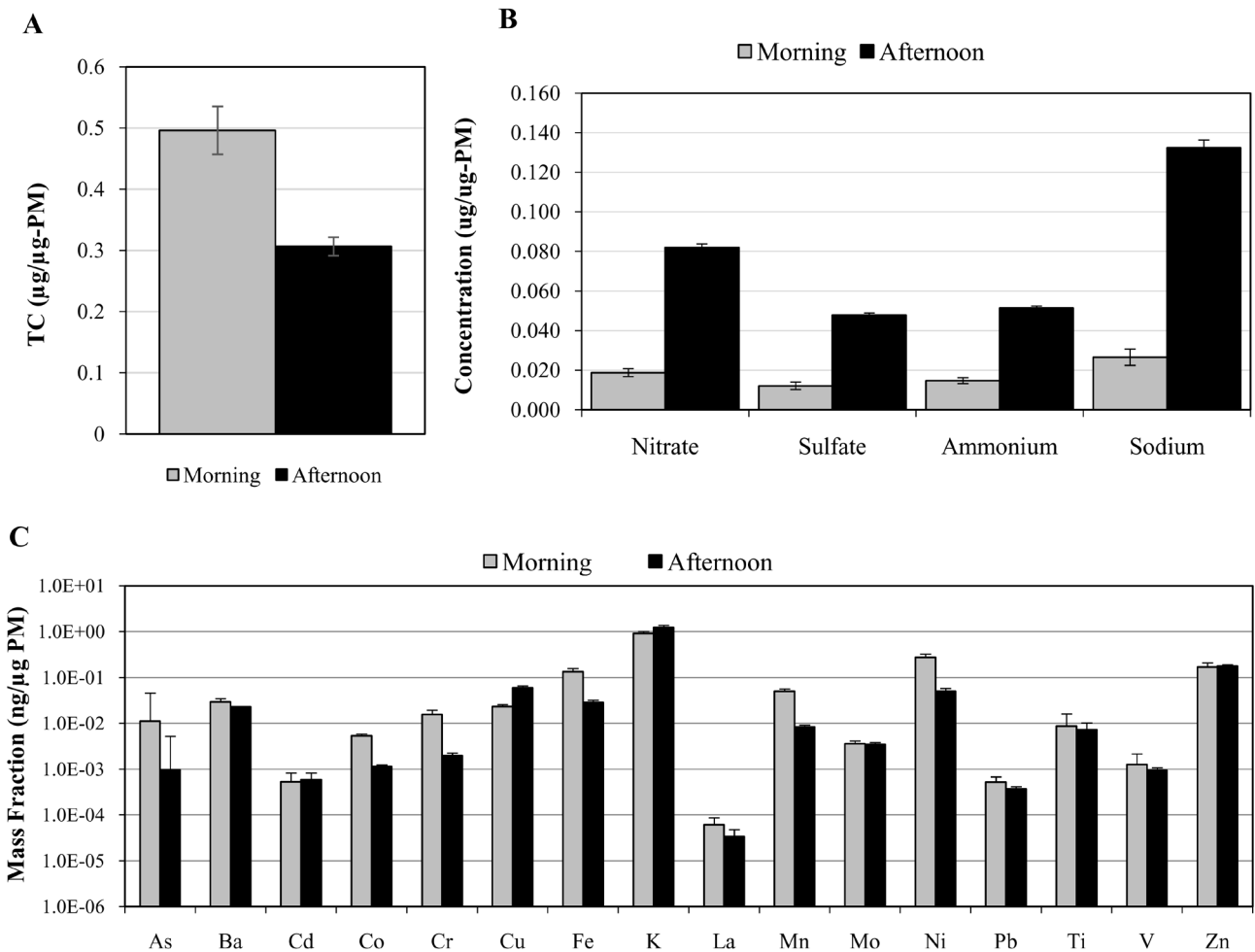
Figure 3. Chemical analyses. Time-integrated PM$_{2.5}$ slurries collected during morning (6–9am) and afternoon (12–4pm) periods analyzed for **A.** Total Carbon (TC), **B.** Inorganic ions (ion chromatography), **C.** Total metals and trace elements (ICP-MS). Mean values presented are based on triplicate analysis of one sample aliquot. Error bars represent laboratory uncertainty values based on contributions of analytical error (standard deviation) and blank subtraction (standard deviation of at least three method blanks).

decreased by 40% from morning (0.50 μg/μg-PM) to afternoon (0.31 μg/μg-PM) (Figure 3A). Mass concentrations of inorganic secondary ions (NO_3^-, SO_4^{2-}, NH_4^+, Na^+) were approximately 5-fold higher in the afternoon as compared to morning slurries (Figure 3B). For the sixteen metals and trace elements analyzed, the am-$PM_{2.5}$ slurry contained higher mass concentrations of several measured elements as compared to the pm-$PM_{2.5}$ slurry (Figure 3C, note log scale; Table S1, Supplementary File 1). Arsenic, chromium, and manganese showed the largest diurnal decline, represented as am-$PM_{2.5}$:pm-$PM_{2.5}$ ratios: arsenic (11.6), chromium (7.9), and manganese (6.0).

Dataset 1. The following raw data sets are provided as comma separated values (.csv) files

http://dx.doi.org/10.5256/f1000research.14836.d203329

PM_Diurnal_Variation_NO_Fig1_DATA

PM_Diurnal_Variation_qPCR_Fig2_DATA

PM_Diurnal_Variation_TC_Fig3A_DATA

PM_Diurnal_Variation_Ions_Fig3B_DATA

PM_Diurnal_Variation_Metals_Fig3C_DATA

Discussion

Diurnal variations in urban $PM_{2.5}$ oxidative and proinflammatory activity showed consistent decreases from morning to afternoon sampling periods in two independent *in vitro* assays using the BV-2 microglia cell line. The collection of total $PM_{2.5}$ as an aqueous slurry was enabled by direct aerosol-into-liquid sampling that more efficiently captures water-insoluble components of ambient $PM_{2.5}$ than traditional filter-based sampling methods used in several prior studies (e.g. Saffari *et al.*, 2015; Verma *et al.*, 2009). These slurry samples are more representative of the full range of ambient PM components and their toxicities than filter-trapped and water eluted PM. Additionally, the results of the NO assay and the qPCR assay for inflammatory gene responses extend findings from the widely used dithiothreitol (DTT) and alveolar macrophage (dichlorodihydrofluorescein, DCFH) assays for oxidative potential, which can be confounded by oxidative recycling from transition metals (Forman & Finch, 2018). Our findings, that primary $PM_{2.5}$ results in a greater oxidative and proinflammatory response than secondary $PM_{2.5}$, are contrary to expectations based on prior reports that secondary, photo-oxidized PM exhibits greater oxidative activity than primary PM.

While there may be a concern that the concentrations of $PM_{2.5}$ treatments used in the *in vitro* assays do not reflect the exact concentrations of $PM_{2.5}$ reaching microglia in the brain following ambient exposures, these assays do serve as a useful model for how brain cells in living organisms would respond to $PM_{2.5}$ at the given concentrations (i.e. 1, 5, 10, and 20 μg/mL). The focus of the paper was to investigate the differences between morning (primary-dominated) and afternoon (secondary-dominated) $PM_{2.5}$, rather than to quantify actual exposure concentrations, and subsequent CNS concentrations,

that would be considered harmful. We modeled these interactions between $PM_{2.5}$ and microglia using concentrations that are below the threshold for cell death, as evaluated by the MTT assay. While there is evidence that particles can directly enter the brain through the olfactory tract (Oberdörster *et al.*, 2004), and thus perhaps maintain higher concentrations than $PM_{2.5}$ passing through the periphery, the concentration of particles interacting directly with brain cells via this route has not been quantified, and thus comparisons to these results could not be made.

Previous studies of diurnal variations in PM composition and oxidative activity have not been consistent and were limited in using only simple assays of oxidative potential (i.e. DTT and DCFH) on filter-captured PM. Relying solely on oxidative potential measures such as the DTT and DCFH assays provides us with only an imprecise measure of cellular oxidative and proinflammatory activity that lacks specificity. The current study improves on the experimental design of past studies by utilizing direct measures of acute oxidative stress and inflammation, including free radical production induced by PM as nitric oxide (NO) and cellular proinflammatory mRNA responses. Additionally, by using the direct aerosol-into-liquid method to collect aqueous slurries in our study, water-insoluble PM species were more efficiently captured, providing samples more representative of the full range of ambient PM components and their toxicities.

Further insight into the sources of particulate toxicity may be gleaned by the apportionment of redox properties to its water soluble and insoluble chemical components, including water-soluble and water-insoluble organic carbon (WSOC and WIOC, respectively). WSOC species are generally defined as hydrophilic, while WIOC are hydrophobic (Turpin & Lim, 2001). Wang *et al.* (2013b) collected aqueous $PM_{2.5}$ slurries by a similar aerosol-into-liquid sampling method, and found that increased WIOC content in $PM_{2.5}$, relative to WSOC content, was highly correlated with redox activity on a per mass basis, indicating a greater intrinsic toxicity of WIOC as compared to WSOC. While this study was limited by its use of the DCFH assay, the greater oxidative potential associated with increased WIOC mass concentrations was attributed to organic compounds such as PAHs, as well as iron and other transition metals.

Our results indicate that morning $PM_{2.5}$, which contains a greater proportion of water-insoluble species, may be intrinsically more toxic and induce greater cellular oxidative stress, than afternoon $PM_{2.5}$ samples that contain a larger mass fraction of oxidized, water-soluble species that are products of photochemical reactions in the atmosphere (Seinfeld & Pandis, 2016), including the inorganic secondary ions NO_3^-, SO_4^{2-}, NH_4^+, and Na^+. The mechanisms underlying the greater toxicity of primary, morning $PM_{2.5}$ may involve non-polar WIOC components, such as PAHs, being able to more easily permeate the hydrophobic lipid-bilayer of cell membranes to trigger the formation of intracellular oxidative species and induce proinflammatory cytokine formation via an acute oxidative stress response.

Primary, traffic-derived $PM_{2.5}$ also consists of greater concentrations of redox active and other toxic metals, as compared to the bulk of secondary $PM_{2.5}$, which consists largely of hydrophilic products of photochemical oxidation. The metals and trace elements we found to be more prevalent in the morning slurry sample included the heavy metals vanadium, chromium, nickel, and arsenic, which are emitted by vehicles both as fuel combustion products as well as remnants of motor oil degradation (Geller *et al.*, 2006), copper, which is associated with vehicular brake wear (Garg *et al.*, 2000; Sanders *et al.*, 2003; Sternbeck *et al.*, 2002), and zinc, which is primarily a product of tire deterioration (Singh *et al.*, 2002). Elevated levels of these metals in both collection periods correspond to vehicular emissions as the major source of primary particles in close proximity to the I-110 freeway. We believe the higher proportions of these metals and WIOC components in primary $PM_{2.5}$ dominant in the morning hours, as compared to photo-oxidized secondary $PM_{2.5}$ prevalent in the afternoon, are responsible for the diurnal variation in acute oxidative stress observed in the current study.

Summary and conclusions

The data presented in this study demonstrate that urban $PM_{2.5}$ collected during the morning rush hour (6–9am), when primary, traffic-derived PM emissions are dominant, induces greater oxidative and proinflammatory responses in cells as compared to $PM_{2.5}$ collected in the afternoon (12–4pm), which contains a higher proportion of photo-oxidized, secondary PM products. Two *in vitro* assays of the cellular inflammatory response consistently demonstrated greater oxidative and proinflammatory activity due to primary (morning) $PM_{2.5}$ exposure. We attribute this effect to the greater transition metal and water-insoluble organic carbon (WIOC) content of primary $PM_{2.5}$, two classes of PM components that increase toxicity (Cho *et al.*, 2005; Hu *et al.*, 2008; Li *et al.*, 2009; Shirmohammadi *et al.*, 2015; Tao *et al.*, 2003; Zhang *et al.*, 2008). Our study also improves upon previous research of diurnal variations in PM-induced oxidative stress by utilizing a unique aerosol-into-liquid PM collection

system that more efficiently captures water insoluble components, thus providing complete aqueous PM samples more representative of ambient PM.

This research will ultimately help us gain a more complete understanding of the complex nature of particulate matter and how its composition and proinflammatory effects change over time due to photochemical aging in the atmosphere. The Southern California climate of Los Angeles with abundant sunshine, compounded with dense vehicular traffic, generates ubiquitous primary and secondary PM throughout the year. Identifying the health effects of these pollutants is critical as we strive to understand the underlying mechanisms of PM-induced oxidative stress, neuroinflammation and associated morbidity. Our findings may help in further elucidating the role of PM in the etiology, onset and development of widespread, chronic diseases that plague urban populations, including cancer, cardiac and respiratory distress, and neurodegenerative disorders such as Alzheimer's disease.

Data availability

Dataset 1: The following raw data sets are provided as comma separated values (.csv) files: 10.5256/f1000research.14836.d203329 (Lovett *et al.*, 2018)

PM_Diurnal_Variation_NO_Fig1_DATA

PM_Diurnal_Variation_qPCR_Fig2_DATA

PM_Diurnal_Variation_TC_Fig3A_DATA

PM_Diurnal_Variation_Ions_Fig3B_DATA

PM_Diurnal_Variation_Metals_Fig3C_DATA

Supplementary material

Table S1. Average concentrations and uncertainty values of total carbon, inorganic ions, metals and trace elements in ambient $PM_{2.5}$ slurry samples collected during morning and afternoon periods.

Click here to access the data.

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Kent E. Pinkerton

Center for Health and the Environment, University of California, Davis, Davis, CA, USA

The authors have adequately addressed all remaining concerns presented by the reviewers. This manuscript has full approval for indexing.

Competing Interests: No competing interests were disclosed.

Referee Expertise: Inhalation toxicology of gases, particles and fibers

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 2

Referee Report 03 September 2018

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Kent E. Pinkerton

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The research article by Lovett and colleagues, Diurnal variation in the proinflammatory activity of urban fine particulate matter ($PM_{2.5}$) by *in vitro* assays", is a fascinating and provocative article, however, not without some controversy in its interpretation.

The authors state ambient $PM_{2.5}$ undergoes diurnal changes in chemical composition due to photochemical oxidation. In this study the authors examined the relationships between oxidative activity and inflammatory responses associated with these diurnal chemical changes. Because secondary PM contains a higher fraction of oxidized PM species, the authors hypothesize $PM_{2.5}$ collected during afternoon hours induce a greater inflammatory response than primary, morning $PM_{2.5}$.

The methods used for PM collections methods are highly appropriate and well executed. The in vitro biological methods implemented with immortalized microglia cells are logical and clearly described. The authors state microglia were used for *in vitro* assays of oxidative and inflammatory responses to PM2.5

exposures because of their increasingly recognized role in environmental neurotoxicology. These are all correct statements. The difficulty comes in the interpretation of the findings.

Specific Comments:

1. It is unclear how the dose of PM delivered to microglia in vitro would compare to the in vivo setting where PM and/or oxidation reaction products would need to be transported either through the olfactory epithelium of the nasal cavity and/or via the blood passing through the lungs. The authors need to provide further clarification as to the credibility of using microglia cells in culture to mimic neurotoxicology. The authors need to clarify these concerns.

2. A five week sampling campaign was conducted by the authors during late summer (August and September) of 2016, ensuring maximum UV sunlight exposure to enhance photochemical oxidation reactions seems reasonable. However, how was the stability of PM samples maintained over such a prolonged period of sampling of five weeks? How stable are photochemical oxidation reactions?

3. $PM_{2.5}$ slurry samples were assayed with immortalized BV-2 microglia. It is unclear in the text what was the concentration of $PM_{2.5}$ slurry samples used for these assays. Please state in the text.

4. Based on Figure 1, were 1, 5 and 20 um/ul of $PM_{2.5}$ used? How do these doses compare to the concentration of $PM_{2.5}$ actually reaching microglia cells in vivo?

5. Nitric oxide (NO) was assayed in BV-2 cell media by the Griess reagent at 30, 60 minute and 24 hour timepoints. A $NaNO_2$ stock solution used to allow the creation of a standardization curve to determine the NO concentration in each treatment well from measured absorbance data. The authors observed a dose-dependent NO response to $PM_{2.5}$ treatments relative to control at all timepoints (30 min., 60 min., 24 hr.). This assay demonstrated greater effects for morning $PM_{2.5}$ than for afternoon $PM_{2.5}$. This assay is quite remarkable, meritorious and clearly illustrated. The interpretation of these findings need to be clearly stated.

6. Summary and Conclusion. The first portion of this section is nicely written to state "urban $PM_{2.5}$ collected during the morning rush hour (6–9am), when primary, traffic-derived PM emissions are dominant, induces greater oxidative and proinflammatory responses in cells as compared to $PM_{2.5}$ collected in the afternoon (12–4pm), which contains a higher proportion of photo-oxidized, secondary PM products". It is unclear whether the authors have provided conclusive evidence of these diurnal differences in terms of PM chemistry. Please clarify.

7. Summary and Conclusion. The authors state, "Two *in vitro* assays of the cellular inflammatory response consistently demonstrated greater oxidative and proinflammatory activity due to primary (morning) $PM_{2.5}$ exposure. We attribute this effect to the greater transition metal and water-insoluble organic carbon (WIOC) content of primary $PM_{2.5}$, two classes of PM components that increase toxicity. Again, how conclusive is the chemical analysis of PM from these two periods for $PM_{2.5}$ collected over a period of five weeks?

8. Summary and Conclusion. The authors state, "This research will ultimately help us gain a more complete understanding of the complex nature of particulate matter and how its composition and proinflammatory effects change over time due to photochemical aging in the atmosphere. The

Southern California climate of Los Angeles with abundant sunshine, compounded with dense vehicular traffic, generates ubiquitous primary and secondary PM throughout the year. Identifying the health effects of these pollutants is critical as we strive to understand the underlying mechanisms of PM-induced oxidative stress, neuroinflammation and associated morbidity". This is a laudatory conclusion made by the authors using in vitro cells that may or may not represent in vivo conditions of cell response and/or PM dose delivered to the nervous system. The authors need to clearly state the advantages of their study, along with the limitations for interpretation and extrapolation to actual in vivo conditions of exposure.

9. Summary and Conclusion. The authors state, "Our findings may help in further elucidating the role of PM in the etiology, onset and development of widespread, chronic diseases that plague urban populations, including cancer, cardiac and respiratory distress, and neurodegenerative disorders such as Alzheimer's disease". Should the authors acknowledge the limitations of the methods used in this study, this concluding statement is reasonable.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
No

Competing Interests: No competing interests were disclosed.

Referee Expertise: Inhalation toxicology of gases, particles and fibers

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 24 Sep 2018
Christopher Lovett, University of Southern California, USA

Authors' responses to specific comments of Dr. Kent Pinkerton:

Comment 1: *It is unclear how the dose of PM delivered to microglia* in vitro *would compare to the* in vivo *setting where PM and/or oxidation reaction products would need to be transported either through the olfactory epithelium of the nasal cavity and/or via the blood passing through the*

lungs. The authors need to provide further clarification as to the credibility of using microglia cells in culture to mimic neurotoxicology. The authors need to clarify these concerns.

Authors' Response: While we acknowledge the limitations of *in vitro* assays, we believe the reliability of the *in vitro* assays in characterizing neurotoxicology, as compared to *in vivo* experiments, has been adequately documented in the several studies cited in the paper (e.g. Morgan, et al., 2011; Davis et al., 2013; Cheng et al., 2016b; Woodward et al., 2017b). Additionally, the specific use of cultured BV-2 cells (microglia) successfully in *in vitro* assays of neurotoxicity has also been demonstrated, as cited in the paper (e.g. Gresa-Arribas et al., 2012; Eun et al., 2017).

Comment 2: *A five-week sampling campaign was conducted by the authors during late summer (August and September) of 2016, ensuring maximum UV sunlight exposure to enhance photochemical oxidation reactions seems reasonable. However, how was the stability of PM samples maintained over such a prolonged period of sampling of five weeks? How stable are photochemical oxidation reactions?*

Authors' Response: The PM samples were collected daily over the course of 5 weeks using the direct aerosol-into-liquid sampling system (Wang et al., 2013a). At the end of each sampling period, morning and afternoon, each daily aqueous sample was added to a total sample collection bottle kept under refrigeration. Once collected and refrigerated, these cumulative aqueous samples were used in the *in vitro* experiments discussed in the paper. It is possible that changes in PM composition might occur during sampling, but this is the advantage of using the direct aerosol-into-liquid system as opposed to filter-based sampling. PM is collected directly into an aqueous suspension and does not have to undergo an aqueous extraction and re-suspension process, which thereby significantly reduces the possibility of any artifact formation. The benefits of this collection method compared to conventional filter samplers are discussed in several prior publications (Zhao et al., 2005; Wang et al., 2013b; Saarikoski et al., 2014).

Added text (Methods: Particulate sample collection):
"At the end of each morning and afternoon daily sampling period, each aqueous slurry sample was added to its corresponding total sample collection bottle that was kept refrigerated. At the end of the 5-week sampling period, these continuously refrigerated, cumulative aqueous slurry samples were then used in the *in vitro* assays. While it is possible that changes in PM composition might occur during sampling, the advantage of using the direct aerosol-into-liquid system is that PM is collected directly into an aqueous suspension and does not undergo an aqueous extraction and re-suspension process, thereby significantly reducing the possibility of any artifact formation. The benefits of this collection method compared to conventional filter sampling systems has been discussed extensively in the literature (e.g. Zhao et al., 2005; Wang et al., 2013b; Saarikoski et al., 2014)."

Additional References (added to paper):
Zhao, Y., Bein, K. J., Wexler, A. S., Misra, C., Fine, P. M., & Sioutas, C. (2005). Field evaluation of the versatile aerosol concentration enrichment system (VACES) particle concentrator coupled to the rapid singleparticle mass spectrometer (RSMS3). *Journal of Geophysical Research: Atmospheres*, *110*(D07S02).

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& Jimenez, J. L. (2014). Evaluation of the performance of a particle concentrator for online instrumentation. *Atmospheric Measurement Techniques*, *7*(7), 2121-2135.

<u>Comment 3</u>: *$PM_{2.5}$ slurry samples were assayed with immortalized BV-2 microglia. It is unclear in the text what was the concentration of $PM_{2.5}$ slurry samples used for these assays. Please state in the text.*

Authors' Response: In the Methods section, the concentrations of $PM_{2.5}$ slurry samples used in each assay are stated, including the nitrite assay (Methods section: Nitrite Assay), which used 1, 5 and 20 µg/mL, and the qPCR assay (Methods section: Quantitative Polymerase Chain Reaction (qPCR)), which used 10 µg/mL.

<u>Comment 4</u>: *Based on Figure 1, were 1, 5 and 20 µg/ml of $PM_{2.5}$ used? How do these doses compare to the concentration of $PM_{2.5}$ actually reaching microglia cells* in vivo*?*

Authors' Response: In the Nitrite Assay (Figure 1), concentrations of 1, 5 and 20 µg/mL were used. In the qPCR assay (Figure 2), the $PM_{2.5\text{-}}$ concentration used was 10 µg/mL. We do not know the concentration of $PM_{2.5}$ reaching microglia in the brain following ambient exposure, however the *in vitro* assays were used as a model for how brain cells respond to $PM_{2.5}$ at the given concentrations. The focus of the paper was to investigate the differences between morning (primary-dominated) and afternoon (secondary-dominated) $PM_{2.5}$, rather than quantifying the actual exposure concentrations, and resulting CNS concentrations, that would be considered harmful. We modeled these interactions between $PM_{2.5}$ and microglia using concentrations that are below the threshold for cell death, as evaluated by the MTT assay. While there is evidence that particles can directly enter the brain through the olfactory tract (Oberdörster et al., 2004), the concentration of particles interacting directly with brain cells via this route has not been quantified, and thus comparisons could not be made.

Added text (Discussion section: paragraph 2):
"While there may be a concern that the concentrations of $PM_{2.5}$ treatments used in the *in vitro* assays do not reflect the exact concentrations of $PM_{2.5}$ reaching microglia in the brain following ambient exposures, these assays do serve as a useful model for how brain cells in living organisms would respond to $PM_{2.5}$ at the given concentrations (i.e. 1, 5, 10, and 20 µg/mL). The focus of the paper was to investigate the differences between morning (primary-dominated) and afternoon (secondary-dominated) $PM_{2.5}$, rather than to quantify actual exposure concentrations, and subsequent CNS concentrations, that would be considered harmful. We modeled these interactions between $PM_{2.5}$ and microglia using concentrations that are below the threshold for cell death, as evaluated by the MTT assay. While there is evidence that particles can directly enter the brain through the olfactory tract (Oberdörster et al., 2004), and thus perhaps maintain higher concentrations than $PM_{2.5}$ passing through the periphery, the concentration of particles interacting directly with brain cells via this route has not been quantified, and thus comparisons to these results could not be made."

Additional Reference (added to paper):
Oberdörster, G., Sharp, Z., Atudorei, V., Elder, A., Gelein, R., Kreyling, W., & Cox, C. (2004).

Translocation of inhaled ultrafine particles to the brain. *Inhalation Toxicology*, *16*(6-7), 437-445.

Comment 5: *Nitric oxide (NO) was assayed in BV-2 cell media by the Griess reagent at 30, 60 minute and 24 hour timepoints. A NaNO$_2$ stock solution used to allow the creation of a standardization curve to determine the NO concentration in each treatment well from measured absorbance data. The authors observed a dose-dependent NO response to PM$_{2.5}$ treatments relative to control at all timepoints (30 min., 60 min., 24 hr.). This assay demonstrated greater effects for morning PM$_{2.5}$ than for afternoon PM$_{2.5}$. This assay is quite remarkable, meritorious and clearly illustrated. The interpretation of these findings need to be clearly stated.*

Authors' Response: To clarify these findings, we offer the flowing additional text:

Added text (Results: Nitric Oxide (NO)):
"The acute effects of PM exposure seen within the first hour of exposure, at 30 and 60 minutes post-treatment, are due to direct NO induction, while the sustained effect still measurable after 24 hours indicates that there has been upregulation of the iNOS enzyme that produces NO. Thus, this overall effect is two-fold, with the increase in NO secretion due to PM$_{2.5}$ exposure mediated by two distinct mechanisms, acute and delayed."

Comment 6: *Summary and Conclusion. The first portion of this section is nicely written to state "urban PM$_{2.5}$ collected during the morning rush hour (6-9am), when primary, traffic-derived PM emissions are dominant, induces greater oxidative and proinflammatory responses in cells as compared to PM$_{2.5}$ collected in the afternoon (12-4pm), which contains a higher proportion of photo-oxidized, secondary PM products." It is unclear whether the authors have provided conclusive evidence of these diurnal differences in terms of PM chemistry. Please clarify.*

Authors' Response: It is well-known that over the course of daytime sunlight exposure, photochemical reactions occur, and secondary PM components, largely products of primary emissions oxidation, are formed such that secondary PM has a different chemical composition than primary PM. This is discussed in several places in the text, and relevant studies were cited (e.g. Forstner et al., 1997; Grosjean & Seinfeld, 1989; Seinfeld & Pandis, 2016). That the observed diurnal variations in cellular oxidative stress, as measured by two distinct *in vitro* assays, are related to higher concentrations of PM components associated with primary PM (i.e. transition metals and WIOC) was also discussed, and several studies were cited in support of this conclusion (e.g. Tao et al., 2003; Li et al., 2009; Cho et al., 2005; Zhang et al., 2008; Hu et al., 2008; Shirmohammadi et al., 2015).

Comment 7: *Summary and Conclusion. The authors state, "Two* in vitro *assays of the cellular inflammatory response consistently demonstrated greater oxidative and proinflammatory activity due to primary (morning) PM$_{2.5}$ exposure. We attribute this effect to the greater transition metal and water-insoluble organic carbon (WIOC) content of primary PM$_{2.5}$, two classes of PM components that increase toxicity." Again, how conclusive is the chemical analysis of PM from these two periods for PM$_{2.5}$ collected over a period of five weeks?*

Authors' Response: We feel that we have provided ample and solid evidence of the

compositional differences between these two sets of samples (am-$PM_{2.5}$ and pm-$PM_{2.5}$). We respectfully disagree that we have not provided clear evidence of these compositional differences of the two sets of samples based on the chemical analyses, specifically regarding both their organic carbon content as well as the toxic trace elements and metals components, as can be seen in Figure 3, as well as in the supplemental chemical analyses data files. If the concern is the lengthy time-integrated sample collection period of 5 weeks leading to sample instability or degradation, the aerosol-into-liquid samples are exceptionally stable given the removal of a filter-extraction step in the analysis process, particularly when kept refrigerated, as discussed above in our response to comment 2.

Comment 8: *Summary and Conclusion. The authors state, "This research will ultimately help us gain a more complete understanding of the complex nature of particulate matter and how its composition and proinflammatory effects change over time due to photochemical aging in the atmosphere. The Southern California climate of Los Angeles with abundant sunshine, compounded with dense vehicular traffic, generates ubiquitous primary and secondary PM throughout the year. Identifying the health effects of these pollutants is critical as we strive to understand the underlying mechanisms of PM-induced oxidative stress, neuroinflammation and associated morbidity". This is a laudatory conclusion made by the authors using* in vitro *cells that may or may not represent* in vivo *conditions of cell response and/or PM dose delivered to the nervous system. The authors need to clearly state the advantages of their study, along with the limitations for interpretation and extrapolation to actual* in vivo *conditions of exposure.*

Authors' Response: One methodological advantage of this study, as stated in the text, is the use of the direct aerosol-into-liquid sampler, which allows us to capture more water-insoluble PM species that may not be eluted during water extraction of filters. Another advantage of this study is that we use two separate *in vitro* assays of proinflammatory biomarkers (the nitrite assay and qPCR). Additionally, while there are limitations in extrapolating *in vitro* findings to processes occurring in living organisms, the focus of this study was to compare the relative proinflammatory effects of direct cellular exposure to morning and afternoon $PM_{2.5}$ rather than to precisely quantify these effects *in vivo*. Additionally, studies of direct interactions between $PM_{2.5}$ and microglia *in vivo* have not been conducted for comparison with our (and numerous other) *in vitro* studies.

Comment 9: *Summary and Conclusion. The authors state, "Our findings may help in further elucidating the role of PM in the etiology, onset and development of widespread, chronic diseases that plague urban populations, including cancer, cardiac and respiratory distress, and neurodegenerative disorders such as Alzheimer's disease". Should the authors acknowledge the limitations of the methods used in this study, this concluding statement is reasonable.*

Authors' Response: Regarding the limitations of methods used in this study, the responses to comments and additional text incorporated into the revised manuscript, as detailed in the responses to comments above, should provide sufficient to address these concerns.

Competing Interests: No competing interests.

Referee Report 30 July 2018

Ning Li (iD)

Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine (CVM),
Michigan State University, East Lansing, MI, USA

General comments

This study examined the relationship between diurnal changes in the chemical properties and the
pro-oxidant and pro-inflammatory activities of PM2.5 collected in Los Angeles area. The hypothesis was
that compared to primary, morning PM (am-PM), secondary PM in the afternoon (pm-PM) would induce a
stronger inflammatory response in microglial cells. PM were collected using an aerosol-into-liquid
collection system. Cellular endpoints included nitric oxide induction and the expression of IL-1b, IL-6 and
MCP-1 genes. Characterization of PM included analyses of metal and trace elements, total carbon and
inorganic ions. The findings were contrary to the authors' expectation. Morning PM had stronger effects in
inducing NO production and up-regulating IL-1b, IL-6 and MCP-1 gene expression than pm-PM. It was
concluded that the diurnal differences between am-PM and pm-PM may be caused by the greater content
of transition metals and water-insoluble organic carbon of am-PM (primary PM). This work has two
strengths: 1) diurnal changes in the chemical properties and adverse health effects of ambient PM have
not been well studied and 2) the use of aerosol-into-liquid collection system reduces the loss of PM
components.

Specific comments

1. Fig. 1. The highest PM concentration was 20 µg/ml. The authors indicated that "The 10 µg/ml dose
   was chosen as below threshold for metabolic impairment based on prior studies from our group".
   Was metabolic impairment assessed at 20 µg/ml? If yes, was there any cellular injury?
2. Were endotoxin levels in these PM samples measured? Was there any difference between
   am-PM and pm-PM?
3. The authors explained the rationale for not using DTT and DCF-DA assays. What was the rationale
   for selecting NO instead of other indicators (e.g., HO-1 or GSH/GSSG) to assess oxidative stress?
4. Fig. 2. What are the reasons that there is no * above pm-PM2.5 in Figures 2A (IL-1b) and 2C
   (MCP-1)? Were pm-PM2.5-induced increases of IL-1b and MCP-1 significantly different from
   respective control? What is the p-value of am-PM2.5 vs. pm-PM2.5 in Fig. 2A?
5. Fig. 3A, 3B and 3C (As, Co, Cr, Fe, Mn and Ni). Are the differences between am-PM and pm-PM
   statistically significant?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: air pollution and allergic airway inflammation

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 31 Jul 2018
Christopher Lovett, University of Southern California, USA

Authors' responses to specific comments of Dr. Ning Li:

1. *Fig. 1. The highest PM concentration was 20 µg/ml. The authors indicated that "The 10 µg/ml dose was chosen as below threshold for metabolic impairment based on prior studies from our group." Was metabolic impairment assessed at 20 µg/ml? If yes, was there any cellular injury?*

Response: The MTT assay was conducted with BV-2 cells and included four doses (1, 5, 10, 20 µg/mL) of PM2.5 slurry samples, followed by 24 hours incubation time. Significant reductions in mitochondrial activity occurred only at the highest PM2.5 dose, 20 µg/mL, in both am-PM2.5 and pm-PM2.5 samples. However, this activity was still above the 50% threshold. Additionally, given strong cell responses at 20 µg/mL for NO and cytokines, we infer that cells were viable at the highest dose.

2. *Were endotoxin levels in these PM samples measured? Was there any difference between am-PM and pm-PM?*

Response: Prior studies using the Limulus assay did not detect endotoxin (Woodward et al., 2017) in ambient PM samples collected at the same central Los Angeles location. Additionally, all sample collection and measurement equipment was routinely sanitized with 70% ethanol solution prior to use each week, per our strict laboratory hygiene protocols.

3. *The authors explained the rationale for not using DTT and DCF-DA assays. What was the rationale for selecting NO instead of other indicators (e.g., HO-1 or GSH/GSSG) to assess oxidative stress?*

Response: Nitric oxide (NO) secretion, measured by the Griess assay, was chosen as a biomarker of the oxidative stress response based on its reliability as an index of PM-induced oxidative stress. Our research group has previously used this measure in several published studies of nPM exposure *in vitro* (e.g. Davis et al., 2013; Cheng et al., 2016).

4. *Fig. 2. What are the reasons that there is no * above pm-PM2.5 in Figures 2A (IL-1b) and 2C (MCP-1)? Were pm-PM2.5-induced increases of IL-1b and MCP-1 significantly different from respective control? What is p-value of am-PM2.5 vs. pm-PM2.5 in Fig. 2A?*

Response: In Figure 2A (IL-1β), the response to pm-PM2.5 was 3.3-fold above control, with marginal significance (p = 0.14). The IL-1β response to am-PM2.5 was 1.5-fold above the pm-PM2.5 response (not significant, p = 0.41). In Figure 2C (MCP-1), the response to pm-PM2.5 was 1.3-fold above control (not significant, p = 0.44).

5. *Figs. 3A, 3B and 3C (As, Co, Cr, Fe, Mn and Ni). Are the differences between am-PM and pm-PM statistically significant?*

Response: The chemical data presented in Figures 3A, 3B, and 3C (total carbon, inorganic ions, total metals/trace elements) include error bars representing laboratory measurement uncertainty. However, because only one time-integrated slurry sample (collected over several hours, from either 6-9am or 12-4pm, during each weekday for 5 weeks) was analyzed in each condition (n = 1), it was not possible to do an ANOVA to determine the statistical significance of differences in concentrations of various chemical species between the am-PM2.5 and pm-PM2.5 samples.

Competing Interests: No competing interests were disclosed.

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