

# Comparison of Acute Health Effects From Exposures to Diesel and Biodiesel Fuel Emissions

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**Objective:** To investigate the comparative acute health effects associated with exposures to diesel and 75% biodiesel/25% diesel (B75) blend fuel emissions. **Methods:** We analyzed multiple health endpoints in 48 healthy adults before and after exposures to diesel and B75 emissions in an underground mine setting—lung function, lung and systemic inflammation, novel biomarkers of exposure, and oxidative stress were assessed. **Results:** B75 reduced respirable diesel particulate matter by 20%. Lung function declined significantly more after exposure to diesel emissions. Lung inflammatory cells along with sputum and plasma inflammatory mediators increased significantly to similar levels with both exposures. Urinary 8-hydroxydeoxyguanosine, a marker of oxidative stress, was not significantly changed after either exposure. **Conclusions:** Use of B75 lowered respirable diesel particulate matter exposure and some associated acute health effects, although lung and systemic inflammation were not reduced compared with diesel use.

Diesel engines are widely used in on- and off-road applications including personal vehicles, trucks, buses, trains, ships, underground mining, construction, and agriculture. Exposure to diesel engine emissions is associated with chronic bronchitis, respiratory tract infections, asthma exacerbation, and increased cardiovascular morbidity and mortality,<sup>1–4</sup> and in 2012 diesel emissions were classified by the International Agency for Research on Cancer as a group 1 carcinogen in humans.<sup>5</sup> Given the health effects of diesel emissions and ubiquitous environmental exposures, reducing engine emissions has become a public health priority.

In recent years, alternative fuels such as biodiesel have been introduced in attempts to reduce diesel particulate matter (DPM) emissions. Often used as a blend with diesel to facilitate use in current engines, biodiesel has been shown to reduce respirable particulates,<sup>6</sup> dependent on fuel formulation, pollution control devices, and engine operating conditions.<sup>7,8</sup> Despite the increasing usage of biodiesel, there is a lack of information on the human health effects of exposure to these emissions, and recent in vitro and in vivo studies suggest

## Learning Objectives

- Discuss the use of biodiesel-containing fuel blends as a potential approach to reducing health harms by lowering respirable diesel particulate matter.
- Summarize the effects of exposure to B75 fuel blend versus diesel on emissions, lung function, and inflammatory biomarkers.
- Discuss the research and practical implications for the concept of using biodiesel fuel blends to reduce diesel particulate exposure and adverse health effects.

that exposure to biodiesel particulates may be more toxic than diesel particulates at equivalent concentrations.<sup>9–12</sup>

This study was conducted to compare the acute human health effects related to exposures to emissions from diesel and a 75% biodiesel/25% diesel (B75) blend fuel in an underground mining setting, where DPM concentrations are among the highest reported.<sup>13</sup> The null hypothesis was that switching to B75 would not reduce the adverse health effects compared with exposures to diesel emissions.

## MATERIALS AND METHODS

### Subjects

Human subject recruitment and testing procedures were approved by the University of Arizona (UA) institutional review board. Subjects were recruited from the UA campus. Inclusion criteria included being at least 18 years of age. Exclusion criteria included recent (within 4 days) diesel exhaust or other significant occupational inhalation exposure, smoking, a diagnosis of asthma, heart disease, diabetes, hypertension, renal or hepatic failure, a difference in blood pressure greater than 15 mm Hg between the arms, baseline forced expiratory volume in one second (FEV<sub>1</sub>) divided by forced vital capacity (FVC) less than 0.7, or current respiratory illness.

### Load-Haul-Dump Training and Baseline Testing

After written consent was obtained, subjects were scheduled for load-haul-dump (LHD) vehicle driver's training a minimum of 96 hours prior to baseline testing, which was completed at least 72 hours prior to the first emissions exposure. Baseline testing consisted of blood pressure measurement, phlebotomy, pulmonary function testing, and sputum induction. Blood pressure was measured in both arms using an automated sphygmomanometer (OMRON, Bannockburn, IL). Blood samples were collected in serum clot activator, heparin, sodium citrate, and ethylenediaminetetraacetic acid tubes. The serum tube was allowed to clot for 30 minutes at room temperature prior to centrifugation. All of the tubes were initially centrifuged at 1000 × g for 15 minutes. The heparin and sodium citrate tubes were decanted and a second 10-minute centrifugation step at 10,000 × g was added to obtain complete platelet removal. Serum and plasma were decanted and stored immediately at –80°C until assayed. Pulmonary function testing was performed following American Thoracic Society (ATS) standards in a sitting position using an EasyOne spirometer (nidd Medical Technologies, Andover, MA). Forced ex-

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piratory volume in one second, FVC, and age- and height-adjusted percentage predicted values were recorded. A minimum of three trials were performed, with a maximum of eight trials at any one sitting. Testing was continued until the two largest FEV<sub>1</sub> values were within 0.150 L of each other, with the same process followed for FVC. Sputum induction was performed using DeVilbiss Ultra-Neb 99HD ultrasonic nebulizers (Somerset, PA) with 3% saline for 30 minutes, as previously described.<sup>14</sup> Samples were diluted with 10% Sputolysin (Calbiochem, San Diego, CA) in phosphate buffered saline and incubated at room temperature for 15 minutes with gentle mixing by inversion every 5 minutes. A 500  $\mu$ L aliquot was removed prior to centrifugation; 50  $\mu$ L of sample was mixed with 50  $\mu$ L Trypan Blue stain (Sigma Chemical Co, St Louis, MO) prior to total cell counting performed using a hemocytometer. The remaining aliquot was mixed with an equal amount (450  $\mu$ L) of preservative (Histochoice, AM-RESCO, Solon, OH), and 500  $\mu$ L of the mixture was cytocentrifuged, stained with Diff-Quik (Dade Behring AG, Switzerland), and analyzed using the first 100 white cells counted, excluding epithelial cells. The remaining sample was centrifuged at 1900 rpm for 20 minutes, and the supernatant and cell pellet were stored at  $-80^{\circ}\text{C}$  until assayed.

### Preexposure Testing on Emissions Exposure Day

On the emissions exposure day, subjects were instructed to fast for at least 6 hours prior to arriving at the UA San Xavier Mining Laboratory. First morning void urine samples and all subsequent voids continuing through the end of the day's testing were refrigerated at  $4^{\circ}\text{C}$  until processing. Baseline exhaled carbon monoxide (CO), expressed as percentage carboxyhemoglobin (%COHb), and baseline fraction of exhaled nitric oxide (FENO) testing were performed following the ATS recommendations and standards, respectively, using the microCO Breath Carbon Monoxide Monitor (Micro Direct, Lewiston, ME) and a NIOX MINO (Aerocrine, Inc, New Providence, NH). After a 20-minute rest in a supine position and ultrasound testing for brachial artery flow-mediated dilation (results to be reported separately),<sup>15</sup> subjects were supplied a carbohydrate meal (cereal, 2% milk, and energy bars) and water.

### Fuels, Equipment, and Study Site

The fuels, equipment, and study site description have previously been reported.<sup>16</sup> Briefly, ultra-low sulfur #2 diesel (Arizona Petroleum, Tucson, AZ) was used for diesel exposure testing, and B75 was prepared by mixing the aforementioned diesel fuel at 25% by volume with a soy methyl ester biodiesel fuel (ASTM D6751-compliant; Arizona Petroleum, Tucson, AZ). Exposure to vehicle emissions was evaluated during mucking activities in the San Xavier Mining Laboratory. A 2005 Wagner B10-203 LHD vehicle with open cab and diesel oxidation catalyst, but no diesel particulate filter, was used for the study and operated on a 35 m all-underground route with a 9.5% grade. As in typical underground mining, the mucking path and muck pile were sprayed with water to limit airborne dust exposures.

### Diesel and B75 Emission Exposures

Exposure-related health effects to diesel and B75 fuel emissions were measured under the same engine operating conditions. All protocols performed by the subjects during their first fuel appointment day at the mine were repeated using the second fuel with a minimum of 2 weeks between exposures. In 2013, the first fuel was diesel. In 2014, with one exception, the first fuel was B75. The fuel tanks were emptied between each fuel type, and the LHD operated for approximately 1 hour with the "new" fuel to ensure all remnants of the previous fuel were removed. For each fuel type, two research subjects alternately mucked (110 minutes) and closely observed (80 minutes) LHD operation, with a 10-minute break during each 200-minute exposure rotation. Methods for personal integrated sam-

ple collection, monitoring, and analysis of particulates and gases have previously been reported.<sup>16</sup>

### Post-Diesel and Post-B75 Emission Exposure Testing

At the end of the 200-minute exposure period, the subjects repeated the exhaled CO monitoring immediately on exiting the mine. The FENO measurement was repeated approximately 5 minutes after exiting the mine. Subjects repeated the 20-minute rest prior to the measurement of postexposure flow-mediated dilation. The subjects were then supplied a small lunch consisting of a sandwich, fruit, and water and returned to the UA campus for a blood draw at 2 hours after exiting the mine (5.5 hours after initial exposure). At 5 hours after exiting the mine (8.5 hours after initial exposure), the subjects returned again to the UA campus for the remainder of the biological sample testing, including blood pressure, spirometry, sputum induction, and urine collection, conducted in the same fashion as during baseline and/or preexposure testing.

### Biological Assays

Urine samples were allowed to return to room temperature, mixed well, and an aliquot of urine was transferred to a 15 mL tube, centrifuged at  $1000 \times g$  for 10 minutes, and the supernatant was stored at  $-80^{\circ}\text{C}$  until assayed. Specific gravity (SG) was measured on the unspun urine (10SG Urine Reagent Strips, Fisherbrand, Hanover, Germany). First catch (baseline) and last catch of the day (generally 8 to 10 hours postexposure) urine was used for 8-hydroxydeoxyguanosine (8-OHdG) analysis. All sputum and plasma samples were allowed to thaw to room temperature, vortexed, and then briefly centrifuged to pellet any precipitates prior to assays. Endothelin-1 (ET-1), soluble P-selectin, and tenascin-C (TN-C) were assayed with heparin plasma samples, whereas high sensitivity interleukin-6 (IL-6), high sensitivity interleukin-8 (IL-8), matrix metalloproteinase-9 (MMP-9), myeloperoxidase (MPO), growth-regulated alpha protein (GRO- $\alpha$ ), and matrix metalloproteinase-8 (MMP-8) were assayed with sputum samples, and creatinine and urinary 8-OHdG were assayed with urine samples. The level of each sputum analyte was adjusted to total protein for normalization. Standards, controls, and samples were assayed in duplicate using the enzyme-linked immunosorbent (ELISA) assay kits developed by R&D Systems, Inc (Minneapolis, MN) with two exceptions—8-OHdG was assayed in triplicate using the ELISA kit supplied by Japan Institute for the Control of Aging (Fukuroi, Japan) and TN-C high molecular weight variants were assayed in duplicate using the ELISA kit supplied by IBL America, Inc (Minneapolis, MN). Absorbance for standards, controls, and samples were obtained using an automated microplate reader (Model ILx808, BioTek Instruments, Inc, Winooski, VT) and concentrations determined from the standard curve using a four-parameter algorithm for best fit, as determined by the BioTek KC4 automated software program (Winooski, VT).

### LC-MS/MS Analysis

See Supplemental Material (available at <http://links.lww.com/JOM/A199>) for detailed methods. Sputum and plasma samples from six subjects (three males and three females) were analyzed for potential novel biomarkers of effect after diesel and B75 exposure. Proteins were extracted, reduced with dithiothreitol, alkylated with iodoacetamide, and then digested with Lys-C/trypsin mix (Promega, Madison, WI) following manufacturer's recommendations. The resulting peptide samples were desalted using solid-phase extraction and concentrated to dryness prior to analysis by high-resolution liquid chromatography-mass spectrometry/mass spectrometry for protein identification using a LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an Advion nanomate ESI source (Advion, Ithaca, NY). Three proteins (GRO- $\alpha$ , MMP-8, and TN-C) considered prime candidate biomarkers were

further validated in the entirety of the sputum and plasma samples using ELISA.

### Statistical Analysis

All manual results were double entered into a spreadsheet and the results checked for accuracy. Industrial hygiene measurements were time weighted over an 8-hour exposure period (TWA<sub>8</sub>), as previously described.<sup>16</sup> The TWA<sub>8</sub> exposure means were first compared across fuel types using the Kruskal-Wallis rank test with the Bonferroni correction (STATA 12.0, StataCorp, College Station, TX). Those analytes with significant differences across post-diesel and post-B75 sampling were further analyzed using the Wilcoxon signed-rank test for paired analysis. Health data (exhaled CO and nitric oxide [NO], cell counts, and ELISAs) were also compared for statistical significance using the Wilcoxon signed-rank test. Spirometry data were normally distributed; therefore, a paired *t* test was used for statistical analysis. Data were analyzed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, www.graphpad.com). Statistical differences were measured for post-diesel or post-B75 compared with baseline values and also post-diesel compared with post-B75 values. For all analyses, statistical significance was taken as a two-tailed *P* value of *P* < 0.05. Data were expressed as median (interquartile range) or mean ± standard deviation, as appropriate.

## RESULTS

### Study Participants

Characteristics of the 48 study participants, predominantly UA students, are summarized in Table 1. The average age was 25.7 years, approximately one third of the subjects were female, and three quarters were non-Hispanic white.

### Diesel and B75 Emission Composition

Respirable diesel particulate matter (rDPM) sampling performed during mucking demonstrated a median of 336.40 µg/m<sup>3</sup> for diesel exposures and 267.80 µg/m<sup>3</sup> for B75 exposures, a 20% reduction (Table 2). There were no statistical differences between the fuel types when comparing acetaldehyde, formaldehyde, or NO levels. Nitrogen dioxide (NO<sub>2</sub>) and CO were significantly reduced in B75 compared with diesel exposures.

### Lung Function and Exhaled CO and NO

At baseline 45 (94%) of the 48 subjects met the ATS standard for the top two FEV<sub>1</sub> and FVC measurements being within 0.150L. Post-exposure, 43 (90%) of the 48 subjects using diesel fuel and 39 (81%) of the 48 subjects using biodiesel met the ATS stan-

dards. Among all subjects, FEV<sub>1</sub> was significantly reduced after both diesel and B75 exposures compared with baseline (Table 3). Post-diesel FEV<sub>1</sub> was also significantly lower than post-B75 FEV<sub>1</sub>. FVC was only significantly reduced after diesel exposure. In addition, post-diesel FVC was significantly reduced compared with post-B75. FEV<sub>1</sub>/FVC was significantly reduced after both diesel and B75 exposures. Both %COHb and FENO significantly increased in subjects after both exposures (Table 4). There were no significant differences in the median levels of post-diesel %COHb or FENO compared with post-B75.

### Lung Inflammatory Cell Infiltration

Sputum total white blood cell, neutrophil, and macrophage counts increased after emission exposures to both fuels, and lymphocytes increased after diesel, but not B75 exposures (Table 5). When comparing post-diesel with post-B75 cell counts, there were no significant differences in any cell type.

### Sputum and Plasma Inflammatory Mediators

Sputum and plasma fluid-phase mediators selected a priori (IL-6, IL-8, MMP-9, MPO, and ET-1) increased from baseline after both exposures (Table 6). P-selectin decreased in the plasma after both exposures. There were no significant differences found in these levels when comparing post-diesel with post-B75.

TABLE 1. Study Participants

Characteristic	
Participants (n)	48
Mean age (yrs) (min-max)	25.7 (19–53)
Sex	
Male	31 (64.6%)
Female	17 (35.4%)
Race	
White, non-Hispanic	36 (75.0%)
Hispanic	5 (10.4%)
African	4 (8.3%)
Asian	2 (4.2%)
Other	1 (2.1%)
Mean height (cm), SD	174.5 (9.2)
Mean weight (kg), SD	76.9 (17.2)
Mean body mass index (kg/m <sup>2</sup> ), SD	25.2 (5.2)
SD, standard deviation.	

TABLE 2. Analyte TWA<sub>8</sub> Exposure Concentrations<sup>a</sup>

Analyte	Diesel	B75	<i>P</i>
rDPM (µg/m <sup>3</sup> ) <sup>b</sup>	336.40 (226.13–432.00)	267.80 (159.10–378.30)	0.0780
Acetaldehyde (ppm) <sup>c</sup>	0.04 (0.03–0.04)	0.03 (0.02–0.04)	0.1922
Formaldehyde (ppm) <sup>c</sup>	0.10 (0.07–0.12)	0.10 (0.08–0.12)	0.7648
NO (ppm) <sup>d</sup>	10.27 (7.45–12.03)	12.54 (6.32–15.15)	0.1647
NO <sub>2</sub> (ppm) <sup>d</sup>	1.58 (1.16–1.89)	1.19 (0.91–1.54)*	0.0423
CO (ppm) <sup>d</sup>	13.39 (7.45–19.73)	8.87 (6.88–15.55)*	0.0182

\**P* < 0.05 for comparison by the Wilcoxon signed-rank test.

<sup>a</sup>Data are presented as median (interquartile range). The differences in *n* resulted from equipment malfunction.

<sup>b</sup>*n* = 47.

<sup>c</sup>*n* = 40.

<sup>d</sup>*n* = 44.

CO, carbon monoxide; NO, nitric oxide; NO<sub>2</sub>, nitrogen dioxide; rDPM, respirable diesel particulate matter; TWA<sub>8</sub>, time-weighted over an 8-hour exposure period.

**TABLE 3.** Changes in Lung Function ( $n = 48$ )<sup>a</sup>

Parameter	Baseline	Post-Diesel	Post-B75	<i>P</i> <sup>b</sup>
FEV <sub>1</sub> (L)	4.05 ± 0.65	3.85 ± 0.64***	3.96 ± 0.66***	0.0051
% Predicted FEV <sub>1</sub>	98.46 ± 11.02	93.65 ± 10.59***	96.10 ± 11.14***	0.0047
FVC (L)	4.86 ± 0.83	4.74 ± 0.81**	4.83 ± 0.83	0.0263
% Predicted FVC	99.17 ± 11.42	96.67 ± 9.76**	98.48 ± 10.88	0.0148
FEV <sub>1</sub> /FVC (%)	83.77 ± 5.60	81.60 ± 6.16***	82.32 ± 6.10**	0.2006

\*\*\* $P < 0.01$ ; \*\*\*\* $P < 0.001$  compared with baseline using the paired  $t$  test.

<sup>a</sup>Data are presented as mean ± SD.

<sup>b</sup>The paired  $t$  test comparing post-diesel with post-B75.

FEV<sub>1</sub>, forced expiratory volume in one second; FVC, forced vital capacity.

**TABLE 4.** Carboxyhemoglobin ( $n = 33$ ) and Fraction of Exhaled Nitric Oxide ( $n = 45$ )<sup>a</sup>

Parameter	Diesel Baseline	Post-Diesel	B75 Baseline	Post-B75	<i>P</i> <sup>b</sup>
% COHb	0.5 (0.3–0.5)	0.6 (0.6–1.0)***	0.3 (0.3–0.6)	0.6 (0.5–1.0)**	0.1421
FENO (ppb)	19.0 (13.0–29.0)	23.0 (18.0–33.0)***	18.0 (13.0–31.0)	22.0 (17.0–32.0)*	0.4659

\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  for comparison with baseline by the Wilcoxon signed-rank test.

<sup>a</sup>Data are presented as median (interquartile range). The differences in  $n$  resulted from equipment malfunction.

<sup>b</sup>Comparing post-diesel with post-B75 using the Wilcoxon signed-rank test.

COHb, carboxyhemoglobin; FENO, fraction of exhaled nitric oxide.

**TABLE 5.** Sputum Total and Differential Cell Counts ( $n = 48$ )<sup>a</sup>

Cell (10 <sup>6</sup> /mL)	Baseline	Post-Diesel	Post-B75	<i>P</i> <sup>b</sup>
Total WBC	1.06 (0.50–1.78)	2.59 (0.97–4.91)***	2.59 (1.46–4.53)***	0.6729
Neutrophil	0.40 (0.15–0.65)	1.31 (0.45–2.63)***	1.32 (0.72–2.30)***	0.6731
Macrophages	0.58 (0.26–1.16)	1.12 (0.47–2.16)***	1.15 (0.57–2.11)***	0.6880
Lymphocytes	0.01 (0.00–0.02)	0.02 (0.00–0.04)*	0.01 (0.00–0.03)	0.1993

\* $P < 0.05$ ; \*\*\* $P < 0.001$  compared with baseline by the Wilcoxon signed-rank test.

<sup>a</sup>Data are presented as median (interquartile range).

<sup>b</sup>Comparing post-diesel with post-B75 using the Wilcoxon signed-rank test.

WBC, white blood cells.

**TABLE 6.** Sputum and Plasma Inflammatory Mediators ( $n = 48$ )<sup>a</sup>

Analyte	Baseline	Post-Diesel	Post-B75	<i>P</i> <sup>b</sup>
Sputum				
IL-6 (pg/μg)	0.1 (0.0–0.1)	0.3 (0.1–0.7)***	0.3 (0.1–0.8)***	0.8350
IL-8 (pg/μg)	3.7 (2.9–6.3)	5.8 (3.4–9.7)***	7.5 (4.3–10.5)***	0.0840
MMP-9 (ng/μg)	0.7 (0.4–1.2)	1.7 (0.9–3.4)***	1.8 (1.2–2.9)***	0.9878
MPO (ng/μg)	0.8 (0.5–1.5)	1.7 (0.6–3.4)***	1.6 (0.9–2.5)***	0.6219
MMP-8 <sup>c</sup> (ng/μg)	0.5 (0.3–0.8)	1.2 (0.6–2.2)***	1.1 (0.8–2.1)***	0.7030
GRO-α <sup>c</sup> (pg/μg)	18.3 (9.4–45.8)	19.7 (9.2–48.3)	23.8 (13.4–76.3)**	0.0859
Plasma				
ET-1 (pg/mL)	1.5 (1.3–1.8)	1.6 (1.3–2.2)**	1.6 (1.4–1.9)*	0.6350
P-selectin (ng/mL)	39.5 (32.4–49.7)	33.6 (29.2–40.4)***	32.4 (25.7–40.5)***	0.4301
TN-C <sup>c</sup> (ng/mL)	64.3 (45.0–82.7)	67.6 (46.6–94.6)**	71.8 (50.4–88.3)	0.3356

\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  for comparison with baseline using the Wilcoxon signed-rank test.

<sup>a</sup>Data are presented as median (interquartile range). The sputum protein levels were normalized to total protein.

<sup>b</sup>Comparing post-diesel with post-B75 using the Wilcoxon signed-rank test.

<sup>c</sup>Protein was identified as a candidate biomarker using proteomic strategies.

IL-6, interleukin-6; IL-8, interleukin-8; MMP-8, matrix metalloproteinase-8; MMP-9, matrix metalloproteinase-9; MPO, myeloperoxidase; GRO-α, growth-regulated alpha protein; ET-1, endothelin-1; TN-C, tenascin-C.

Sputum levels of each analyte, not adjusted for total protein, are included in Supplemental Digital Content Table S1 (available at <http://links.lww.com/JOM/A200>).

### Novel Biomarker Discovery

After proteomic analysis, a total of 848 sputum and 407 plasma proteins were identified. Using label-free spectral counting for relative quantitation, 42 and 32 candidate protein markers in the sputum and plasma, respectively, were identified that met the previously defined criteria (see Supplemental Material Methods [available at <http://links.lww.com/JOM/A199>] and Supplemental Digital Content Table S2 [available at <http://links.lww.com/JOM/A201>] and Digital Content Table S3 [available at <http://links.lww.com/JOM/A202>]). Two sputum (MMP-8 and GRO- $\alpha$ ) and one plasma (TN-C) candidate markers were further validated in all samples using ELISA (Table 6). Sputum MMP-8 significantly increased after both exposures. Sputum GRO- $\alpha$  was only significantly elevated in post-B75 exposures, although there were no significant differences when comparing post-diesel to post-B75 levels. Plasma TN-C was significantly increased after diesel exposure, and, albeit not quite significantly, in the post-B75 exposures. There were no significant differences in post-diesel and post-B75 TN-C levels.

### Urinary 8-OHdG

8-hydroxydeoxyguanosine levels normalized to creatinine (ng 8-OHdG/mg creatinine), or SG did not significantly increase or decrease from baseline after exposures to diesel or B75. There were no significant changes in 8-OHdG when comparing post-diesel and post-B75 levels (Table 7).

### Additional Analyses

A subanalysis limited to subjects without any reported medical conditions or medication use ( $n = 42$ ) had similar outcomes to the analyses using all subjects, with the following exceptions. Sputum IL-8 increased significantly higher post-B75 compared with post-diesel exposures ( $P = 0.041$ , median values 6.7 and 5.5 pg/ $\mu$ g, respectively). Plasma ET-1 lost statistical significance comparing baseline and post-B75 ( $P = 0.093$ , median values 1.4 and 1.6 pg/mL, respectively) but remained significant when comparing baseline with post-diesel ( $P = 0.028$ , median values 1.4 and 1.5 pg/mL, respectively). When normalizing urinary 8-OHdG to SG, the level decreased significantly after diesel exposures compared with baseline levels ( $P = 0.045$ , median values 9.0 and 11.1 ng, respectively).

## DISCUSSION

In this study, switching to B75 from diesel fuel led to a 20% reduction in rDPM exposure. This was consistent with previous reports demonstrating a reduction in DPM using biodiesel blends,<sup>6,8,17</sup> although at least one study reported no difference in DPM.<sup>18</sup> The mixed results can likely be attributed to the fuel blend, engine operating conditions, and pollution control devices.<sup>7,19</sup> This study did not find significant increases in formaldehyde when switching from diesel to a biodiesel blend, which is inconsistent with a previous report<sup>18</sup>; however, CO levels were reduced significantly, which has

been reported.<sup>20</sup> In addition, there were no statistical differences in acetaldehyde or oxides of nitrogen (sum of NO and NO<sub>2</sub>) in this study when switching to a biodiesel blend from diesel, consistent with what has been previously reported.<sup>18,20</sup>

The respirable particulate concentrations reported in this study are at the high end of reported ambient exposures, including those found most commonly in occupational settings.<sup>13,21</sup> Nevertheless, the particulate concentrations are in the maximum range of those reported for highly polluted cities, such as Beijing.<sup>21,22</sup> At these elevated exposure concentrations, significant reductions in lung function as well as increases in both respiratory and systemic inflammation were observed. The marked inflammation is a likely pathway for lung cancer, other adverse respiratory effects, and cardiovascular disease; all of which are known sequelae of chronic DPM exposure.<sup>1-4,23</sup> The increased concentration seen postexposure for most of the inflammatory biomarkers did not differ significantly between the two fuels, despite the 20% reduction in rDPM observed with B75 use. These findings bring into question the assumption that reductions in DPM concentrations from the use of alternative fuels will necessarily lead to decreased chronic toxicity.

Lung function was one of the health endpoints for which a significant difference was found comparing post-diesel and post-B75 values. Although acute changes in spirometry are not typically reported in studies where subjects are exposed to diluted diesel exhaust using controlled exposure chambers,<sup>24-27</sup> at least one previous study demonstrated exposure to diesel exhaust decreased peak expiratory flow.<sup>28</sup> In general, previous studies had a lower number of subjects, different exposure durations and environments, and lower particulate concentrations; all of which are plausible reasons for the observed variability.

Exhaled CO (expressed as %COHb) increased to a small extent with exposure to emissions from both fuel types. Percentage COHb data were missing for 15 subjects due to equipment malfunction. Comparing subjects with and without full %COHb measurements, sex, but not age or ethnicity, varied significantly between the two groups. It is possible that the 15 missing values in the study could have had a small effect on the %COHb levels that were reported, but given the relatively minor increase with both fuel types, the effect of the missing data is not likely to be biologically important.

In addition, this study revealed that FENO, a marker of airway inflammation, increased significantly and to the same levels after emission exposures to both fuel types, comparable with previous findings of FENO increasing after acute diesel exhaust exposures.<sup>24</sup> FENO has also been shown to increase in asthmatic children from urban air pollution with positive correlations between the concentration of pollutants and the level of airway inflammation.<sup>29</sup>

Diesel exhaust has been shown to increase inflammatory cell recruitment into the airways.<sup>25-27</sup> These increases were typically measured 6 to 24 hours after initial diesel exposure, consistent with the approximately 8.5 hours after initial exposure to each fuel type in this study. We observed similar increases in cell counts for neutrophils and macrophages after exposures to diesel and B75, although lymphocytes increased significantly after diesel, but not B75, exposures.

**TABLE 7.** Urine 8-Hydroxydeoxyguanosine ( $n = 47$ )<sup>a</sup>

Analyte	Diesel Baseline	Post-Diesel	B75 Baseline	Post-B75	P <sup>b</sup>
8-OHdG/creatinine	6.4 (4.8–8.3)	7.1 (5.0–8.4)	7.0 (6.1–8.2)	6.5 (5.1–8.9)	0.9031
8-OHdG/SG	9.5 (5.0–16.7)	8.7 (3.6–13.4)	12.2 (8.4–18.4)	9.9 (3.5–17.3)	0.1985

<sup>a</sup>Data are presented as median (interquartile range). The 8-OHdG levels were normalized to creatinine levels (ng/mg) and specific gravity (SG). None of the baseline to postexposure comparisons are significant ( $P < 0.05$ ) using the Wilcoxon signed-rank test.

<sup>b</sup>Comparing post-diesel with post-B75 using Wilcoxon signed-rank test.  
8-OHdG, 8-hydroxydeoxyguanosine; SG, specific gravity.

Interleukin-6 and IL-8 are cytokines released by several cell types during an inflammatory response. The mRNA and protein expression of these two cytokines has been studied *in vitro* and *in vivo*<sup>30–32</sup> to measure the level of inflammation from exposures to diesel particulates and air pollution. In addition, the increased airway release of IL-6 and IL-8 has been seen in human studies analyzing acute diesel exhaust exposures.<sup>28,33,34</sup> Accordingly, we observed a significant increased release of both IL-6 and IL-8 in the sputum after diesel and B75 exposures. The extent of IL-6 release was similar for both fuel types, and IL-8 was slightly (but not significantly) higher for B75 exposures.

Matrix metalloproteinase-8 (secreted exclusively by neutrophils) and MMP-9 (secreted by many cell types) are involved in the remodeling of the extracellular matrix under normal physiological processes and also inflammation and metastasis. Matrix metalloproteinase-8 has been used as a biomarker of inflammation and cardiovascular disease.<sup>35</sup> Nevertheless, the acute elevation of MMP-8 observed in response to diesel and B75 emission exposures is a novel finding. The increased levels measured in this study are likely related to the neutrophil infiltration observed in the lung. Induced sputum MMP-9 levels have been shown to be correlated with lung function and airway inflammation, displaying an inverse relationship with FEV<sub>1</sub> and a significant correlation with total white cells, neutrophils, and IL-8.<sup>36</sup> This suggests that MMP-9 may be a promising marker of airway effect for DPM exposure.

Myeloperoxidase is an enzyme that has potent pro-oxidative and proinflammatory properties and is typically released from activated neutrophils; accordingly, its levels have been used as a marker of inflammation and cardiovascular disease. In a recent study analyzing mouse lung and liver toxicity after equivalent doses of diesel or biodiesel emissions, MPO levels displayed a greater dose-related increase after biodiesel, compared with diesel, emission exposures.<sup>37</sup> In this study, MPO levels in the sputum increased to similar levels after diesel and B75 exposures, despite the lower rDPM exposure with B75 use.

Growth-regulated alpha protein- $\alpha$  preferentially chemoattracts and activates neutrophils. This protein is inducible by tumor necrosis factor- $\alpha$  and interleukin-1. Previous human studies have shown a nonsignificant increase of GRO- $\alpha$  after acute diesel exhaust exposures.<sup>33,38</sup> In this study, GRO- $\alpha$  was identified in the sputum via proteomic strategies as a candidate biomarker being elevated after B75 exposures. When ELISA analysis was performed on all sputum samples, GRO- $\alpha$  was significantly elevated after only B75 emission exposures, also a novel finding.

Endothelin-1 is an endothelin-derived vasoconstrictor peptide that is an inflammatory mediator. Increased levels of ET-1 have been reported in cardiovascular and inflammatory lung diseases.<sup>39,40</sup> A previous human study showed increases in plasma ET-1 levels after acute exposures to diesel exhaust,<sup>41</sup> suggesting an early endothelial response and vasoconstriction. This study demonstrated similar significant increases in plasma ET-1 levels after diesel and B75 exposures, suggesting that the level of vascular dysfunction is similar from exposures to both fuel types.

P-selectin is responsible for mediating the rolling of leukocytes over vascular surfaces during early stages of inflammation. In chamber studies of acute human exposure to filtered diesel exhaust (300  $\mu\text{g}/\text{m}^3$  for 1 hour), plasma P-selectin levels were not significantly altered at 2 or 6 hours postexposure,<sup>42</sup> but increased significantly at 24 hours postexposure.<sup>43</sup> These results are inconsistent with this study findings, where P-selectin decreased 5.5 hours after initial exposure to both fuel types. Different exposure environments, engine, and engine operating conditions may be plausible explanations for the inconsistencies between the studies. It has also been shown that exercise training can reduce plasma inflammatory mediators such as P-selectin.<sup>44–46</sup> Although participation in this study required moderate physical exertion during mucking, it

is unclear if this level of exertion caused the decrease in plasma P-selectin.

Tenascin-C is an extracellular matrix protein important not only in tissue injury and repair but also in disease states such as chronic inflammation and tumorigenesis. In addition, TN-C is capable of inducing proinflammatory cytokines.<sup>47</sup> High circulating concentrations of plasma TN-C have been reported in association with mortality and cardiovascular disease in chronic kidney patients.<sup>48</sup> In this study, TN-C was identified in the plasma via proteomic strategies as a novel biomarker candidate that increased after exposures to diesel and B75 emissions. These increased TN-C plasma levels may be due to lung and/or endothelial damage.

This study is the first human study to use proteomic strategies to reveal novel biomarkers of emission exposures to both diesel and B75. The candidate biomarkers in the sputum and plasma (MMP-8, GRO- $\alpha$ , and TN-C) were validated by ELISA. The most comparable previous study was performed on rat bronchoalveolar lavage fluid after different exposure concentrations and durations to diesel exhaust particles<sup>49</sup>; this study revealed a total of 65 proteins using liquid chromatography–mass spectrometry analysis on whole and weak cation exchange extracted bronchoalveolar lavage fluid, with two distinct proteins (anaphylatoxin C3a and calgranulin A) appearing post-exposure at all diesel exhaust particles doses.

The accumulation of rDPM in the lung leads to a large inflammatory response, as seen in this study and many others. It is well established that inflammatory events lead to an increase in reactive oxygen species that can damage proteins and DNA. A classic biomarker of oxidative stress to DNA is urinary 8-OHdG.<sup>50</sup> 8-OHdG is an oxidized DNA nucleoside that is eliminated in the urine after excision by DNA repair enzymes. Increased levels of 8-OHdG have been measured after a minimum of 3 month's occupational exposures to ambient PM<sub>2.5</sub> in Taiwanese traffic conductors.<sup>51</sup> In this study, urine 8-OHdG did not increase significantly 8 to 10 hours after initial diesel or B75 exhaust exposure.

This study has several important limitations. It measured health effects reflecting the use of diesel and an alternative (B75) fuel while operating the same vehicle, producing different rDPM concentrations, and therefore did not compare the toxicity of the same concentration of rDPM from each fuel type. Multiple health parameters were assessed, but each at a single time point after a single 200-minute exposure. More prolonged exposure periods, or the measurements of health parameters at other time points, would potentially produce different results. In addition, only one blend of biodiesel (B75) and type (soy methyl ester) was evaluated in this study, whereas there are various biodiesel blends in use. Also, the use of different pollution control devices and engine operating conditions will affect the overall toxicity of the exhaust produced, so it is important not to generalize the study findings beyond the use of diesel engines with only a diesel oxidation catalyst. A cross-over study design was used, rather than randomizing by fuel type for each day of testing, which may have introduced bias. Finally, this study concentrated on markedly elevated exposure concentrations, and the effects of lower concentrations would be expected to result in less marked health effects, which could potentially vary to a greater or lesser degree by fuel type.

## CONCLUSIONS

In this study, we evaluated multiple health parameters after acute exposure to diesel and B75 emissions. Although lung function was affected to a greater degree by diesel emissions, many biomarkers of effect including measures of inflammation and oxidative stress were similar when comparing the two fuel types, despite the 20% reduction in rDPM achieved by the use of B75. Additional studies are needed to evaluate the potential differential health effects from emissions of these fuels at both lower concentrations and for more chronic

exposure periods, as well as for a larger selection of biodiesel fuel sources, blend concentrations, and pollution control devices. This study highlights the need to further evaluate the health effects associated with alternative fuels, and not assume that reduction in rDPM alone will lead to reduced health effects.

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