

## Clinical Study

# Inhalation of Nebulized Diesel Particulate Matter: A Safety Trial in Healthy Humans

Sandra C. Dorman,<sup>1</sup> Kaylyn M. Sutcliffe,<sup>1</sup> Jacques Abourbih,<sup>2</sup> and Stacey A. Ritz<sup>2</sup>

<sup>1</sup> School of Human Kinetics, Laurentian University, 935 Ramsey Lake Road, Greater Sudbury, ON, Canada P3E 2C6

<sup>2</sup> Medical Sciences Division, Northern Ontario School of Medicine, ON, Canada P3E 2C6

Correspondence should be addressed to Sandra C. Dorman; [sdorman@laurentian.ca](mailto:sdorman@laurentian.ca)

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Diesel particulate matter (DPM) has adverse health effects. Examining the underlying pathophysiological mechanisms would be facilitated by the introduction of an exposure method that is safe, portable, and cost-effective. The purpose of this study was to establish a novel method to study DPM exposure via nebulization and an inhalation dose that was safe, yet capable of eliciting an inflammatory response. Ten participants enrolled in this nonblinded, nonrandomized study. Subjects inhaled nebulized 0.9% saline and increasing doses of DPM suspended in 0.9% saline (75, 150, and 300  $\mu\text{g}$ ) in a sequential manner. FEV<sub>1</sub> was measured repeatedly during the first 2 h after exposure and blood, oximetry, sputum, and heart rate were taken before, 2 h, and 24 h after inhalation challenge. DPM inhalation was well-tolerated at all doses. A decrease in FEV<sub>1</sub> was observed after each inhalation challenge (including saline). Inhalation of 300  $\mu\text{g}$  DPM produced a significantly different FEV<sub>1</sub> response curve. An increase in particle inclusion-positive sputum macrophages for all DPM doses confirmed that the nebulized particles were reaching the lower airways. Serum GM-CSF was elevated after exposures to 150 and 300  $\mu\text{g}$  DPM. No other inflammatory changes were detected. DPM inhalation via nebulizer is a safe method of delivering low doses of DPMs in healthy people.

## 1. Introduction

In a variety of epidemiological and experimental studies, researchers have demonstrated that respiratory exposure to airborne pollutants is associated with systemic sequelae that can not only have effects on the respiratory tract itself but also create fertile conditions for the development of atherosclerosis, plaque destabilization, atherothrombosis, and consequent cardiovascular events [1–3]. Diesel particulate matter (DPM) is a significant component of airborne particulate matter, as diesel engines are widely used in modern industry and transportation; DPM contains substances that can pose a risk to human health, and the particles themselves are readily respirable and penetrate into the alveolar spaces. DPM is widely used as an experimental model for particulate air pollution for several reasons: they are a common real-life pollutant; they are chemically and physically well-characterized; standardized material is available; and the existing literature on DPM exposure (in cultures, animal models, and humans)

gives us a broad knowledge base from which to base our protocols and expectations [4–7].

There are a few existing models for human experimental exposures to DPMs. The most common protocol is to direct exhaust from a diesel engine into an exposure chamber. Although very effective, the disadvantage of this system is the elaborate, fixed setup, which is expensive to build and operate. Nasal instillation of DPM is a less expensive method which induces local inflammation in the nasal lavage fluid [6], increases in IgE [5, 7], and augments allergen-induced responses in the nasal passages [7]. This method is inexpensive and simple but does not deliver the particles to the lower respiratory tract.

Nebulization of particles has not been described in the literature for use in human subjects, although it is frequently used in animal studies. If established as safe and effective, inhalation of nebulized DPM would be an inexpensive and simple method to facilitate health research on the effects of particulates in the lungs and systemically. In this safety trial,

TABLE 1: Subject characteristics.

Subject	FEV <sub>1</sub>	FVC	Gender	Age
1	3.1	3.8	F	35
2	3.9	5.6	M	38
3	3.2	4.1	F	32
4	3.2	3.8	F	34
5	3.7	4.5	F	38
6	3.2	3.5	F	19
7	3.4	4.1	F	19
8	3.7	4.1	F	21
9	2.4	3.2	F	38
10	3.9	4.5	F	23

we examined whether DPM exposure via nebulization is safe for experimental study, aiming to identify the lowest effective dose of DPMs capable of reliably eliciting inflammatory responses in the airways and blood.

## 2. Methods

**2.1. Subjects.** Twelve healthy subjects were enrolled in the study (female: 10; male: 2). Prior to study entry subjects were examined by a physician to confirm their overall good health. This included a physical exam and blood work, assessing C-reactive protein (CRP) levels, kidney and liver function, and (for female subjects) a pregnancy test. Subjects were excluded from the study if their baseline CRP levels were above 3 mg/L, they were pregnant, or for any other health consideration identified by the examining physician. Subjects were nonsmokers, were not using any medication other than birth control, and had not experienced respiratory infection within the previous six weeks. Two subjects (1 male; 1 female) were excluded during screening due to high levels of serum CRP. All subjects were instructed to refrain from taking over-the-counter nonsteroidal anti-inflammatories for two weeks prior to and during the course of the study. See Table 1 for subject characteristics.

**2.2. Study Design.** As it was primarily a safety trial, this study was a nonrandom, nonblinded design consisting of four arms. Subjects were required to come into the laboratory on thirteen separate sessions. Visit one was the screening visit, with the following twelve sessions comprising the four arms of the study. For each arm, subjects visited the laboratory for three consecutive days. Each arm was separated by a minimum of 7 days and all visits occurred in the morning within 1 hour of each other to minimize circadian variations. A physician was onsite during all study visits and examined patient results prior to continuing with the next inhalation challenge.

For each arm of the study, on study day 1 (baseline), subjects were asked if they had any medical issues since the last visit and it was confirmed that they had not taken any medications prior to their sessions. Baseline spirometric measurements (FEV<sub>1</sub>, FVC), pulse rate, and pulse oximetry were taken followed by sputum induction and blood sampling.

On day 2, baseline spirometry was repeated, followed by the inhalation of the nebulized saline or DPM. After inhalation challenge, pulse rate, pulse oximetry, spirometric measurements, and a symptoms questionnaire were measured for 2 h, at which time blood sampling and sputum induction were performed. On day 3, all the measurements taken on day 1 were repeated.

This study protocol was approved by the Laurentian University Ethics Board, and all subjects provided written, informed consent prior to participation in the study.

**2.3. Diesel Particulate Matter.** Standardized DPM (SRM2975) was obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA). The standardized DPM was collected from the exhaust of a diesel forklift and hot bag filter system, as described in the certificate of analysis for this material [8]. DPM was suspended in 3 mL of 0.9% saline. DPM solutions were diluted and sonicated using a Sonic Dismembrator Model 500 (Fisher Scientific) 20 m prior to inhalation challenge. The top dose used in our study, 300 µg, was selected based on the work of Diaz-Sanchez et al., who administer this amount to human subjects by intranasal instillation and is roughly equivalent to breathing Los Angeles air for 24 h or 30 s of breathing standing in close proximity to an operating diesel engine [7].

**2.4. Inhalation Challenge.** The AeroEclipse II Breath Actuated Nebulizer (Monaghan Medical Corporation, Plattsburgh, NY, USA) was used for inhalation challenge. 3 mL of either 0.9% saline or a mixture of 0.9% saline with DPM doses of 75 µg, 150 µg, and 300 µg (DPM75, DPM150, and DPM300) was placed into the nebulizer cup (final concentrations of 25, 50, and 100 µg/mL, resp.). The nebulizer was set to “breathe actuated” mode to ensure subjects inhaled the entire dose. The flow meter, attached to medical air, was set to 50 P.S.I. with a flow of 8 liters per minute. Subjects wore a nose plug and were asked to inhale and exhale through the nebulizer at a normal breathing pace until the entire solution was gone.

**2.5. Oximetry & Pulse Rate.** Oximetry and pulse rate were measured using a SuperSpiro Spirometer that was equipped with a Nonin SpO<sub>2</sub> probe (Micro Medical Ltd., Kent, UK). Oximetry and pulse rate were measured concurrently on day 1 and at 2 min, 30 min, 2 hours, and 24 hours after inhalation.

**2.6. Spirometry.** Spirometry was performed with a SuperSpiro Spirometer V1.05 (Micro Medical Ltd., Kent, UK) according to the American Thoracic Society standards [9]. FEV<sub>1</sub> and FVC were repeated a minimum of three times, selecting the best effort. Spirometry was also performed after each inhalation challenge and at 10, 20, 30, 45, 60, 90, and 120 min after inhalation challenge.

**2.7. Symptoms Questionnaire.** An eight-question symptom score questionnaire was administered at 2 h after inhalation, querying the subjects’ experience of: headache, nausea, dizziness, difficulty concentrating, fatigue, weakness, heart rate, and dyspnea.

TABLE 2: Total cell counts following inhalation of saline, DPM75, DPM150, and DPM300 ( $\times 10^6$  cells/g).

	Baseline	2 h	24 h
Saline	7.5 $\pm$ 2.78	4.7 $\pm$ 1.43	5.1 $\pm$ 1.25
DPM 75	6.2 $\pm$ 1.36	3.6 $\pm$ 0.79	5.3 $\pm$ 0.10
DPM 150	13.0 $\pm$ 9.11	3.4 $\pm$ 0.59	3.7 $\pm$ 0.12
DPM 300	5.3 $\pm$ 1.00	5.5 $\pm$ 1.55	9.0 $\pm$ 0.35

Values given as mean  $\pm$  SEM. No significant differences between time or groups was found.

**2.8. Sputum Induction/Processing.** Sputum was induced by inhalation of a hypertonic saline mist and processed according to Pin et al. [10] and modified according to Pizzichini et al. [11]. Briefly, subjects inhaled 3, 4, and 5% aerosolized saline for 7 min each, expectorating between each, until sufficient sample was obtained. Sputum plugs were selected from the expectorate, and if sufficient quantity was obtained (approximately 200 mg), cell smears were prepared and stained with DiffQuik (Fisher Scientific). Differential cell counts were performed by counting 400 nonsquamous cells from duplicate slides. In a subsequent reading of the same slides, macrophages were subdivided into those with no visible particle inclusions (negative), those with fewer than 20 inclusions (low-positive), and those with more than 20 inclusions (high-positive), as described by Mukae et al. [12]. All counts were performed by a technician blinded to the subject and exposure status.

**2.9. Peripheral Blood Collection/Analysis.** Venous blood samples were obtained at baseline and 2 and 24 hours after inhalation challenge. Complete blood counts (CBC), erythrocyte sedimentation rate (ESR), international normalized ratio (INR), and C-reactive protein (CRP) measurements were performed by LifeLabs Medical Laboratory Services. Blood serum was separated by centrifugation and stored at  $-80^{\circ}\text{C}$ . IL-6, GM-CSF, and IL-8 protein levels were quantified using commercially available ELISAs (eBioscience, USA, and BD OptEIA, Canada, resp.). The limits of detection for IL-6 and GM-CSF were approximately 2 pg/mL. The limit of detection for IL-8 was approximately 3.1 pg/mL. Values below the limit of detection were assumed to be 0 pg/mL for statistical analysis.

**2.10. Statistics.** Summary statistics were expressed as mean  $\pm$  SEM. Data were analyzed using repeated measures (rm) ANOVA (between group analysis: saline versus DPM75 versus DPM150 versus DPM300; within group analysis: before versus 2 hr after versus 24 hr after inhalation). Statistical significance was accepted as  $P < 0.05$ .

### 3. Results

**3.1. Inhalation-Induced Airway Responses.** Baseline FEV<sub>1</sub> values were similar on all study days. During the two hours after inhalation challenge, there was a significant decrease in the mean maximal fall in FEV<sub>1</sub> from baseline after all challenges ( $P < 0.05$ ) (Figures 1(a)–1(d)). The percent mean maximal falls in FEV<sub>1</sub> from baseline were  $3.6 \pm 0.97\%$ ,  $3.0 \pm 0.62\%$ ,  $4.7 \pm 0.95\%$ , and  $6.2 \pm 1.81\%$ , for saline, DPM75,

DPM150, and DPM300, respectively. All of the maximum falls in FEV<sub>1</sub> occurred within 45 min of inhalation. These declines were transient for all doses, with FEV<sub>1</sub> statistically indistinguishable from baseline by 90 min after inhalation. When comparing the mean maximal fall in FEV<sub>1</sub> between inhalation challenges, the 300  $\mu\text{g}$  dose was statistically significantly different from the saline and 75  $\mu\text{g}$  doses (Figure 1(e)) ( $P < 0.05$ ).

#### 3.2. Sputum Cell Counts

**Total Cell Counts.** Baseline values were similar on all study days. Following inhalation challenge, there were no significant changes in the total cell counts at 2 or 24 hours compared to baseline for any of the challenges. There were no significant differences in total cell counts between groups (Table 2).

Following inhalation challenge, there were no significant differences in the total number of or in the percent change in neutrophils, macrophages, or eosinophils at 2 or 24 hours compared to baseline for all of the challenges (data not shown). There were no significant differences in the number or percentage of these cells between groups.

**3.3. Sputum Macrophage Particle Inclusions.** The majority of sputum macrophages (80–90%) had no visible particle inclusions in all groups at all time points (data not shown). The majority of sputum macrophages with visible particle inclusions fell into the “low-positive” category (i.e., fewer than 20 inclusions) with the proportion of “high-positive” (i.e., more than 20 inclusions) sputum macrophages under 0.5% of total sputum macrophages (data not shown). Thus, for the purposes of analysis, the “low-positive” and “high-positive” macrophages were grouped together (Figure 2).

At baseline, approximately 10–13% of sputum macrophages had particle inclusions (Figure 2). After inhalation challenge with DPM, the proportion of macrophages with particle inclusions increased significantly at 2 h compared to baseline ( $P < 0.05$ ), approaching 20% for all DPM doses. At 24 h after DPM exposure, particle inclusions in sputum macrophages remained slightly elevated but remained statistically significant only for the 150  $\mu\text{g}$  dose.

#### 3.4. Blood Analysis

**White Blood Cell Count.** Baseline values were similar on all study days. Following inhalation challenge, there were no significant changes in WBC at 2 or 24 hours compared to baseline for all of the challenges and no significant differences between groups (data not shown).

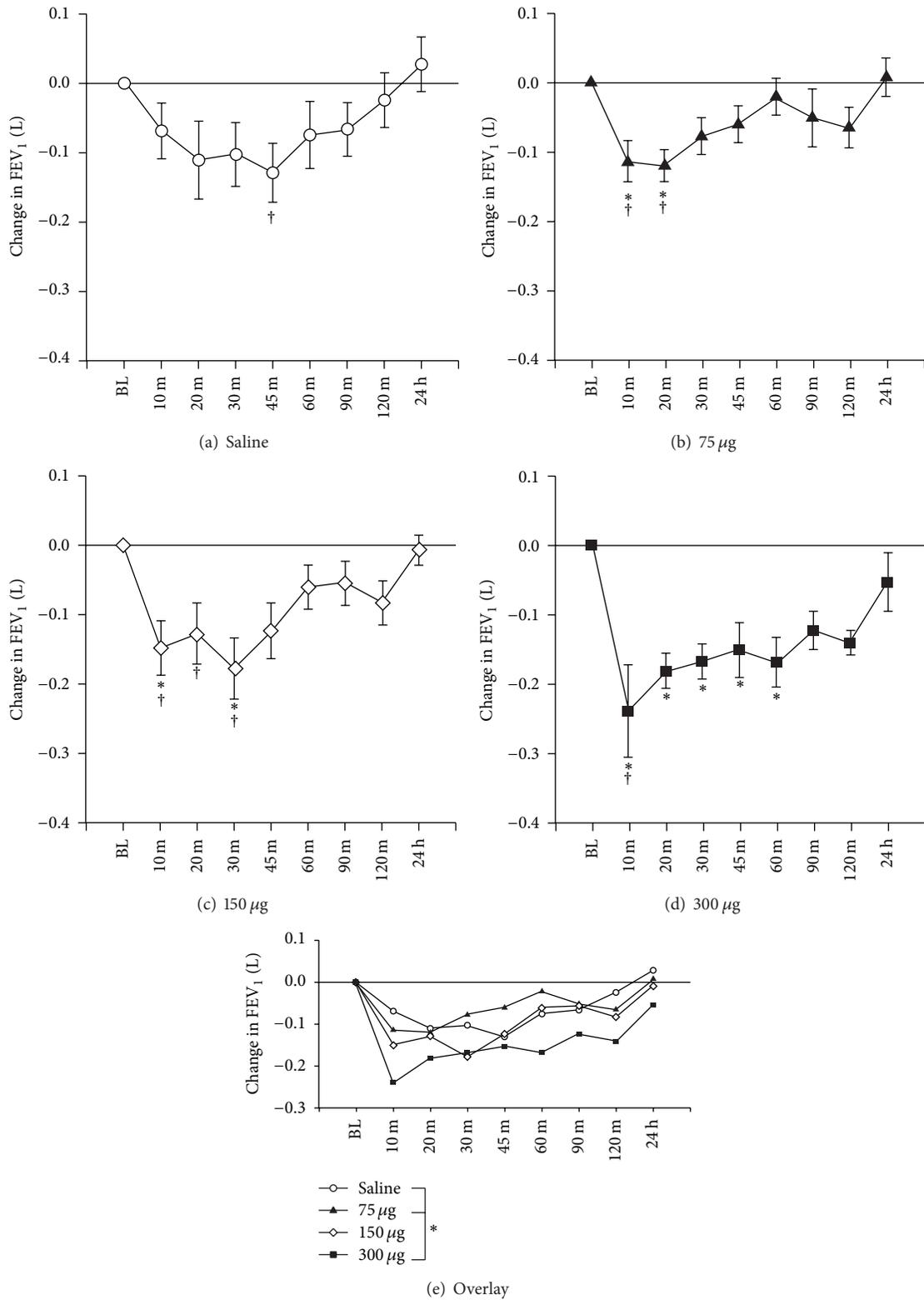


FIGURE 1: Change in forced expiratory volume per second (FEV<sub>1</sub>) following inhalation of saline (a), 75 µg (b), 150 µg (c), or 300 µg DPM (d). Data in (a)–(d) are shown as mean ± SEM. (e) is an overlay of the curves from (a)–(d) for comparison of means. In (a)–(d), \* significant decrease in FEV<sub>1</sub> from baseline (BL) by 1-way repeated measures ANOVA; † significant difference from 24 h by 1-way repeated measures ANOVA. In (e), \* significant difference between the doses by repeated measures ANOVA.

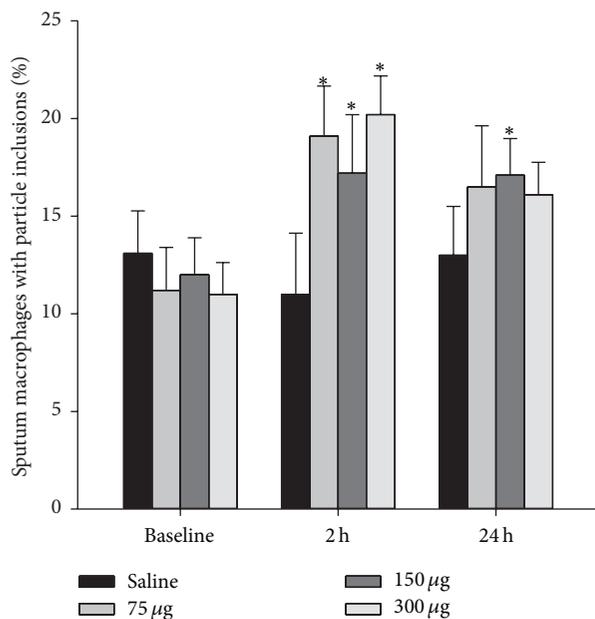


FIGURE 2: Percent of sputum macrophages with particle inclusions at baseline (BL), 2 h, and 24 h after exposure to saline or varying doses of DPM. Data are shown as mean  $\pm$  SEM. \* Significant change from baseline by repeated measures ANOVA.

Following inhalation challenge, there were no significant differences in the total number of neutrophils, monocytes, eosinophils, or platelets at 2 or 24 hours compared to baseline for all of the challenges and no significant differences between groups (data not shown).

Baseline values for erythrocyte sedimentation rate, prothrombin time, and C-reactive protein were similar on all study days. Following inhalation challenge, there were no significant changes in either measure at 2 or 24 hours compared to baseline for all of the challenges and no significant differences between groups (data not shown).

### 3.5. Sputum Supernatant and Serum Assays

**Interleukin-8 in Sputum Supernatant.** Baseline values were similar on all study days. Following inhalation challenge, there were no significant changes in IL-8 at 2 or 24 hours compared to baseline for saline, DPM150, or DPM300. There was a significant decrease from baseline at the 2 h time point for DPM75 ( $P < 0.05$ ) (data not shown). When comparing inhalation challenges, we found a significant decrease in IL-8 at 2 h after inhalation of DPM300 compared to saline, but not for DPM75 and DPM150. We found a significant decrease in IL-8 at 24 h after inhalation of DPM75 compared to saline and DPM150; no other significant difference between groups at 24 h after inhalation challenge was found (data not shown).

**Interleukin-8 in Serum.** In the serum, baseline values were similar on all study days. Following inhalation challenge, there was a significant decrease in IL-8 from baseline for DPM150 at 2 h and 24 h (BL:  $6.5 \pm 1.78$ ; 2 h:  $2.3 \pm 0.61$ ; 24 h:  $1.5 \pm 0.66$ ) but not for DPM75 or DPM300 (data not

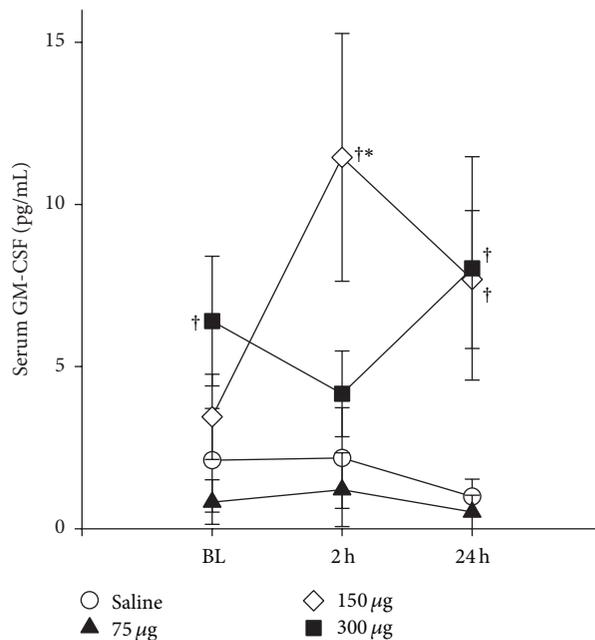


FIGURE 3: Serum granulocyte macrophage colony-stimulating factor (GM-CSF) levels measured by ELISA at baseline (BL), 2 h, and 24 h after exposure to saline or varying doses of DPM. Data are shown as mean  $\pm$  SEM. \* Significant difference from baseline and † significant difference from saline at a given timepoint, as determined by repeated measures ANOVA.

shown). When comparing inhalation challenges, there was significantly less IL-8 at 2 and 24 h after inhalation of DPM150 compared to saline, DPM75, and DPM300; there were no other significant differences in IL-8 between groups.

**Interleukin-6 in Sputum Supernatant.** There were no significant changes in IL-6 at 2 or 24 h compared to baseline for any dose, nor were there any differences between inhalation challenges between groups.

**Interleukin-6 in Serum.** There were no significant changes in IL-6 at 2 or 24 h compared to baseline for any dose, nor were there any differences between inhalation challenges between groups.

**Granulocyte/Macrophage-Colony Stimulating Factor in Sputum Supernatant.** There were no significant changes in GM-CSF at 2 or 24 hours compared to baseline for any dose, nor were there any differences between inhalation challenges between groups.

**Granulocyte/Macrophage-Colony Stimulating Factor in Serum.** The baseline value for DPM300 was significantly greater than the saline and DPM75 dose (Figure 3). At 2 h after inhalation of DPM150, we found a significant increase in GM-CSF compared to saline, DPM75, and DPM300. At 24 h after inhalation of DPM150 and DPM300, we found a significant increase in GM-CSF compared to saline and DPM75.

**3.6. Oximetry & Pulse Rate.** Baseline values were similar on all study days. Following inhalation challenge, there were no significant changes in blood oxygen saturation or pulse rate at 2 min, 30 min, 2 h, or 24 h compared to baseline for all of the challenges (data not shown).

**3.7. Symptom Questionnaire.** None of the subjects reported any serious adverse symptoms during the saline inhalation challenge. Following inhalation challenge of DPM75, 3 subjects answered positively to the symptoms questionnaire: 1 subject said that they felt both mild nausea and had a mild headache, 1 subject said they felt mild dizziness, and 1 subject said they experienced mild weakness. At DPM150 1 subject answered positively to the symptoms questionnaire, saying they experienced mild fatigue. At DPM300, 4 subjects answered positively to the symptoms questionnaire: 1 experienced mild headache and mild fatigue; 1 experienced mild headache, mild weakness, and mild difficulty breathing; 1 experienced mild fatigue and mild difficulty concentrating; and 1 respondent experienced mild fatigue. None of these symptoms for any of the 4 arms were statistically significant. One symptom that was not on the questionnaire that was spontaneously reported by subjects was a scratchy throat: 6 out of 10 subjects reported a mild scratchy throat at DPM300.

## 4. Discussion

Our findings establish that inhalation of nebulized DPM mixed in saline via a nebulizer is a safe and effective method for research on the local and systemic effects of particulate matter in healthy human subjects. Inhalation of DPM using this method induced declines in FEV<sub>1</sub> and some mild symptoms such as nausea, dizziness, fatigue, headache, and a scratchy throat. We also saw evidence that the DPM reached the lower airways through an increase in the positive macrophage particle inclusions and evidence of systemic effects with an increase in GM-CSF in the blood serum. To our knowledge, this is the first published study of the isolated effects of DPM using nebulization as a delivery method. As a pilot study, with safety being the paramount concern, we used a nonrandom, nonblinded design, so that we could establish the tolerability of each dose in each subject before escalating the dose further.

We observed small but significant decreases in FEV<sub>1</sub> following all inhalation challenges, which were transient and returned to baseline by the 24 h time point. We were somewhat surprised to see a small decrement in FEV<sub>1</sub> after inhalation of saline, as we would expect that in healthy individuals, we would not see any change in FEV<sub>1</sub> after isotonic, hypotonic, or hypertonic saline inhalations [13]. However, it is known that inhalation of saline can cause airway constriction in people with asthma [14]. Our subjects were screened for airway disease by questionnaire, but we did not perform methacholine challenge on them to test for airway hyperresponsiveness. Thus, it is possible that a subset of our subjects had undiagnosed asthma and were thus hyper-responsive, resulting in the observed decrement in FEV<sub>1</sub> after inhalation of saline only. Inhalation of nebulized DPM resulted in a small but statistically significant drop in FEV<sub>1</sub> in

healthy humans; it is reasonable to hypothesize that there may be a more severe problem in subjects who have an underlying pulmonary disease or airway hyperresponsiveness [15, 16]. We therefore would recommend that, in addition to the safety measures that we took during this study, all subjects be prescreened with methacholine challenge, particularly if this method was to be performed in a population with allergy or asthma.

Our findings indicate that DPM exposure has acute effects on lung function of healthy individuals, in contrast with what has been reported by others [4, 17–20]. In contrast, lung function declines have been documented in people with asthma exposed to DPM. For example, McCreanor et al. [15] observed a decrease in FEV<sub>1</sub> in mild and moderate asthmatic subjects who were exposed to diesel exhaust for 2 h in a street setting. Interestingly, the FEV<sub>1</sub> drop that we observed at the 300 µg dose of DPM was comparable in magnitude to that found by McCreanor et al. (6.1% versus 6.2%, resp.). In a real-life setting, people do inhale concentrations of particulates, similar to the doses we used, in short periods of time, for example, standing behind a bus when it is started or in some occupational settings. Thus, the ability of DPM to induce a decrease in FEV<sub>1</sub> in healthy humans in a real life setting is unclear, but our findings indicate that it is able to do so, at least under experimental conditions.

The doses used in this study did not elicit a lung inflammatory response as measured in induced sputum, in contrast with other human exposure studies [4, 17–20]. Nightingale et al. [4] were the first group to look at the isolated health effects of DPM in the lower airways after a controlled inhalation exposure in humans and measured an inflammatory response in sputum. However, differences in experimental design may explain this disparity, as our subjects received a single acute dose of an exact dose of DPM by nebulization, while in the Nightingale study, subjects inhaled a lower concentration over a 2 h time period, resulting in a similar overall dose but inhaled over very different periods of time. It may be that nebulization over a short period results in a different pattern of deposition of particles in the airways as compared to inhalation of a similar dose in an exposure chamber over a longer period of time. An alternative explanation is that we did not look at an appropriate time point. Nightingale et al. saw changes at 4 hours that did not persist at 24 hours after inhalation. We measured airway inflammation at 2 and 24 hours after inhalation, and it is possible that inflammatory changes occurred between these time points that we did not capture.

The lack of inflammation we observed cannot be attributed to a failure of the nebulized DPM to reach the lower airways, as we measured a significant increase in particle-containing macrophages in the sputum after DPM exposure. This not only demonstrates that the particles reached the alveoli but is also significant because alveolar macrophages phagocytosing particulate matter can release cellular mediators, which can stimulate the bone marrow, indirectly signaling an inflammatory response [12]. Others have suggested that alveolar macrophages can release some of their lysosomal contents (ROS) when in direct contact with particles or during phagocytosis, which can also lead to

inflammation indirectly [21]. Although we did not measure a cellular inflammatory response after any dose of DPM inhaled, it seems likely that the dose of particulate that reached the lungs was not sufficient to stimulate the alveolar macrophages to a significant extent; this is supported by other research that shows a dose response associated with macrophage responses to DPM [12]. In this study, only a tiny fraction of alveolar macrophages were “high-positive” for particle inclusion, even after the DPM300 inhalation challenge ( $0.2 \pm 0.08\%$  at 2 h, compared to 0 in the saline group). Induction of measureable inflammatory responses may require a larger dose or longer exposure in order to stimulate airway resident cells such as alveolar macrophages (or airway epithelial cells) to release proinflammatory mediators or ROS and generate an inflammatory response.

Not surprisingly in the absence of notable local inflammation, inhalation of nebulized DPM did not induce discernable systemic cellular inflammation after inhalation of DPM. However, we did see a significant increase in serum levels of GM-CSF at 2 h following inhalation of DPM150, and serum GM-CSF levels were significantly elevated 24 h after exposure to DPM150 and DPM300 (Figure 3). Our observations are consistent with *in vitro* studies examining the effects of DPM, which have shown increases in GM-CSF [22]. Moreover, Van Eeden et al. have measured increased levels of circulating GM-CSF in healthy soldiers exposed to forest fires with the predominant pollutant being particulate matter [23]; the increases we observed were small in comparison; however, our DPM doses are also relatively low and DPM has a different chemical composition than the particulates derived from forest fires. However, given the lack of cellular inflammation at the time points we measured, these small increases in GM-CSF may not be sufficient to induce a measureable inflammatory response.

This increase in serum GM-CSF, however, was not accompanied by an increase in sputum supernatant GM-CSF or in granulocyte levels in the sputum or blood. Increased levels of GM-CSF generally have not been detected in the supernatant following sputum induction [24], although increased levels have been evident when using bronchoscopy after inhalation of similar DPM doses [25]. Alternatively, it is possible that our inability to measure changes in granulocytes may be due to the time points selected for sampling or simply that these doses of DPM had a relatively small effect on the levels of GM-CSF produced.

The increased level of GM-CSF present at baseline in the DPM300 might suggest that the increase in serum GM-CSF persisted for at least one week after inhalation and that our wash-out period needed to be longer. However, given that there were no other indicators of a persistent inflammatory response, we suspect that the more likely explanation is that some of our subjects were exposed to an unidentified stimulus outside the laboratory that increased GM-CSF levels.

The presence of GM-CSF is of interest to us because GM-CSF is a cytokine that stimulates bone marrow hematopoietic stem cells to produce increased numbers of neutrophils, eosinophils, basophils, and monocytes, in addition to promoting dendritic cell maturation and antigen presentation.

Thus, GM-CSF-rich airway environments have been hypothesized to promote the development of an immune response by creating conditions conducive to  $T_H2$ -cell differentiation and that exposure to DPM and other particulates could support the differentiation of  $T_H2$ -cells through the induction of GM-CSF [26, 27], which can promote  $T_H2$ -cell proliferation by enhancing the ability of dendritic cells to generate antigen specific B and T-cell responses [28]. Our observation further supports the existing literature demonstrating that DPM exposure can increase expression of GM-CSF.

Finally, this study showed that DPM may act as an irritant, as some of our subjects complained of mild symptoms immediately after exposure that were resolved by the 2 h time point. Rudell et al. [20] looked at diesel exhaust as a whole and found that it caused similar symptoms, potentially due to both the gaseous and particulate components of diesel exhaust, which are known irritants. In contrast, Nightingale et al. [4], who examined DPM alone, reported no adverse symptoms; they attributed this to the lack of gaseous components that they believed were more likely to act as an irritant. Our study contradicts this and suggests that the DPM alone is also able to induce mild symptoms even in the absence of the gaseous components of diesel exhaust. Our subjects also spontaneously reported an itchy throat, with 6 out of 10 subjects complaining of this symptom after DPM300 exposure. Throat irritation is noted in other studies [20, 29]. The number of subjects who reported an itchy throat suggests that this should be monitored in future studies. We cannot rule out the possibility that the unblinded nature of our study design meant that participants knew what dose they were inhaling, thereby influencing the symptoms reported. Given the imperative to safety in this pilot study this could not be helped, as it was important to escalate from low to higher doses, but future studies at these doses would benefit from being blinded.

## 5. Conclusion

Overall, this novel method of inhaling nebulized DPM mixed in saline proved to be a safe and effective way to examine the effects of DPM and our protocol provides a framework for future research. Inhalation of nebulized DPM delivered the particles to the lower airways and elicited transient decrements in  $FEV_1$ . However, even at the highest dose of DPM administered in this study, we did not observe cellular inflammatory responses in the airways and blood and measured only very small changes in proinflammatory cytokine levels. For studies aimed at examining inflammatory responses, higher doses may be required but should be tested carefully for safety, particularly in subjects who may have airway hyperreactivity. Understanding the mechanisms through which airborne particulates influence inflammation and lung function will help us to understand the correlation between particulate exposure and increases in cardiopulmonary morbidity and mortality that have been demonstrated epidemiologically. It may also help us identify the threshold dose that is able to elicit an inflammatory response in healthy and susceptible populations, which could be a critical

consideration in defining emission standards and workplace policies.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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