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| 14. ABSTRACT<br>Respiratory symptoms are frequently reported in personnel deployed to the Middle East. This project characterized the respiratory toxicity of inhaled Iraqi sand (IS). Adult rats underwent a 6-wk inhalation to air or mainstream cigarette smoke (MSCS) (3h/d, 5d/wk) that included exposure to IS or crystalline silica (1 mg/m <sup>3</sup> , 19h/d, 7d/wk) or air during the last w weeks. Assessments included motor activity, whole-body plethysmography, cytological and biochemical analysis of bronchoalveolar lavage fluid, lung metal burden, nasal and lung pathology and changes in lung protein and gene expression. A number of metals including nickel, manganese, and vanadium concentrations were seen in IS-exposed rats, suggesting that several metals present in IS are bioavailable. Rats exposed to IS only developed mild inflammation in the anterior nose and lung. Silica inhalation was associated with some pulmonary responses that were not seen in IS-exposed rats, such as mild laryngeal and tracheal inflammation, mild tracheal epithelial hyperplasia, and elevated lung silica concentrations. MSCS inhalation with or without co-exposure to MSCS and silica has more widespread airway lesions when compared with rats exposed to MSCS only. Silica exposed rats had more robust |                      |                            |                                          |                                                         |                                                           |  |
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RESEARCH ARTICLE

# Biological responses in rats exposed to cigarette smoke and Middle East sand (dust)

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## Abstract

Respiratory symptoms are frequently reported in personnel deployed to the Middle East. This project characterized the respiratory toxicity of inhaled Iraqi sand (IS). Adult rats underwent a 6-wk inhalation to air or mainstream cigarette smoke (MSCS) (3 h/d, 5 d/wk) that included exposure to IS or crystalline silica (1 mg/m<sup>3</sup>, 19 h/d, 7 d/wk) or air during the last 2 weeks. Assessments included motor activity, whole-body plethysmography, cytological and biochemical analysis of bronchoalveolar lavage fluid, lung metal burden, nasal and lung pathology, and changes in lung protein and gene expression. A number of metals including nickel, manganese, vanadium, and chromium were detected in IS. Elevated lung parenchyma aluminum, silica, barium, manganese, and vanadium concentrations were seen in IS-exposed rats, suggesting that several metals present in IS are bioavailable. Rats exposed to IS only developed mild inflammation in the anterior nose and lung. Silica inhalation was associated with some pulmonary responses that were not seen in IS-exposed rats, such as mild laryngeal and tracheal inflammation, mild tracheal epithelial hyperplasia, and elevated lung silica concentrations. MSCS inhalation with or without co-exposure to either IS or silica resulted in changes consistent with pulmonary inflammation and stress response. Rats exposed to MSCS and silica had more widespread airway lesions when compared with rats exposed to MSCS only. Silica-exposed rats had more robust pulmonary gene expression and proteomic responses than that seen in IS-exposed rat. Our studies show that the respiratory toxicity of IS is qualitatively similar to or less than that seen following short-term silica exposure.

**Keywords:** Particle toxicity, nanoparticles, rat, *in vivo*, *in vitro*, pulmonary toxicity, cytotoxicity, dust inhalation

## Introduction

Airborne particulate matter (PM) has been linked to a range of serious respiratory and cardiovascular health problems. It is well recognized that deposition of a variety of PM sources in the lung can result in the generation of inflammatory cytokines and the subsequent development of lung injury (Dreher et al., 1997; Gavett et al., 1997; Kodavanti et al., 1999; Scapellato and Lotti, 2007). The key health effects associated with exposure to ambient PM include: increased risk of myocardial infarction,

premature mortality, decreased lung function and aggravation of asthma, chronic bronchitis, and other respiratory and cardiovascular disease (Gordon, 2007; Simkhovich et al., 2008). Recent epidemiologic studies estimate that exposures to PM among the general US population may result in tens of thousands of excess deaths per year, and many more cases of illness (Simkhovich et al., 2008).

There are both natural and anthropogenic PM sources. Natural PM sources include volcanic emissions, forest and grassland fires, and dust storms. Currently a large

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number of U.S. troops (~100 K) are deployed to Iraq and Afghanistan, where sand and dust storms are a frequent occurrence, especially during the spring and summer months. There is also a sizable Iraqi population that is also at risk. Neither population at risk routinely wear adequate respiratory protection. Airborne particulate levels (as  $PM_{10}$ ; i.e. particle size  $<10\ \mu m$ ) in such regions can exceed  $10,000\ \mu g/m^3$ , significantly higher than the  $150\ \mu g/m^3$  threshold set for a 24 h period by either the military exposure guideline or National Ambient Air Quality Standard, even in the absence of dust storms (Engelbrecht et al., 2009).

Inhalation of sand dust has been associated with a variety of adverse health effects. Deployment to Iraq and Afghanistan is associated with increased reporting of respiratory symptoms in ground-based military personnel (Smith et al., 2009; Weese & Abraham, 2009). Desert inhabitants can also develop Desert Lung Syndrome, a rare non-progressive non-occupational dust pneumoconiosis resulting from silica-containing dust depositing in the lungs (Bar-Ziv & Goldberg, 1974). This syndrome generally develops after years of heavy exposure to sand particles (Nouh, 1989). An acute desert-related lung disease described as Desert Storm pneumonitis was found to occur following inhalation of fine Saudi dust and pigeon droppings (Korenyi-Both et al., 1992; 1997). Sporadic cases of severe acute eosinophilic pneumonitis with unknown etiology have also been reported among several U.S. military personnel deployed to Southwest Asia (Shorr et al., 2004). Asian sand dust exposure is associated with increased daily mortality in Seoul, Korea, and Taipei, Taiwan (Kwon et al., 2002) and cardiovascular and respiratory dysfunction in Taipei (Bell et al., 2008).

Given the potential for significant sand dust exposure among deployed military personnel, it is critical to determine the biologic plausibility of Iraqi sand (IS) exposure as a cause for increased morbidity seen in military personnel with pulmonary disease. In pilot studies examining the respiratory toxicity of IS, young male Sprague-Dawley rats given a single intratracheal high dose of saline-suspended IS (up to  $15\ mg/kg$  body weight) developed alveolitis, alveolar hyperplasia, pneumonitis, and eosinophilic infiltration in the lungs by 3–7 days after particle instillation (Wilfong et al., 2011).

The goal of this project is to further characterize the respiratory toxicity of inhaled IS and to determine whether exposure to mainstream cigarette smoke (MSCS) could exacerbate IS particle-induced effects in lungs of exposed rats. This is particularly important since smoking and other tobacco use among active duty members of the U.S. Military remains higher than that seen in the general population especially during deployments (Poston et al., 2008). Our hypothesis was that the results of this animal bioassay would corroborate known human epidemiological data on US personnel deployed to the Middle East.

## Materials and methods

### Experimental design

All animals were initially acclimatized for 2 weeks before exposure. Animals were randomly divided into six groups ( $n = 17$  animals/group) namely: Air-Air, Air-IS, Air-Silica, MSCS-Air, MSCS-IS, and MSCS-Silica (Figure 1). The two-part nomenclature used to designate treatment groups includes identification of their initial exposure (Air or MSCS) followed by the particle challenge used (Air [none], Silica, or IS). Equal groups of rats were initially exposed (nose-only) to either MSCS or clean air for 3 h/day for 4 weeks. Afterwards, animals were returned to their housing cages, which were contained within  $1\text{-m}^3$  inhalation chambers supplied with clean air. Once the 4-week pre-exposure was completed, animals were exposed each day for 3 h (5 day/wk) to clean air or MSCS via nose-only inhalation followed by whole-body exposure to either IS ( $1\ mg/m^3$  nominal concentration), silica (as a reference material,  $1\ mg/m^3$  nominal concentration), or clean air for approximately 19 h (7 days/week) for 2 weeks. An exposure day began with the 3 h MSCS or clean air nose-only exposures at 1030, followed by IS, silica, or air exposure that occurred from 1500 until 1000 the following day. The gaps in time between nose-only and whole-body exposures were used for animal husbandry tasks.

### Materials

Reference cigarettes (3R4F reference cigarettes) were obtained from the University of Kentucky Tobacco

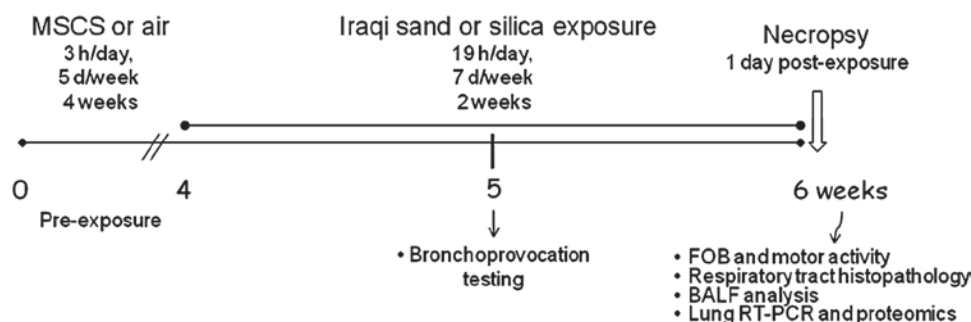


Figure 1. Experimental design overview, showing pre-exposure to mainstream cigarette smoke (MSCS) or air, Iraqi sand (IS) or silica. A total of 6 exposure groups were used: Air-Air, Air-IS, Air-Silica, MSCS-Air, MSCS-IS, and MSCS-Silica. Experimental end points and time line are also depicted.

Research and Development Center (Lexington, KY). Purified, high quality, natural crystalline silica sand was obtained as Min-U-Sil®5 from U. S. Silica (Berkeley Springs, WV). This product has a specific gravity of 2.65 g/mL with 97% of the mass associated with particle diameters less than 5 µm.

### *Iraqi sand*

Surface sand samples were collected at Camp Victory, a US Army base situated on the grounds of the Baghdad international airport. Sand samples were collected for the Naval Medical Research Unit/Environmental Health Effects Laboratory by military personnel under the guidance of US Army Public Health Command. The surface dust (upper 10 mm of soil) was collected in an area no larger than 15.24 m × 15.24 m, and sampling was confined to local soil containing no fill material, rocks, or stained or contaminated soil. Samples were then transferred to plastic zip top bags for transportation. The sand samples were irradiated at the Armed Forces Radiobiology Research Institute (Bethesda, MD), using a Cobalt-60 (<sup>60</sup>Co) source for 4 h to eliminate microbiological flora. The sand was sieved to remove pebbles, twigs, and other large objects. The resultant sand mixture was then ground with a mortar and pestle to break up clumps of sand. This ground-up material was then used to produce the sand aerosol. A representative sample of the Camp Victory sand was sent to Dr. Jose A. Centeno of the Armed Forces Institute of Pathology (AFIP) for high-resolution inductively coupled plasma-mass spectrometry (HR-ICP-MS) analysis of the inorganic component of sand (see Tissue chemical analyses for additional methods). Soil samples (0.2 g) were prepared for analysis using nitric acid digestion in a microwave system following U.S. Environmental Protection Agency Method 3051a which is designed to provide a rapid multi-element acid leach digestion. Results of this analysis are presented in Table 1.

### *Inhalation chambers*

The nose-only MSCS exposures were conducted using a Cannon-style nose-only exposure system (Lab Products, Seaford, DE). The nose-only exposure system was a dynamic, non-rebreathing system that could hold up to 52 animals. Open nose-only tubes were used to hold the animals during the MSCS exposure. Whole-body

exposures were conducted in three 1-m<sup>3</sup> stainless steel and glass inhalation exposure chambers with silicone door seals, cage units, and three catch pans in place. Each 1-m<sup>3</sup> chamber was contained within an 8 m<sup>3</sup> chamber. The outlet air from the 1-m<sup>3</sup> chamber was connected via a manifold to the 8 m<sup>3</sup> chamber exhaust. Temperature and relative humidity were measured near the center top of the 1-m<sup>3</sup> chamber by a temperature and humidity sensor (Series 200, Rotronic Instruments Corp., Huntington, NY) connected to the Continuum Building Automation System (Andover Controls Corporation, TAC, Carrollton, TX). The supply air flow in the 1-m<sup>3</sup> chamber was monitored by measuring the pressure drop across an orifice located in the inlet line of the 1-m<sup>3</sup> chamber.

### *Cigarette smoke aerosol generation system*

Exposure atmospheres were generated using a cigarette smoking machine developed at The Hamner Institutes for Health Sciences. The cigarette smoking machine consisted of 6 air-operated vacuum pumps (Baker Air & Hydraulics, Greenville, SC) connected to a manifold. Air pressure was applied to the air-operated vacuum pumps to create an air flow that drew the smoke through a lit cigarette into the manifold. The MSCS was mixed with HEPA-filtered air and directed into the air supply of the nose-only exposure system.

### *Sand and silica aerosol generation systems*

The crystalline silica or sand exposure atmospheres were generated by aerosolizing either silica or IS using separate dry powder generators (Wright Dust Feeder Model WDF-II, Waltham, MA). The aerosol outputs of the Wright Dust Feeder (WDF) were directed into separate 38 L pressure vessels which acted as settling chambers. Particles leaving the mixing chamber were delivered to the exposure chamber. The IS and silica samples were packed in the small WDF generation cup at a force of 2000 and 1000 pounds, respectively, using a hydraulic press (Model No. 3912, Carver, Inc., Wabash, IN). The air delivery pressure through the WDF was maintained at 6 (silica) or 26 psi (IS) during generation. The WDF rotation speeds were adjusted to produce a proper particle concentration when mixed with the inlet air stream to the 1-m<sup>3</sup> inhalation exposure chamber.

Table 1. Compositional (ICP-MS) analysis of the Iraqi sand sample used for the inhalation exposure.

| Analyte | Concentration (ppm) | Analyte | Concentration (ppm) | Analyte | Concentration (ppm) | Analyte | Concentration (ppm) | Analyte | Concentration (ppm) |
|---------|---------------------|---------|---------------------|---------|---------------------|---------|---------------------|---------|---------------------|
| Ca      | 83745               | Eu      | 533                 | P       | 31.4                | Ce      | 3.25                | Cd      | 0.26                |
| Fe      | 33850               | Sr      | 273                 | Tb      | 28.7                | U       | 3.18                | Mo      | 0.21                |
| Al      | 30867               | Ni      | 187                 | Tl      | 28.2                | Cs      | 2.81                | Dy      | 0.20                |
| Mg      | 25977               | Cr      | 174                 | Co      | 23.6                | Zr      | 2.60                | Th      | 0.05                |
| K       | 5108                | Ba      | 131                 | Pb      | 16.1                | Gd      | 1.42                | Er      | 0.01                |
| Na      | 2603                | V       | 88.5                | B       | 15.3                | W       | 0.96                |         |                     |
| S       | 1734                | Zn      | 67.9                | Ag      | 15.0                | Be      | 0.94                |         |                     |
| Mn      | 696                 | Cu      | 36.7                | As      | 8.62                | Nd      | 0.75                |         |                     |
| Ti      | 598                 | Rb      | 31.5                | Sm      | 4.40                | La      | 0.51                |         |                     |



### Atmosphere monitoring

Exposure atmospheres on each nose-only tower were analysed using gravimetric filters at a port on the tower. Periodically, a sample of the atmosphere was pulled through an aerodynamic particle sizer (APS, Model 3020, TSI, Inc., St. Paul, MN). The instrument measured aerodynamic particle size distribution. Exposure atmospheres were also monitored by an optical particle monitor (RAM-S, MIE, Inc., Billerica, MA). The particle size distribution data using the optical particle sizer were collected from each of the chambers during the exposures on Mondays and Fridays of the 2-week period.

### Animals

Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulations, relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals in facilities that are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All experimental protocols were approved by the Institutional Animal Care and Use Committees at The Hamner Institutes for Health Sciences and the Naval Health Research Center, San Diego. Eight week-old, male CD (Sprague-Dawley) rats [CrI:CD(SD)BR rats, Charles River Laboratories, Wilmington, MA] were used and maintained in the AAALAC-accredited animal facility at The Hamner Institutes for Health Sciences. Facility rooms were regulated at a constant temperature ( $22 \pm 2^\circ\text{C}$ ) and humidity ( $50 \pm 10\%$  relative humidity) and 12-h light:12-h dark cycles. Rats were randomized into groups such that body weights (mean  $\pm$  SEM) of the groups were equivalent at the onset of the experiment. Animals had access to food and water *ad libitum* while housed in their domiciliary housing, and also during whole-body exposures. The only time animals did not have access to food and water was during the nose-only exposure to MSCS or clean air. Body weights were measured prior to placement on the study, weekly during exposures, and on the days of neurobehavioral testing.

### Functional observational battery (FOB) and motor activity assessments

FOB and motor activity testing were performed at the end of the sand or air exposure. All FOB assessments were performed by the same technician throughout the study. The technician that conducted the FOBs was unaware of the animal's exposure. All FOB testing was completed during the light phase of the animals daily diurnal cycles. Observations were made: (1) while the rat was in the observation cage, (2) during removal of the rat from the observation cage, (3) while the rat was being held and examined for clinical observations, (4) as the animal moved freely about the open field, and (5) during manipulative tests.

Motor activity was measured during ten 6-min intervals for a total of 60 min using an automated cage rack

photobeam activity system (San Diego Instruments, San Diego, CA). White noise was generated using a Coulbourn Instruments (Allentown, PA) white noise generator. Mean ( $\pm$ SEM) white noise levels of  $67.9 \pm 0.8$  dBA and room illumination of approximately  $2.3 \pm 0.3$  foot candles were maintained in the motor activity testing laboratory during testing. Mean and SEM values were calculated for total motor activity and ambulations for each 6-min interval during the measurement period.

### Bronchoprovocation testing

Following the first week of sand-exposures, a subset of eight animals from each of the six exposure groups were randomly chosen for a one-time (end of week 5) pulmonary function testing procedure. The measurements were not terminal and each animal was returned to continue participation in the study. This testing involves use of a whole-body plethysmograph, a device in which the rats are free to roam about, yet, at the same time, have non-invasive measurements made of their pulmonary function. The bronchoprovocation involved exposure to methacholine as previously described (Delorme & Moss, 2002). Briefly, animals were placed into individual whole-body plethysmographs (Model 3213, Buxco Electronics Inc., Wilmington, NC) and baseline physiological measurements (respiratory rate, tidal volume, and airway resistance) recorded for a 3-min period. After baseline measurements, animals were exposed to methacholine (Sigma-Aldrich, St. Louis, MO) aerosols of increasing concentrations (0, 2.5, 20, 80 or 320 mg/mL). Methacholine aerosols were generated with a Collison 3 jet nebulizer (BGI Incorporated, Waltham, MA). The nebulizer was operated at a pressure of 21 psi that produced a flow output of approximately 8 L/min. The aerosol was delivered to 8 whole-body plethysmographs via an inner plenum from a Cannon Nose-only exposure tower. The exhaust on each plethysmograph was controlled by a vacuum rotameter on a Bias Flow Regulator (Buxco Electronics Inc., Wilmington NC) and set at approximately 1 L/min. Each exposure was for 3 min followed by clean air and the recording of physiological measurements for another 3 min. Whenever possible, the concentration of methacholine that induced a 200% increase in airway resistance ( $PC_{200}$ ) was calculated for each animal and averaged for each exposure group. Physiological data were captured by a pressure transducer, amplified, digitally recorded, and analysed by the BioSystem XA software (version 2.7.9, Buxco Electronics Inc, Wilmington, NC). Data included estimation of the enhanced pause (Penh), a unit-less index that in some cases may reflect airway hyper-reactivity. For example, Bergren (2001) has shown that changes in Penh correlate with changes in pulmonary resistance in MSCS-exposed rodents. Correlation of Penh with changes in airway resistance, however, remains controversial (Bates et al., 2004).

### Airway histopathology

One day after the last sand exposure, necropsy was performed. Rats were anesthetized with sodium

pentobarbital (intraperitoneal injection, approximately 30 mg/kg) and exsanguinated by transection of the abdominal aorta. The trachea was exposed and the lungs were filled with 10% neutral buffered formalin (NBF) at approximately 30 cm water pressure. Afterwards, the nasal cavities were flushed (retrograde through the trachea) with NBF. Lungs with trachea and larynx attached were excised and placed in NBF. Following fixation, the larynx, trachea and left lung were changed to 70% ethanol, gross trimmed, routinely processed to paraffin block, sectioned (5  $\mu$ m), and stained with hematoxylin and eosin (H & E). A single cross section of larynx that included the base of the epiglottis was reviewed histologically. Sections of the trachea at the thyroid and more posterior were examined. A single lobe of lung (the left lobe) was evaluated histologically.

The heads were removed, skinned, trimmed of excess tissue and placed in NBF. Respiratory tract tissues were fixed in NBF for approximately 48 h. Following fixation, the heads were rinsed under running tap water for at least 30 min and decalcified in Immunocal™ Formic Acid Bone Decalcifier (Decal Chemical Corp., Tallman, NY) for 7–14 days. Noses were sectioned transversely to provide sections of the nasal cavity at six standard levels (Morgan, 1991), routinely processed and embedded in paraffin, sectioned (5  $\mu$ m), deparaffinized, and stained with H & E. Nose sections from nose tips and Levels I, II, III, IV and V were evaluated in animals from each dose group. Diagnoses were made at each level dependent on the type of epithelium affected. Transitional and respiratory epithelium were combined to economize the number of diagnoses made and to better illustrate the changes occurring at each nose level without being overly complex. Similarly, the diagnosis of olfactory epithelial degeneration was combined with olfactory epithelial necrosis.

During the light microscope examination, histopathologic diagnoses for tissues of each animal were recorded. Microscopic findings were graded using a subjective grading scale (P=present [nongradable], 1=minimal, 2=slight/mild, 3=moderate, 4=moderately severe, 5=severe/high). All tissues were evaluated by a veterinary pathologist.

#### **Bronchoalveolar lavage fluid (BALF) collection**

Rats were anesthetized with sodium pentobarbital (intraperitoneal injection, approximately 30 mg/kg) and exsanguinated by transection of the abdominal aorta. The right middle and caudal lobes of the lung were isolated and ligated prior to collection of BALF. The right caudal lung lobe was collected for gene expression studies, while the right middle lobe was used for analytical chemical evaluations. The lungs were lavaged three times using an endotracheal tube with calcium/magnesium (Ca/Mg)-free phosphate-buffered saline (28 mL/kg body weight). Total cell counts of the BAL fluid were performed using an electronic cell counter (Model ZBI; Coulter Electronics, Hialeah, FL). Differential cell counts were

determined for cytocentrifuge preparations (Cytospin Model II; Shandon Pittsburgh, PA) stained with Diff-Quick (American Scientific Co., Sewickley, PA) by enumeration of 200 cells per slide. The BALF was centrifuged at 500g for 10 min at 4°C; supernatants were analysed for total protein (Coomassie plus reagent; Pierce and Co., Rockford, IL) and lactate dehydrogenase (LDH) (Assay kit 228; Sigma Chemical Co. St. Louis, MO) concentrations using an automated centrifugal spectrophotometer (Cobas Fara II; Hoffman-LaRoche, Branchburg, NJ).

#### **Tissue chemical analyses**

The right middle lobe was placed into acid-washed tissue cups, quickly frozen in liquid nitrogen, and stored at –70°C until ready for analysis. Tissue samples were sent to Dr. Jose A. Centeno of the AFIP for high-resolution ICP-MS analysis of the inorganic component of sand particulates in tissues. Digestion was performed using ultra-pure trace element grade 70% HNO<sub>3</sub> solution under a pressure controlled microwave digestion system (MarsXpress, CEM Inc, Mathews, North Carolina, USA), and then reconstituted with 2% HNO<sub>3</sub>. After extraction, the samples were analysed by ICPMS-MS (Finnigan Element II, Thermo Scientific Inc, Bremen, Germany) with Indium (<sup>115</sup>In) as an internal standard. Calibration was performed with multi-element standard solutions. Each sample was diluted up to 14 mL and analysed by Element II for the following elements employing medium-resolution mode: Mg, Al, Si, Ca, V, Cr, Mn, Fe, Co, Cu, Zn, Rb, Sr, Zr, Mo, Cd, Ba, W, Tl, Pb, and U. Analytical results are listed on Table 2 for metals with an overall significant treatment effect (ANOVA,  $p < 0.05$ ) followed by a significant ( $p < 0.05$ ) post-hoc pair wise comparison (Tukeys). Tissue concentrations for each element are expressed on a wet-weight basis.

#### **RNA isolation**

A sample of lung was also collected ( $n=5$  rats/group) for pulmonary gene expression. Total lung RNA was isolated from tissues snap-frozen in liquid nitrogen using TriReagent (Sigma Chemical Co., St. Louis, MO). RNA was further purified with Qiagen Rneasy minicolumns (Qiagen, Valencia, CA) and resuspended in 50  $\mu$ L diethylpyrocarbonate-treated water according to the manufacturer's protocol. RNA quality was assessed by spectrophotometry and with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). All samples had a 28S/18S ratio  $\geq 2.0$  and were stored at –80°C for gene chip analysis or their use in real-time polymerase chain reaction.

#### **Microarray hybridizations and data analysis**

Probe preparation and hybridization to the microarray was performed in The Hamner Institutes for Health Sciences' Gene Expression Core Facility using standard Affymetrix procedures. Double-stranded cDNA was synthesized from RNA using an oligo-dT24-T7. Biotinylated cRNA was synthesized from an aliquot of the cDNA template using the T7 RNA Transcript Labeling Kit (ENZO

Table 2. Mean ( $\pm$ SD) metal concentrations in lung tissue following exposure to air, main stream cigarette smoke (MSCS), silica, or Iraqi sand.

| Analyte  | Air-Air           | Air-Iraqi sand    | Air-Silica                     | MSCS-Air          | MSCS-Iraqi sand               | MSCS-Silica                    |
|----------|-------------------|-------------------|--------------------------------|-------------------|-------------------------------|--------------------------------|
| Al (ppm) | 1.11 $\pm$ 2.51   | 13.0 $\pm$ 2.95*  | 1.77 $\pm$ 5.36                | 1.14 $\pm$ 1.93   | 12.9 $\pm$ 2.02*              | 1.62 $\pm$ 1.46                |
| Ba (ppb) | 12.41 $\pm$ 18.73 | 32.7 $\pm$ 33.40* | 20.0 $\pm$ 13.90               | 15.2 $\pm$ 18.25  | 43.3 $\pm$ 21.73*             | 34.0 $\pm$ 26.32*              |
| Cd (ppb) | 2.75 $\pm$ 1.52   | 3.02 $\pm$ 1.20   | 2.18 $\pm$ 1.67                | 44.4 $\pm$ 11.90* | 43.9 $\pm$ 10.15*             | 46.0 $\pm$ 8.83*               |
| Fe (ppm) | 96.1 $\pm$ 32.43  | 114 $\pm$ 20.01   | 99.9 $\pm$ 25.18               | 102 $\pm$ 28.31   | 136 $\pm$ 21.97*              | 120 $\pm$ 17.25*               |
| Mg (ppm) | 139 $\pm$ 34.17   | 156 $\pm$ 19.23   | 134 $\pm$ 22.36                | 148 $\pm$ 35.93   | 166 $\pm$ 14.07*              | 156 $\pm$ 14.50                |
| Mn (ppb) | 139 $\pm$ 47.32   | 280 $\pm$ 57.21*  | 150 $\pm$ 35.33                | 199 $\pm$ 54.38*  | 338 $\pm$ 32.18* <sup>‡</sup> | 223 $\pm$ 56.34* <sup>‡</sup>  |
| Mo (ppb) | 70.0 $\pm$ 19.00  | 72.9 $\pm$ 11.84  | 69.4 $\pm$ 20.10               | 92.4 $\pm$ 23.35* | 97.6 $\pm$ 15.12*             | 98.6 $\pm$ 14.21*              |
| Si (ppm) | 3.08 $\pm$ 5.65   | 31.3 $\pm$ 8.44*  | 66.1 $\pm$ 26.36* <sup>†</sup> | 3.35 $\pm$ 3.19   | 33.3 $\pm$ 5.41*              | 61.2 $\pm$ 21.46* <sup>†</sup> |
| V (ppb)  | 2.27 $\pm$ 3.21   | 26.3 $\pm$ 5.17*  | 2.64 $\pm$ 2.7                 | 3.35 $\pm$ 2.76   | 26.3 $\pm$ 4.33*              | 4.000 $\pm$ 5.38               |

Data shown for metals with an overall significant treatment effect (ANOVA,  $p < 0.05$ ) followed by a significant ( $p < 0.05$ ) post-hoc pair wise comparison (Tukeys).

\* $p < 0.05$  (versus Air-Air exposed control).

<sup>†</sup> $p < 0.05$  (versus similar sand-exposed control).

<sup>‡</sup> $p < 0.05$  (versus similar air-exposed control).

Diagnostics, Farmingdale, NY). The labeled cRNA was then fragmented, hybridized to an Affymetrix Rat Genome 230 2.0 Array (Affymetrix, Santa Clara, CA), and stained using phycoerythrin-conjugated streptavidin (Molecular Probes, Eugene, OR). Gene expression results have been deposited in the National Center for Biotechnology Information (NCBI) Expression Omnibus database (GSE28804).

Expression values for probe sets were calculated using the robust multichip average (RMA) method (Irizarry et al., 2003a,b) implemented in the Bioconductor Package within R statistical software. Genes that were differentially expressed (Lockhart et al., 1996; Fodor et al., 1993; Fodor et al., 1991) between the groups of interest were determined by an ANOVA ( $F$ - or  $t$ -test) analysis of the data, performed in R using the affyImGUI package (Wettenhall et al., 2006). Using a 0.05% false discovery rate (FDR; Benjamini & Hochberg, 1995) threshold to control for multiple comparisons, 119 genes were found to be differentially expressed between the six exposure groups; reported  $p$  values have been adjusted by the FDR method. Genes identified as statistically significant were subject to an additional filter by selecting only those genes that exhibited a  $\geq 2$ -fold change from the control animals. Probe sets were mapped to genes using the Rat Genome Database (<http://rgd.mcw.edu/>). For probe sets that failed to match a gene, BLAST searches were performed with the target sequence from Affymetrix against the NCBI nr database.

### Proteomic analysis

Four milliliters of BALF was concentrated and buffer exchanged to Buffer A using 5 kDa molecular weight cut-off filters (Agilent, Palo Alto, CA) prior to immunodepletion of high abundant proteins using the Mouse 3, Multiple Affinity Removal System – LC Column (Agilent, Palo Alto, CA) according to manufacturer's recommendations. Fractions containing the unbound proteins were pooled, concentrated, and buffer exchanged to 50 mM ammonium bicarbonate. The proteins were denatured in 5 mM dithiothreitol and 0.1% Rapigest (Waters,

Milford, MA), and then alkylated with iodoacetamide (50 mM final). Two micrograms of sequencing grade trypsin (Promega, Madison, WI) was added prior to an overnight digest at 37°C. The Rapigest was hydrolyzed with formic acid, and the samples were clarified with 0.22  $\mu$ m spin filters, dried under vacuum, and reconstituted in 0.1% formic acid. The peptides were separated using a Waters nanoACQUITY UltraPerformance Liquid Chromatography and analysed on a coupled Waters Q-ToF Premier quadrupole, orthogonal acceleration time-of-flight tandem mass spectrometer as previously described (Lewis et al., 2010). For each treatment group, five samples were analysed. However, for each group except MSCS-IS, one sample was removed from the analysis, because the mass spectral data contained only a small number of intense peaks, unlike the remaining 25 samples.

Mass spectrometry data were processed using ProteinLynx Global SERVER (PLGS) version 2.4 (Waters, Milford, MA). Data preparation parameters were set to the manufacturer's default with the exception of a 785.8426 lock mass for charge 2 and 813.3895 for charge 1. Workflow parameters for database searches were set to the manufacturer's default with the exceptions of setting the false positive rate parameter to 10%, allowing deamidated asparagine and glutamine and oxidated methionine as variable modifications, and enabling PPM calc. An in-house protein identification database was created from all 29,405 rat RefSeq sequences (downloaded from the NCBI) combined with likely contaminant proteins including porcine trypsin and human keratins. For the identifications used in the accurate mass and time (AMT) quantification method (see below), genomic model proteins (XP prefix) were removed from the database to reduce redundant identification, leaving 16,715 rat proteins. To minimize false identification, only proteins identified in at least 3 replicates were considered present. Proteins which shared identical peptides were combined into homology groups based on PLGS ion accounting output. The 239 proteins that were identified formed 132 unique homology groups.



Two different methods were used to quantify changes in protein abundance. In the first, the top 3 most abundant ions from protein identification were used as a measure of the protein's abundance (Silva et al., 2006), and *t*-tests were used to calculate significance. In the second method, AMT were used to align the mass spectral data using Progenesis software (Nonlinear Dynamics, Durham, NC). Peak identifications were imported from PLGS search results, and significance values for matched proteins were calculated by ANOVA. In both methods, comparisons were made between each group and the air only control and between each MSCS group and the MSCS only control. Each protein which changed by 2-fold with a *p* value < 0.01 in any condition was reported as changing, and for these proteins, any condition with a *p* value < 0.05 was also reported as changing.

### Statistical analysis

Data that upon visual inspection were deemed to be potential outliers were subjected to analysis using the Grubbs' test for outliers (Grubbs, 1969). Levene's test for homogeneity (*p* < 0.05), followed by one-way analysis of variance (ANOVA) (*p* < 0.05) and Dunnett's *t*-test (*p* < 0.05) were performed for homogeneous data. A natural log (ln) transformation of the data was used when the Levene's test for homogeneity indicated the data to be non-homogenous. A Levene's test followed by a one-way analysis of variance (ANOVA) (*p* < 0.05) and Dunnett's *t*-test (*p* < 0.05) were performed on the transformed data. In the event that the Bartlett's test on the transformed data indicated non-homogenous data, a Kruskal-Wallis *H*-test (*p* < 0.05) and Wilcoxon 2-sample Rank-Sum test (*p* < 0.05) were used. Categorical FOB data were analysed using a log-linear model (*p* < 0.05). For all analyses, group differences were considered significant if the test's statistical type I error was less than 0.05 (i.e. *p* < 0.05). A nested analysis of motor activity data was performed using a repeated-measures analysis with exposure as a grouping factor and test period and test session time as within-subject factors (MANOVA) (*p* < 0.05).

## Results

### Test atmospheres

Based on mass weight filter measurements, the actual MSCS aerosol concentration (mean ± SD) was  $0.48 \pm 0.076$  mg/L. Based on the aerosol particle sizer results, the mass median aerodynamic diameter (MMAD, mean ± SD) of the MSCS aerosol was determined to be  $1.05 \pm 0.07$  μm ( $1.33 \pm 0.03$  σ<sub>g</sub>). Based on mass weight filter measurements, the actual silica aerosol concentration (mean ± SD) was  $1.153 \pm 0.311$  mg/m<sup>3</sup>. Based on the aerosol particle sizer results, the MMAD (mean ± SD) of the silica aerosol was determined to be  $1.36 \pm 0.02$  μm ( $1.394 \pm 0.007$  σ<sub>g</sub>). Based on mass weight filter measurements, the actual IS aerosol concentration (mean ± SD) was  $0.915 \pm 0.245$  mg/m<sup>3</sup>. Based on the aerosol particle sizer results, the MMAD (mean ± SD) of the sand aerosol

was determined to be  $1.71 \pm 0.06$  μm ( $1.509 \pm 0.008$  σ<sub>g</sub>). Overall mean daily chamber temperatures ranged from 22.4 to 25.3°C, and the relative humidity in the 1-m<sup>3</sup> inhalation chambers ranged from 52 to 64%.

### Clinical effects

A statistically significant decrease in body weight was observed in animals from all MSCS-exposed groups (Figure 2). Exposure to neither silica nor IS was associated with any effect on body weight gain or terminal body weight when compared with air-exposed control animals. The most commonly noted clinical observation was alopecia. Abnormal discoloration of the fur was also seen in MSCS-exposed rats. These changes were also noted during the FOB examination. Other effects seen during the FOB evaluation included a significant decrease in hind limb grip and hind limb splay, staining of the fur and facial crusts, increased grooming behavior, and labored or audible breathing in animals exposed to MSCS when compared with rats exposed to air only (data not shown). FOB evaluations did not reveal any effects associated with exposure to either silica or IS when compared with air-exposed controls (data not shown). Exposure to MSCS either alone or in combination with silica or IS was associated with significantly decreased motor activity (Figure 3). This effect was related to MSCS exposure, since neither IS nor silica (data not shown) inhalation was associated with altered motor activity when compared with air-exposed controls. Likewise, combined MSCS and IS inhalation did not further affect motor activity when compared with animals exposed to MSCS.

### BALF analysis

Data for cytological and biochemical analysis of BALF are presented in Table 3. Exposure to neither silica nor IS was associated with any statistically significant effect

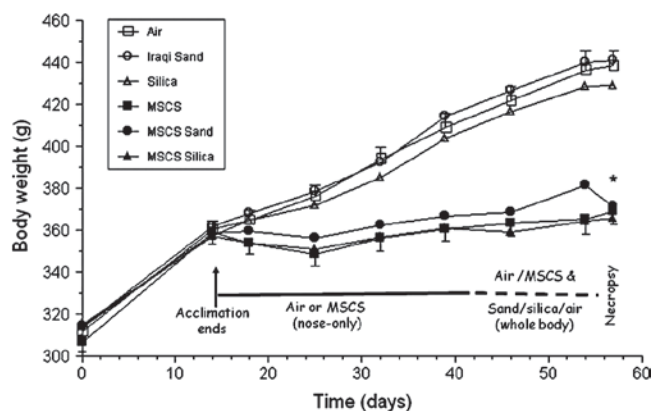


Figure 2. Mean (±SEM) body weights seen in rats exposed initially to either air or main stream cigarette smoke (MSCS) followed by combined air or MSCS exposure with exposure to air, IS, or crystalline silica. Error bars are only shown for the air control (□) and MSCS groups (■). \*Denote start and end periods when statistically significant decreases (*p* < 0.05) in body weights were seen following MSCS exposure.



for any parameter measured. Further, no exposures were associated with any effect on total protein, percent lymphocytes, or total cell counts in BALF. However, MSCS exposure resulted in an increase in the relative numbers of neutrophils and concomitant decrease in the relative numbers of macrophages present in the BALF when compared with air-exposed controls. Co-exposure to MSCS and silica was associated with a statistically significant increase in BALF LDH concentration when compared

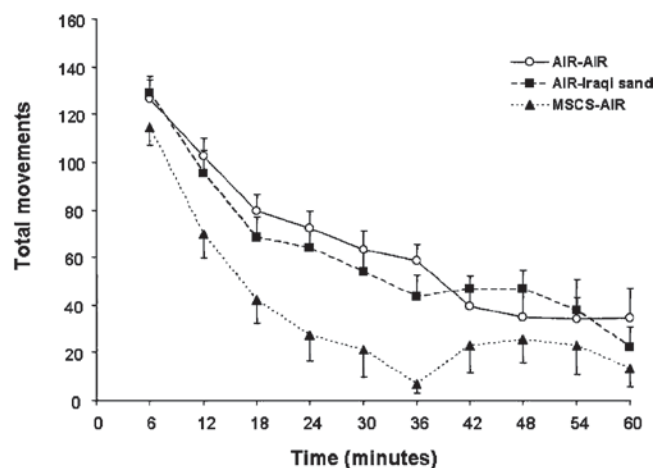


Figure 3. Mean ( $\pm$ SEM) total movement seen in rats exposed to either air, main stream cigarette smoke (MSCS), or IS. A statistically significant decrease ( $p < 0.05$ ) in movement was seen in rats following MSCS exposure. Motor activity assessed one day after the last exposure to sand, silica, or air.

with air-exposed controls. BALF LDH concentrations were unaffected in the remaining exposure groups.

### Whole-body plethysmography

Mean  $\pm$  SEM (group size provided in parentheses)  $PC_{200}$  R concentration results for Air-Air, Air-IS, Air-Silica, MSCS-Air, MSCS-IS, and MSCS-Silica exposure groups are as follows:  $3.18 \pm 0.16$  (7),  $6.22 \pm 2.28$  (8),  $5.73 \pm 1.16$  (8),  $25.53 \pm 8.31$  (7),  $7.63 \pm 3.89$  (2), and  $18.12 \pm 8.78$  (6) mg/mL of methacholine, respectively. A statistically significant increase in the  $PC_{200}$  R concentration was observed in rats exposed to MSCS and either air or silica. Group sizes were insufficient to reach a definitive conclusion for the MSCS-IS exposure group. No statistically significant effect for either silica or IS alone was seen on this parameter. Additional pulmonary function data are presented in Table 4. As noted earlier, a significant decrease in body weight ( $>15\%$ ) was seen in rats exposed to MSCS (all exposure groups) when compared with air-exposed controls. Rats in the MSCS-Air group but not MSCS-IS and MSCS-Silica also had a significant decrease in their baseline respiratory rate when compared with air-exposed controls. Rats exposed to silica also had a marginally decreased ( $p = 0.055$ ) baseline respiratory rate when compared with air-exposed controls. Rats exposed to MSCS-Air had a statistically significant increase in their baseline Penh value when compared with air-exposed controls as did MSCS-IS. Animals exposed to all MSCS groups and challenged with methacholine (at 20 mg/mL)

Table 3. Differential BALF cells and BALF protein concentrations following exposure to air, main stream cigarette smoke (MSCS), silica, or Iraqi dust.

| Endpoint                      | Air-Air            | Air-Iraqi sand   | Air-Silica       | MSCS-Air           | MSCS-Iraqi sand    | MSCS-Silica        |
|-------------------------------|--------------------|------------------|------------------|--------------------|--------------------|--------------------|
| Total cells ( $\times 10^6$ ) | $1.33 \pm 0.10$    | $1.49 \pm 0.14$  | $1.13 \pm 0.13$  | $1.23 \pm 0.19$    | $1.11 \pm 0.10$    | $1.29 \pm 0.23$    |
| Neutrophils (%)               | $0.81 \pm 0.21$    | $1.62 \pm 0.38$  | $3.67 \pm 1.59$  | $20.75 \pm 4.69^*$ | $21.22 \pm 5.15^*$ | $29.64 \pm 6.96^*$ |
| Lymphocytes (%)               | $0.49 \pm 0.13$    | $0.35 \pm 0.09$  | $0.55 \pm 0.16$  | $0.13 \pm 0.08$    | $0.29 \pm 0.11$    | $0.28 \pm 0.10$    |
| Macrophage (%)                | $98.70 \pm 0.18$   | $98.03 \pm 0.36$ | $95.78 \pm 1.54$ | $79.12 \pm 4.68^*$ | $78.49 \pm 5.09^*$ | $70.08 \pm 6.90^*$ |
| Total protein ( $\mu$ g/mL)   | $72.92 \pm 8.64$   | $70.58 \pm 6.22$ | $77.33 \pm 6.39$ | $66.82 \pm 4.85^a$ | $78.70 \pm 5.86$   | $134.45 \pm 33.08$ |
| LDH (U/L)                     | $15.73 \pm 3.23^a$ | $16.25 \pm 2.66$ | $23.58 \pm 3.12$ | $24.17 \pm 5.40$   | $27.20 \pm 2.56$   | $34.64 \pm 5.91^*$ |

Data collected at necropsy (1 day after the last exposure to sand, silica, or air).

LDH, lactate dehydrogenase.

<sup>a</sup>one outlier value was removed from the analysis based upon the Grubb's test for outliers.

\* $p < 0.05$ .

Table 4. Physiological measurements (mean  $\pm$  S.E.M.) collected at baseline and following a brief exposure to methacholine (MCh) at 20 mg/mL.

| Endpoint                        | Air-Air         | Air-Iraqi sand   | Air-Silica           | MSCS-Air          | MSCS-Iraqi sand    | MSCS-Silica        |
|---------------------------------|-----------------|------------------|----------------------|-------------------|--------------------|--------------------|
| Body weight                     | $421.6 \pm 9.4$ | $430.9 \pm 16.1$ | $420.0 \pm 10.4$     | $353.3 \pm 5.6^*$ | $356.9 \pm 12.3^*$ | $361.5 \pm 11.7^*$ |
| Baseline respiratory rate (BPM) | $154 \pm 12$    | $170 \pm 12$     | $109 \pm 12^\dagger$ | $108 \pm 9^*$     | $145 \pm 16$       | $144 \pm 12$       |
| Respiratory rate (BPM) with MCh | $190 \pm 7$     | $206 \pm 12$     | $201 \pm 7$          | $187 \pm 12$      | $180 \pm 10$       | $192 \pm 9$        |
| Baseline tidal volume (mL)      | $1.78 \pm 0.05$ | $1.57 \pm 0.07$  | $1.68 \pm 0.07$      | $1.66 \pm 0.05$   | $1.80 \pm 0.08$    | $1.88 \pm 0.09$    |
| Tidal volume (mL) with MCh      | $2.01 \pm 0.15$ | $2.05 \pm 0.10$  | $1.82 \pm 0.09$      | $1.56 \pm 0.07^*$ | $1.52 \pm 0.08^*$  | $1.54 \pm 0.10^*$  |
| Baseline Penh                   | $0.63 \pm 0.04$ | $0.58 \pm 0.06$  | $0.81 \pm 0.13$      | $1.16 \pm 0.16^*$ | $1.23 \pm 0.11^*$  | $1.10 \pm 0.20$    |
| Penh with MCh                   | $7.27 \pm 1.03$ | $5.09 \pm 0.71$  | $5.69 \pm 0.88$      | $3.75 \pm 0.83^*$ | $2.15 \pm 0.31^*$  | $3.11 \pm 0.42^*$  |

Rats were exposed to either air or main stream cigarette smoke (MSCS) prior to a second 7 day challenge with either air, Iraqi sand, or crystalline silica. Body weights collected within 1–2 days of the pulmonary function testing are also shown.

$n = 8$  rats/exposure group.

\* $p < 0.05$ .

$^\dagger p = 0.055$ .

also had reduced tidal volumes and Penh values when compared with air-exposed controls.

### Histopathology

Airway histopathology data are presented in Table 5. Other than in the lungs, there were no histological findings in rats exposed to filtered air only. Six air-exposed rats had minimal to mild perivascular mixed cell infiltration and two rats had focal inflammatory changes in the alveoli. These lesions were considered within normal limits for this strain and age of animals. As expected, rats exposed to MSCS (all exposure groups) had significant airway epithelial responses. A small amount of inflammation of the transitional/respiratory or squamous epithelium found at the tip of the nares (data not shown) and nasal Level I was occasionally seen. At nasal Levels II (data not shown) and

III, there were moderate to moderately severe squamous metaplasia to transitional/respiratory epithelium that was seen in all MSCS-exposed rats. This was particularly evident on the lateral wall and extended around both the nasoturbinate and maxilloturbinate and sometimes on the nasal septum. Olfactory epithelial degeneration/necrosis was also present in most animals at Levels II and III and was seen in some animals at Levels IV and V. The majority of the MSCS-exposed rats had squamous metaplasia of the nasopharyngeal duct at Level V. There was moderate squamous metaplasia at the base of the epiglottis and the tracheas of this group of rats that was characterized by mild to moderately severe hyperplasia and minimal to mild squamous metaplasia. There was occasionally minimal to mild multifocal inflammation of the alveoli characterized by scattered accumulations

Table 5. Incidence of representative respiratory tract lesions observed in rats following exposure to air, mainstream cigarette smoke (MSCS), silica, or Iraqi sand.

| Lesion                | Site (nasal section indicated by level)   | Air-Air       | Air-Iraqi Sand | Air-Silica      | MSCS-Air        | MSCS-Iraqi Sand | MSCS-Silica     |
|-----------------------|-------------------------------------------|---------------|----------------|-----------------|-----------------|-----------------|-----------------|
| Macrophages           | Alveolus                                  | 3/12<br>(1.0) | 9/12*<br>(1.0) | 8/12*<br>(1.0)  | 11/12*<br>(1.1) | 9/12*<br>(1.4)  | 10/12*<br>(1.9) |
| Inflammation          | Larynx                                    | 1/12<br>(1)   | 2/12<br>(1.0)  | 11/12*<br>(1.0) | 9/11*<br>(1.1)  | 4/12<br>(1.0)   | 5/12*<br>(1.2)  |
|                       | Trachea                                   | 0/12          | 0/12           | 5/12*<br>(1.0)  | 0/12            | 0/12            | 3/12<br>(1.0)   |
|                       | Transitional/respiratory epithelium (I)   | 0/12          | 1/12<br>(2.0)  | 0/12            | 9/12*<br>(1.4)  | 10/12*<br>(1.5) | 5/12*<br>(1.4)  |
| Hyperplasia           | Trachea                                   | 0/12          | 0/12           | 10/12*<br>(1.4) | 11/12*<br>(2.5) | 12/12*<br>(2.0) | 12/12*<br>(2.0) |
|                       | Nasopharyngeal duct (V)                   | 0/12          | 0/12           | 0/12            | 2/12<br>(1.0)   | 8/12*<br>(1.5)  | 3/12<br>(1.7)   |
| Squamous metaplasia   | Larynx                                    | 0/12          | 0/12           | 0/12            | 11/11*<br>(3.0) | 12/12*<br>(3.0) | 12/12*<br>(3.0) |
|                       | Trachea                                   | 0/12          | 0/12           | 0/12            | 11/12*<br>(1.6) | 12/12*<br>(1.1) | 11/12*<br>(1.3) |
|                       | Transitional/respiratory epithelium (I)   | 0/12          | 0/12           | 0/12            | 1/12<br>(2)     | 0/12            | 6/12*<br>(1.8)  |
|                       | Transitional/respiratory epithelium (III) | 0/12          | 0/12           | 0/12            | 11/12*<br>(3.5) | 12/12*<br>(3.0) | 12/12*<br>(3.0) |
|                       | Olfactory epithelium (II)                 | 0/12          | 0/12           | 0/12            | 5/12*<br>(2.0)  | 6/12*<br>(1.5)  | 5/12*<br>(1.8)  |
|                       | Nasopharyngeal duct (IV)                  | 0/12          | 0/12           | 0/12            | 0/12            | 0/12            | 4/12*<br>(2.0)  |
|                       | Nasopharyngeal duct (V)                   | 0/12          | 0/12           | 0/12            | 9/12*<br>(1.9)  | 9/12*<br>(1.6)  | 7/12*<br>(1.9)  |
| Degeneration/necrosis | Olfactory epithelium (II)                 | 0/12          | 0/12           | 0/12            | 10/12*<br>(3.2) | 12/12*<br>(3.7) | 7/12*<br>(3.9)  |
|                       | Olfactory epithelium (III)                | 0/12          | 0/12           | 0/12            | 10/12*<br>(3.8) | 8/12*<br>(4.1)  | 10/12*<br>(3.8) |
|                       | Olfactory epithelium (IV)                 | 0/12          | 0/12           | 0/12            | 5/12*<br>(4.0)  | 7/12*<br>(3.3)  | 7/12*<br>(3.4)  |
|                       | Olfactory epithelium (V)                  | 0/12          | 0/12           | 0/12            | 5/12*<br>(3.0)  | 5/12*<br>(3.6)  | 6/12*<br>(2.8)  |

Severity scores (in parentheses) are given for animals with a statistically significant increase in incidence. Mean severity scores are presented where: 1 = minimal/present; 2 = slight to mild; 3 = moderate; 4 = moderately severe; and 5 = severe.

\* $p < 0.05$  (one-tailed Fisher's Exact).

of lymphocytes, macrophages and neutrophils. In most MSCS-exposed rats, there was a mild diffuse increase in alveolar macrophages containing particles of brown pigment in the cytoplasm. In some animals, there was a perivascular mixed cell infiltration of the lungs (data not shown).

Rats exposed to IS only had minimal changes that were only seen occasionally (Table 5). One rat had some mild inflammatory change in the anterior nose. In the lung, minimal to mild perivascular mixed cell infiltration was present in some rats. Minimal focal alveolar inflammation was occasionally present (3/12 rats). Minimal alveolar macrophages were evident in the majority of rats. Changes seen in rats exposed to MSCS and IS were similar to the changes seen in rats exposed to MSCS only.

The majority of rats exposed to silica only had minimal laryngeal inflammation (Table 5). Other lesions seen in rats exposed to silica only included minimal to mild hyperplasia of the tracheal epithelium, tracheal inflammation, minimal perivascular mixed cell infiltration of the lung (9/12), minimal focal alveolar inflammation, and the presence of minimal increase in alveolar macrophages. With few exceptions, rats exposed to MSCS and silica had lesions that were similar to those seen in rats exposed to MSCS only. Rats exposed to MSCS and silica had squamous metaplasia of the transitional/respiratory epithelium at Level I and nasopharyngeal duct at Level IV, suggesting that this lesion was more widespread in these animals when compared with rats exposed to MSCS only (Table 5).

### Tissue metal analyses

Elemental (ICP/MS) analyses of lung tissues are presented in Table 2. Exposure to IS resulted in elevated lung aluminum, vanadium, silica, barium, and manganese concentrations when compared with animals exposed to air only. As expected, animals exposed to silica also had elevated lung silica concentrations, and the levels seen in these animals were approximately double those seen in animals exposed to IS. Exposure to MSCS only resulted in elevated lung manganese, molybdenum, and cadmium concentrations when compared with air-exposed controls. Lung molybdenum and cadmium concentrations seen in MSCS-exposed rats were unaffected by additional sand or silica exposure. Animals exposed to MSCS and IS had higher lung manganese concentrations than those seen in rats exposed to IS and air. This increase appears to represent an additive response. Lung iron concentrations were also elevated (~20% versus their respective air-exposed control) in rats exposed to combined MSCS and either sand or silica exposure. Combined silica and MSCS exposure resulted in higher lung rubidium concentrations ( $4.31 \pm 0.53$  ppb) when compared to animals exposed to silica and air ( $3.66 \pm 0.65$  ppb).

### Gene expression

Only minimal effects on lung gene expression were seen following IS inhalation (Tables 6 and 7). Exposure to

Table 6. Gene expression changes seen in rats exposed to air, main stream cigarette smoke (MSCS), silica, or Iraqi sand.

| Affymetrix probe set ID | Genbank accession # | Gene Symbol | Description                                                            | Air-Iraqi sand |               | Air-Silica |               | MSCS-Iraqi sand |               | MSCS-Silica |               |
|-------------------------|---------------------|-------------|------------------------------------------------------------------------|----------------|---------------|------------|---------------|-----------------|---------------|-------------|---------------|
|                         |                     |             |                                                                        | p value        | Fold $\Delta$ | p value    | Fold $\Delta$ | p value         | Fold $\Delta$ | p value     | Fold $\Delta$ |
| 1390443_at              | NM_001108312.1      | RGD1563888  | Similar to DNA segment, Chr 16, ERATO Doi 472, expressed               | 0.045          | -2.24         |            |               |                 |               |             |               |
| 1378347_at              | AW252020            | -           | EST: similar to natural killer tumor recognition protein               | 0.018          | -2.03         | <0.001     | 3.28          | 0.005           | 2.23          | <0.001      | 2.99          |
| 1370810_at              | NM_022267.1         | Ccnd2       | cyclin D2                                                              |                |               | 0.003      | 2.44          |                 |               |             |               |
| 1377437_at              | NM_001014171.1      | Veph1       | ventricular zone expressed PH domain homolog 1 (zebrafish)             |                |               | 0.001      | 2.23          |                 |               |             |               |
| 1394171_at              | AI639310            | -           | EST: unknown function                                                  |                |               | <0.001     | 2.46          |                 |               |             |               |
| 1389986_at              | AI008409            | -           | EST: similar to metastasis associated lung adenocarcinoma transcript 1 |                |               | 0.016      | 2.19          |                 |               |             |               |
| 1383052_a_at            | NM_001169120.1      | Zfp91       | zinc finger protein 91                                                 |                |               | <0.001     | 2.01          |                 |               |             |               |
| 1389868_at              | NM_001109292.1      | RGD1564560  | Similar to RCK                                                         |                |               | 0.002      | 2.69          |                 |               | 0.003       | 2.71          |
| 1382809_at              | NM_031147.2         | Cirbp       | Cold inducible RNA binding protein                                     |                |               | <0.001     | 4.40          |                 |               | 0.010       | 3.07          |
| 1384692_at              | AI172110            | -           | EST: unknown function                                                  |                |               | 0.010      | 2.15          |                 |               | 0.001       | 2.50          |

Data shown for expression changes with an overall significant treatment effect (ANOVA,  $p < 0.05$ ) and a 2-fold increase or decrease in expression when compared with rats exposed to air only. None of these genes changed significantly in MSCS-Air treatment group. Analysis performed on samples collected one day after the end of the air, sand, or silica exposure ended.

Table 7. The up-regulated genes in rats exposed to main stream cigarette smoke (MSCS) and either Iraqi sand or silica when compared with rats exposed to MSCS only.

| Probe set ID      | GenBank accession #              | Gene symbol      | Description                                                                  | Biological role                                                  | p value | Fold change |
|-------------------|----------------------------------|------------------|------------------------------------------------------------------------------|------------------------------------------------------------------|---------|-------------|
| MSCS + Iraqi sand |                                  |                  |                                                                              |                                                                  |         |             |
| 1370463_x_at      | NM_001008839.1                   | RT1-CE16         | RT1 class I, locus CE16                                                      | Antigen presentation, MHC class I receptor activity              | 0.049   | 2.35        |
| 1397859_x_at      | NM_001008829.1 // NM_001008830.1 | RT1-A2 // RT1-A3 | RT1 class I, locus A2 // RT1 class I, locus A3                               | Antigen presentation, 89% similar to rat MHC class I RT1 (RT16), | 0.002   | 12.87       |
| 1398594_at        | AI029767                         | -                | EST: similar to Tial cytotoxic granule-associated RNA binding protein-like 1 | Regulator of G-protein signaling                                 | 0.048   | 2.74        |
| MSCS + Silica     |                                  |                  |                                                                              |                                                                  |         |             |
| 1384692_at        | AI172110                         | -                | EST: unknown function                                                        |                                                                  | 0.037   | 3.09        |
| 1392675_at        | AI044784                         | -                | EST: unknown function                                                        |                                                                  | 0.048   | 3.14        |
| 1379101_at        | NM_001107678.1                   | Dhx36            | DEAH (Asp-Glu-Ala-His) box polypeptide 36                                    | ATP binding, ATP-dependent helicase activity                     | 0.028   | 3.11        |
| 1376438_at        | BF416513                         | -                | EST: unknown function                                                        |                                                                  | 0.038   | 2.94        |
| 1383332_at        | BI281615                         | -                | EST: similar to homeodomain interacting protein kinase 2                     |                                                                  | 0.048   | 2.02        |
| 1377651_at        | NM_001107658.1                   | Trio             | Triple functional domain (PTPRF interacting)                                 |                                                                  | 0.044   | 2.23        |
| 1378347_at        | AW252020                         | -                | EST: similar to natural killer tumor recognition protein                     |                                                                  | 0.028   | 3.07        |

Analysis performed on samples collected one day after the end of the air, sand, or silica exposure ended.

silica was associated with a more robust effect on lung gene expression (Table 6). As expected, exposure to MSCS alone or in combination with silica or IS was associated with a variety of lung gene expression changes (see Supplementary Tables 1 and 2) although variation in gene expression was two times greater with exposure to MSCS in combination with silica or IS. Animals exposed to MSCS and either IS or silica had decreased (~2-fold) expression of Kruppel-like factor 2 (lung-KLF2). KLF2 is expressed in lung and is essential for normal blood-vessel integrity and lung development and is also essential for T-cell trafficking (Carlson et al., 2006). Genes showing increased expression levels in MSCS-exposed animals (>2-fold relative to air-exposed controls) were often involved with cell signaling, chemotaxis, chemokine and cytokine signaling and functioning, and other inflammatory processes (Table 7).

### Proteomics

BALF was analysed by quantitative LC-MS analysis to identify proteins changing in abundance in response to the treatments. Of the 139 unique proteins that were identified, 12 were determined to be changing in abundance in response to the exposures (Table 8). There were no significant changes resulting from exposure to IS with either Air or MSCS as the initial treatment, when compared to their respective controls. Two proteins (complement 2; serpinA3I) that were significantly downregulated in the MSCS-IS samples when compared to Air-Air controls, but not significantly in the MSCS-Air or MSCS-Silica samples, are unlikely to truly be a difference caused by IS

exposure. These proteins were in fact downregulated in all three MSCS treatment groups (though not statistically significantly), and their levels were not significantly different between the MSCS-Air and MSCS-IS exposures. We believe their changes in abundance are primarily due to the MSCS treatment alone. The proteins with changes caused by silica exposure are involved in inflammation, oxidative stress, and immune response. By far, the majority and most robust changes in protein abundance were in samples from MSCS-exposed rats and include proteins involved in inflammation, oxidative stress, and detoxification of components of the cigarette smoke. Two proteins, acidic mammalian chitinase (CHIA) and palate, lung, and nasal epithelium associated protein (PLUNC) are involved in allergic airway inflammation mediated by IL-13 (Zhu et al., 2004; Chu et al., 2007). Interestingly, both of these had the same trend in effect across the MSCS treatment groups with MSCS-Air samples showing the least effect, MSCS-IS an intermediate effect and MSCS-Silica the greatest effect. There are also two proteins, annexin A1 and glutathione S-transferase Pi which were up-regulated in response to MSCS exposure, but had a significantly lower abundance in samples from MSCS and silica or MSCS-IS co-exposures.

### Discussion

Several epidemiological studies have evaluated whether respiratory symptom reporting is increased in troops during deployment to the Middle East. Richards et al. (1993) surveyed respiratory complaints in 2598 male ground



Table 8. Proteins identified by mass spectral analysis changing in abundance in BALF following exposure to air, main stream cigarette smoke (MSCS), silica, or Iraqi sand.

| Genbank accession # | Gene symbol | Description                                   | Quant method | Vs Air-Silica |        |          |        |                 |        | Vs Air-Air  |        |          |        |             |        | Vs MSCS-Air |        |             |        |          |        |
|---------------------|-------------|-----------------------------------------------|--------------|---------------|--------|----------|--------|-----------------|--------|-------------|--------|----------|--------|-------------|--------|-------------|--------|-------------|--------|----------|--------|
|                     |             |                                               |              | Air-Silica    |        | MSCS-Air |        | MSCS-Iraqi sand |        | MSCS-Silica |        | MSCS-Air |        | MSCS-Silica |        | MSCS-Air    |        | MSCS-Silica |        | MSCS-Air |        |
|                     |             |                                               |              | p value       | Fold Δ | p value  | Fold Δ | p value         | Fold Δ | p value     | Fold Δ | p value  | Fold Δ | p value     | Fold Δ | p value     | Fold Δ | p value     | Fold Δ | p value  | Fold Δ |
| NP_997469.1         | CHIA        | Acidic mammalian chitinase                    | AMT          |               |        | 0.001    | 2.980  | <0.001          | 3.490  | <0.001      | 4.110  |          |        | <0.001      | 4.110  |             |        | <0.001      | 4.110  |          |        |
| NP_997469.1         | CHIA        | Acidic mammalian chitinase                    | Top3         |               |        | 0.001    | 5.484  | <0.001          | 6.522  | <0.001      | 7.642  |          |        | 0.002       | 7.642  |             |        | 0.002       | 7.642  |          |        |
| NP_114178.1         | Aldh3a1     | Aldehyde dehydrogenase                        | AMT          |               |        | <0.001   | 2.420  | 0.003           | 2.280  | 0.005       | 1.830  |          |        | 0.005       | 1.830  |             |        | 0.005       | 1.830  |          |        |
| NP_071964.2         | Serpina1    | α-1 antiproteinase                            | AMT          | 0.007         | -2.110 |          |        |                 |        |             |        |          |        |             |        |             |        |             |        |          |        |
| NP_037036.1         | Anxa1       | Annexin A1                                    | AMT          |               |        | 0.010    | 2.200  |                 |        |             |        |          |        |             |        | 0.040       | -1.760 |             |        |          |        |
| NP_037036.1         | Anxa2       | Annexin A1                                    | Top3         | 0.008         | 2.122  | 0.042    | 5.416  |                 |        |             |        |          |        |             |        |             |        |             |        |          |        |
| NP_757376.2         | C2          | Complement C2                                 | Top3         | 0.007         | -3.075 |          |        | 0.002           | -4.556 |             |        |          |        |             |        |             |        |             |        |          |        |
| NP_036709.1         | Gstp1       | Glutathione S-transferase Pi                  | AMT          |               |        | 0.010    | 2.080  |                 |        |             |        |          |        |             |        | 0.040       | -1.670 |             |        |          |        |
| NP_476455.1         | Prdx1       | Peroxisedoxin 1                               | Top3         |               |        | 0.009    | 2.439  |                 |        |             |        |          |        |             |        |             |        |             |        |          |        |
| NP_446062.1         | Prdx5       | Peroxisedoxin 5                               | Top3         | 0.004         | 2.191  | <0.001   | 4.256  |                 |        |             |        |          |        | 0.007       | 2.625  |             |        | 0.009       | -1.621 |          |        |
| NP_742028.1         | PLUNC       | Palate, lung, and nasal epithelium associated | AMT          |               |        | 0.030    | -2.640 | 0.004           | -3.530 | 0.005       | -4.060 |          |        | 0.005       | -4.060 |             |        | 0.030       | -1.540 |          |        |
| NP_445937.1         | S100a6      | S100 calcium binding protein A6               | Top3         | 0.002         | 2.241  | 0.044    | 1.792  |                 |        |             |        |          |        |             |        |             |        |             |        |          |        |
| NP_072130.1         | Sec14l3     | SEC14 like protein 3                          | AMT          |               |        |          |        |                 |        |             |        |          |        | 0.005       | -1.900 |             |        |             |        |          |        |
| NP_872280.1         | Serpina3l   | Serine protease inhibitor                     | Top3         |               |        |          |        | 0.008           | -3.539 |             |        |          |        |             |        |             |        |             |        |          |        |

No protein abundances were significantly different between the Air-Iraqi sand and Air-Air samples or the MSCS-Iraqi sand and MSCS-Air samples. Analysis performed on samples collected one day after the end of the air, sand, or silica exposure ended.

troops deployed to Saudi Arabia during Operation Desert Shield. These investigators reported that a significant number of men reported developing sore throat (34.4%), cough (43.1%), and persistent rhinorrhea (15.4%) when deployed. An examination of the local soil revealed that only a small fraction (0.21%) of the dust (sand) found in Saudi Arabia was respirable (<10 μm). A prospective study of military personnel deployed to Iraq and Afghanistan reported that deployed troops had a higher rate of newly reported respiratory symptoms than nondeployers (14% versus 10%), independently of smoking status (Smith et al., 2009). Respiratory symptoms were more frequent with land-based deployment as compared with sea-based deployment, suggesting that exposures to environmental influences (e.g. sand inhalation) and other factors related to ground combat might be important in the etiology of these symptoms.

To characterize the respiratory toxicity of inhaled IS and determine whether cigarette smoking exacerbates IS particle-induced lung disease, laboratory animal inhalation studies were conducted using an inhalation exposure system that would more closely mimic environmental exposures to IS PM than any current protocols. Using this system, a short-term inhalation exposure of young male rats to IS or control silica PM was performed. Animals were pre-exposed nose-only to MSCS or air for 4 weeks prior to and continued during the 2 weeks of near-continuous whole-body IS or control silica PM exposure. The animal bioassay data were used to identify and characterize physiologic, biochemical and structural biomarkers of pulmonary disease or distress in rats and correlate these results with known human epidemiological data on US personnel deployed to the Middle East.

The “grab sample” of sand used in this study was collected from the top soil layer (~10 mm) at a relatively undisturbed area near Camp Victory. This sample is thought to be representative of airborne material that U.S. troops are exposed to at that base. Ambient air monitoring in Iraq has revealed an appreciable quantity of material in the PM<sub>2.5</sub> fraction (Engelbrecht et al., 2009) and is reflected by the sand aerosol used in this study (MMAD = 1.7 μm). The sand samples were gamma irradiated prior to use to eliminate the potential for clinical effects arising from microbiological contaminants present in the sand material. *Asperigillus niger*, *Chrysosporium spp*, *Cryptococcus albiolus*, *Acremonium spp*, and other organisms have been isolated from IS (MB Lyles, personal communication). Nominal IS aerosol concentrations used in the present study (1 mg/m<sup>3</sup>) were intended to mimic exposure concentrations reported during deployment at Camp Victory. For example, PM<sub>10</sub> sampling information obtained from Camp Victory from late 2003 to mid 2005 gave an average of 411 μg/m<sup>3</sup> (USACHPPM, 2007).

The present experiment used pre-exposure to MSCS to induce lung pathology prior to IS or silica exposure. Pre-existing lung disease can exacerbate an individual's response to PM or other air pollutants. For example, individuals with chronic inflammatory lung diseases, such as

asthma and chronic obstructive pulmonary disease are susceptible to the adverse effects of traffic-related PM (Kim et al., 2004; Sint et al., 2008). Epidemiological data suggest that ambient particulate air pollution is associated with greater excess mortality in male smokers compared with never-smokers (Wong et al., 2007). Cigarette smoking has been shown to be a risk factor in Japanese men (Miki et al., 2003; Shintani et al., 2000), individuals exposed to environmental agents (firework smoke) (Hirai et al., 2000), and troops deployed to the Middle East that develop acute eosinophilic pneumonia (Shorr et al., 2004). Pre-exposure to MSCS also increases the pulmonary toxicity of ozone and possibly other air pollutants (Bhalla, 2002; Yu et al., 2002).

A number of metals including calcium, silicon, iron, aluminum, magnesium, potassium, nickel, manganese, vanadium, and chromium were detected in the Iraqi dust sample used in our experiment. Similar analytical chemical results have been reported elsewhere (Perdue et al., 1992). There is growing evidence that the soluble metal component of atmospheric dusts may be responsible for dust-induced pulmonary injury (Chen & Lippmann, 2009). For example, intratracheal instillation of the water soluble component of residual oil fly ash (ROFA) can result in pulmonary inflammation (Dreher et al., 1997) and lung responses vary depending upon the metal content of the ROFA sample (Dreher et al., 1997; Gavett et al., 1997; Kodavanti et al., 1999). IS iron was approximately 50% higher than those reported in acid digested ROFA particles, whereas nickel (Ni) and vanadium (V) were approximately 200- and 500-fold lower, respectively (Costa & Dreher, 1997). Pulmonary toxicity has also been reported with Canadian dusts found in ambient air samples (Adamson et al., 2004; Prieditis & Adamson, 2002). In our study, elevated lung parenchyma aluminum, silica, barium, manganese, and vanadium concentrations were seen in sand-exposed animals when compared with air controls, suggesting that several of the metals present in IS are bioavailable. For example, lung manganese and vanadium concentrations were approximately 2- and 10-fold higher, respectively, in IS exposed animals versus air-exposed controls.

Despite the presence of these metals, the findings of our study indicate that near continuous 2-week exposure of animals to an IS sample collected from Camp Victory did not result in alterations in body weight gain or motor activity, impaired pulmonary function, or airway pathology as assessed using analysis of BALF and airway histopathology. As noted earlier, rats exposed to IS only had infrequent minimal changes in the anterior nose and lung. This minimal toxicological response was also reflected in our lung gene expression and proteomics studies. Two transcribed locus did show decreased expression following IS exposure. While neither probe maps to a rat gene of known toxicological relevance, one targets an expressed sequence tag (EST) with similarity to the Mouse natural killer tumor recognition (Nktr) gene (BLAST score E = 10<sup>-134</sup>) (The rat Nktr gene has yet to be identified, but analysis of synteny suggest that it should

be located in an unsequenced region of chromosome 8). If rat Nktr is indeed being differentially expressed, this might represent an IL-2 mediated effect of PM exposure. This probe set is differentially expressed in all PM exposed treatment groups albeit all show increased expression with the exception of Air-IS treatment group.

Short-term MSCS inhalation with or without co-exposure to either IS or silica resulted in reduced body weight, decreased motor activity, increased relative BALF neutrophil counts, enhanced airway reactivity, nasal and lung inflammation and pathology, lung gene expression changes consistent with pulmonary inflammation and stress response/detoxification, and proteomic changes indicative of an IL-13 response. Several of these effects have been previously noted in rats (Bergren, 2001; Cooper et al., 2010; Friedrichs et al., 2006; Stevenson et al., 2007). Co-exposure to MSCS and IS resulted in a significant up-regulation of only three genes and no proteins when compared with rats exposed to MSCS alone, whereas three proteins were downregulated when compared with rats exposed to Air-Air. The genes, RT1-CE16 and RT1-A2 are within the rat major histocompatibility complex (MHC) class I receptor and MHC class I RT1 families, respectively. These genes are associated with the A allele of the transporter associated with antigen processing (TAP-A), which can transport peptides with basic carboxy-terminal residues. The third gene that was up-regulated in MSCS and IS-exposed rats is an EST with similarity to Tial1, a regulator of G-protein signaling.

In our study, inhalation exposure to crystalline silica was associated with some pulmonary responses that were not seen in IS-exposed rats. Overall, however, responses seen in our study were milder than those reported by others (Castranova et al., 2002; Oberdörster, 1996; Sayes et al., 2007) and may reflect exposure dose and study duration, choice of animal model, or other factors. Animals exposed to silica in our experiment developed mild laryngeal, tracheal inflammation, mild tracheal epithelial hyperplasia, and elevated lung silica concentrations. Lung silica concentrations seen in the silica-exposed rats were approximately double those seen in animals exposed to IS. Certain histopathologic responses were exacerbated by MSCS co-exposure. Rats exposed to MSCS and silica had squamous metaplasia of the transitional/respiratory epithelium at Level I and nasopharyngeal duct at Level IV, suggesting that this lesion was more widespread in these animals when compared with rats exposed to MSCS only.

Silica-exposed rats also had a slightly more robust pulmonary gene expression response than that seen with rats exposed to IS. One change in gene expression of note was a modest (2.44-fold) increase in the differential expression of cyclin D2. The protein encoded by this gene belongs to the highly conserved cyclin family, whose member's functions as regulators of cyclin-dependent kinases and have been previously shown to be affected by silica exposure (Shen et al., 2006, 2008). Exposure to silica, with or without MSCS, also resulted

in a 3 to 4-fold increase in the expression of a gene encoding Cirbp, a rat cold inducible RNA binding protein. Cold inducible RNA binding proteins have been shown to be induced after exposure to a moderate cold-shock and other cellular stresses such as UV radiation or hypoxia (Leonart, 2010). Initially, it was suggested that these proteins have a suppressive rather stimulatory effect on proliferation; however, proliferative and/or proto-oncogenic functions have recently been assigned to CIRP (Leonart, 2010). Animals exposed to silica also had a marginal (2-fold) increase in the transcript encoding ZFP91 (zinc finger protein 91) which may act as a transcription factor and also plays a role in cell proliferation or anti-apoptosis. Exposure to MSCS and silica also resulted in a significant up-regulation of several array probes when compared with rats exposed to MSCS alone. A modest increase (~3-fold) increase in the expression of DHX36 was seen in rats co-exposed to MSCS and silica when compared to animals exposed to MSCS only. The protein encoded by this gene has been shown to enhance the de-adenylation and decay of mRNAs with 3'-UTR AU-rich elements (ARE-mRNA) (Iwamoto et al., 2008). The protein has also been shown to resolve into single strands, the highly stable tetramolecular DNA configuration (G4) that can form spontaneously in guanine-rich regions of DNA (Vaughn et al., 2005). Two proteins, CHIA and PLUNC, known to be regulated by IL-13 (Zhu et al., 2004; Chu et al., 2007), showed increased regulation with co-exposure to MSCS and PM versus MSCS alone, though the changes were only significant for one protein, PLUNC, and then only in the MSCS-Silica treatment (when compared with animals exposed to MSCS alone). This suggests that the presence of silica, and perhaps to a lesser extent IS, may increase the IL-13 response triggered by cigarette smoke. Glutathione S-transferase Pi, which was up-regulated in response to MSCS exposure, had a significantly lower abundance in samples from MSCS and silica co-exposures. While the mechanism of this effect is unclear, it is notable that silica may be repressing the detoxification of cigarette smoke achieved by the glutathione S-transferase.

In conclusion, our studies show that the respiratory toxicity of IS is qualitatively similar to or less than that seen following crystalline silica exposure. Future work is currently underway in our laboratories to investigate the respiratory toxicity of sand collected from other Middle Eastern regions and the role soluble metals found in these samples play in respiratory effects. It remains unknown whether longer-term exposure to the concentrations used in our study may be associated with pulmonary toxicity.

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## Declarations of interest

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