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# Biological effects of inhaled hydraulic fracturing sand dust. IX. Summary and significance

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ABSTRACT

An investigation into the potential toxicological effects of fracking sand dust (FSD), collected from unconventional gas drilling sites, has been undertaken, along with characterization of their chemical and biophysical properties. Using intratracheal instillation of nine FSDs in rats and a whole body 4-d inhalation model for one of the FSDs, *i.e.*, FSD 8, and related *in vivo* and *in vitro* experiments, the effects of nine FSDs on the respiratory, cardiovascular and immune systems, brain and kidney were reported in the preceding eight tandem papers. Here, a summary is given of the key observations made in the organ systems reported in the individual studies. The major finding that inhaled FSD 8 elicits responses in extra-pulmonary organ systems is unexpected, as is the observation that the pulmonary effects of inhaled FSD 8 are attenuated relative to forms of crystalline silica more frequently used in animal studies, *i.e.*, MIN-U-SIL® 5. An attempt is made to understand the basis for the extra-pulmonary toxicity and comparatively attenuated pulmonary toxicity of FSD 8.

## 1. Introduction

Inhalation of crystalline α-quartz (silica) dust in many types of workplaces is well known to cause silicosis, kidney disease, autoimmune disease, lung cancer and increased susceptibility to tuberculosis. The development of pulmonary fibrosis and cancer following silica inhalation have been much studied and well-described, and the mechanisms leading to these conditions have been investigated using animal models and related *in vivo* and *in vitro* experiments. Ordinarily, such studies are performed using crystalline silica of high purity. Previous measurements of respirable dust at unconventional oil and gas well drilling sites during hydraulic fracturing operations indicated that some workers were exposed to levels of silica dust that exceeded established workplace exposure limits (see Fedan (2020) for review). Importantly, fracking sand is a type of industrial sand and some industrial sand-exposed worker populations are at an increased risk for silicosis and lung cancer (Hughes et al., 2001; McDonald et al., 2005; Steenland and Sanderson, 2001; Rando et al., 2018; Vacek et al., 2019). With the emergence of hydraulic fracturing as a mainstay method for oil and gas retrieval, and a rise in the number of workers potentially exposed to dust generated during the handling of sand used in the formulation of fracking fluid, the question arose as to whether the toxicity of inhaled fracking sand dust (FSD) resembles that observed after inhalation of crystalline silica.

The previous eight papers in this series (Fedan et al., 2020; Olgun et al., 2020; Russ et al., 2020; Sager et al., 2020; Krajnak et al., 2020; Sriram et al., 2020; Anderson et al., 2020) described chemical and biophysical properties of nine FSDs, their pulmonary effects after intratracheal instillation, and the effects of inhaled FSD 8 in the pulmonary, cardiovascular and immune systems, brain and kidney. The main findings made in the preceding studies are highlighted in this report. The reader is directed to the individual investigations for full descriptions of the results.

A second purpose of this report is an attempt to reconcile the finding that, at least in the lungs where comparisons may be drawn, the profile of many responses to inhaled FSD in the rat model differ appreciably from those that have been established for inhalation of reference crystalline silica, *i.e.*, MIN-U-SIL® 5 (MIN-U-SIL). That is, the toxicity of inhaled crystalline silica reported in the literature and inhaled FSD 8 are not equivalent at the post-exposure time points investigated, with FSD 8 being milder in its pulmonary effects.

## 2. Methods

The reader is referred to the individual reports for detailed descriptions of the methods used in the seven individual investigations. Male rats were exposed to FSDs by intratracheal instillation or by inhalation; following exposure, a panel of *in vivo* and *in vitro* experiments were performed, as will be described below.

## 3. Summary results and discussion of major findings in the individual investigations

### 3.1. Study I. Scope of the investigation (Fedan, 2020)

Fedan (2020) described the purpose of the overall investigation, which yielded new information about the biological effects of inhaled FSD on several organ systems (see below). The paper also provided a brief review of the literature related to silica dust and fracking sand exposure, which was the impetus to the overall investigation.

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### 3.2. Study II. Particle characterization and pulmonary effects following intratracheal instillation (Fedan et al., 2020)

Nine FSDs collected from drilling sites at several locations in the US were analyzed for their chemical and biophysical properties and their effects one month after intratracheal instillation. Their properties were compared to those of respirable crystalline silica, MIN-U-SIL.

#### 3.2.1. Endotoxin analysis

An analysis of this pro-inflammatory substance revealed that the FSDs contained levels of endotoxin that were below those known to contribute to an inflammatory response.

#### 3.2.2. Scanning electron microscope analysis of FSDs and MIN-U-SIL

The nine neat FSD samples contained angular particles covering a range of sizes, 0.1 to 50 μm. MIN-U-SIL particles were more uniform size ($\leq$ 5 μm).

#### 3.2.3. Dynamic light scattering

Dynamic light scattering was employed to semi-quantitatively characterize the hydrodynamic size distributions of the nine neat FSDs compared to MIN-U-SIL. Mono-modal and bi-modal spectra for size and number were observed among the nine FSDs and MIN-U-SIL. Spectra were generally not concordant among FSDs or with MIN-U-SIL.

#### 3.2.4. X-ray diffraction analysis

The α-quartz content in each FSD sample was assessed and observed to be different among the nine FSDs, with the highest-level present in FSD 8. Cristobalite and tridymite were not detected in any of the FSDs.

#### 3.2.5. Energy dispersive analyses (EDS)

EDS mapping was performed to compare surface elements present in the nine FSDs. Mapping indicated that the surface elemental compositions of the nine FSDs were comprised of many elements that were not uniformly distributed on a given particle. The EDS composite maps were different for the FSDs. Surface regions showing only silica spectra also were noted.

#### 3.2.6. Mineral analysis

Analysis indicated that numerous minerals were associated with FSD particles and MIN-U-SIL. The mineral compositions of the FSDs were different in terms of element identify and abundance, and collectively, differed from MIN-U-SIL. The range of total mineral content was from 8.2 to 32.4 mg/kg.

#### 3.2.7. Electron paramagnetic resonance (EPR) spectroscopy

The EPR spectra indicated the presence of the Si radical signal in MIN-U-SIL but not in the neat FSDs. The signal was "masked" by the minerals present in the dusts. A small Si radical signal appeared after washing FSD 8, but not the others.

#### 3.2.8. Inflammatory responses toxicity end points

*3.2.8.1. Bronchoalveolar lavage markers and cell differentials.* To obtain preliminary results comparing the possible pulmonary toxicity of the nine FSDs, and of the FSD group compared to MIN-U-SIL, 159 μg/rat or 500 μg/rat doses of FSDs or MIN-U-SIL were administered by intratracheal (i.t.) instillation. Thirty days post-exposure, the total numbers of cells, PMNs, alveolar macrophages, and lactate dehydrogenase (LDH) levels in bronchoalveolar lavage, were unaffected by the FSDs. MIN-U-SIL elevated these parameters in a dose-dependent manner.

*3.2.8.2. Lung histopathology.* Examined 30 d post-treatment, histiocytic and neutrophilic alveolitis were found in rats receiving i.t. instillation of 500 μg of MIN-U-SIL and was accompanied by minimal to mild fibrosis. In contrast, histiocytic and neutrophilic alveolitis was limited to one rat in each of four different FSD exposure groups. Alveolar septal fibrosis was identified in 15 of the FSD-exposed rats (9 exposed to 500 μg and 6 exposed to 159 μg). In both MIN-U-SIL- and FSD-exposed rats, the alveolar septal fibrosis was extremely subtle and unlikely to be functionally significant. The lungs from MIN-U-SIL- and FSD-exposed rats in this study had a notable absence of multiple and sometimes coalescing classic silicotic granulomas with prominent collagen fibers (fibrosis) that have been seen in most previous silica instillation studies in rats, albeit at higher doses than those used in this intratracheal instillation study.

### 3.3. Study III. Cytotoxicity and pro-inflammatory responses in murine macrophages (Olgun et al., 2020)

The purpose of this study was to investigate the effects of soluble and non-soluble components in FSD 8 for their ability to promote pro-inflammatory responses in cultured murine macrophages (RAW 264.7 cells) following a 4 h or 24 h incubation with 1 or 5 mg/ml FSD 8.

#### 3.3.1. Active bacterial growth not a likely source of observed inflammatory response

Inasmuch as fracking sand and FSDs are exposed to environmental conditions, the presence of microbial species present in FSD 8 before and after washing in PBS was examined. Bacterial, but not fungal, rDNA was visualized using agarose gel electrophoresis. To characterize the bacterial composition of the FSD 8, the amplified DNA was sequenced to taxonomically identify the bacteria and determine if there were species unique to the FSD. Twenty species were identified, six of which followed extraction in PBS. Four species were unique to the washed FSD 8 but did not appear in great abundance to suggest active growth.

#### 3.3.2. Cellular viability

FSD 8 particles reduced cell viability following 24 h incubation with the macrophages.

#### 3.3.3. Electron spin resonance (ESR)

ESR analysis performed to evaluate the presence of free radicals indicated that .OH radicals were detected in an acellular system using Fenton-like reactions with suspensions of FSD 8.

#### 3.3.4. Intracellular reactive oxygen species (ROS)

Soluble and insoluble components of FSD 8 stimulated intracellular ROS levels following a 4 h incubation.

#### 3.3.5. DNA damage: comet assay

An increase in the percentage of DNA in comet tails was observed following incubation with FSD 8, indicative of damage to DNA.

#### 3.3.6. Cytokine production

TNFα, IL-6 and endothelin-1 responses were elicited by FSD 8 particle suspensions. TNFα responses were associated with cell blebbing.

#### 3.3.7. Enhanced dark-field microscopy (EDM)

EDM indicated that FSD 8 particles were engulfed in macrophages following incubation with the cells.

### 3.4. Study IV. Pulmonary ventilatory and non-ventilatory effects (Russ et al., 2020)

The purpose of this study was to investigate the effects of inhaled FSD 8 on the ventilatory and non-ventilatory functions in lung and airways *in vivo* and *in vitro*, with emphasis on organ and cell function.

#### 3.4.1. Inhalation exposures

The FSD 8 whole-body inhalation exposure system and chemical

characteristics of FSD were described in this paper. Male rats were exposed to filtered air or to FSD 8 aerosol at either 10 or 30 mg/m$^3$ for 6 h/d for four consecutive days or a single exposure for some experiments. [Female rats were not included in the studies. Approximately 19.1% of oil and gas extraction workers in all job categories are women (U.S. Department of Labor, 2019); however, NIOSH field staff report women are rarely found at drilling rigs, the site of potential FSD exposures.] Post-exposure end-points were examined at days 1, 7 and 27 in papers four through eight and, in one series of experiments, at 90 d post-exposure.

*3.4.2. Particle size characterization*

Neat FSD 8 particles ranged in physical size (< 0.5 μm to >10 μm); 0.5–1 μm particles were the most prevalent. In the inhalation exposure chamber the mass median aerodynamic particle diameter of FSD 8 was 1.75 ± 2.4 μm. The count median aerodynamic particle diameter of FSD 8 was 227.0 ± 1.7 nm. The particles were in the respirable range.

FSD exposure dose calculations are given in this report.

*3.4.3. Lung burden of FSD 8 and clearance from the lung*

Enhanced dark-field microscopic imaging of sections from lung tissue at 1, 7, 27, and 90 d post-exposure to a single 6-h exposure to 10 or 30 mg/m$^3$ FSD 8 was used to calculate the half-life ($t_{1/2}$) of the particles in the lungs. FSD 8 was observed in the lungs at 1 d post-exposure. Later, FSD 8 was found within alveolar macrophages. FSD 8 clearance from the lungs occurred over a calculated clearance $t_{1/2}$ of 11.3 d. After a single exposure to 30 mg/m$^3$ FSD 8, no dust was measurable gravimetrically in the lungs of rats 0 h and 1 d post-exposure.

*3.4.4. Tracheal particle deposition in vivo*

Particle deposition in the trachea was assessed, inasmuch as tracheal epithelial ion transport was affected by FSD 8 exposure (see below). Particles were relatively few but were present in clusters or as isolated particles 1 d after a single exposure to 30 mg/m$^3$ FSD 8 but were rarely observed at 7 and 27 days.

*3.4.5. Lung mechanics in vivo*

Respiratory system resistance and elasticity, tissue damping, tissue elasticity, Newtonian resistance and hysteresivity were unaffected by FSD 8 inhalation.

*3.4.6. Airway reactivity to methacholine in vivo*

Airway reactivity to inhaled methacholine (MCh) was increased modestly 7 d after inhalation of 10 mg/m$^3$ FSD (lung compliance) and 7 d after inhalation of 30 mg/m$^3$ (lung resistance), following 4-d exposures.

*3.4.7. Airway epithelium integrity in vitro*

In the isolated, perfused trachea, the modulatory role of the airway epithelium on reactivity of the smooth muscle to MCh was heightened (10 mg/m$^3$, 7 d after 4 d of exposure) and lessened (30 mg/m$^3$, 1 d after 4 d of exposure), signifying that the epithelium's effect on reactivity was increased and decreased, respectively, depending on FSD 8 dose and post-exposure period. Whether these changes resulted from alterations in diffusion barrier effectiveness and/or variations in the release of epithelium-derived relaxing factor is not known.

*3.4.8. Airway efferent motor nerve activity in vitro*

Neurogenic contractile responses of the smooth muscle in isolated tracheal strips were unaffected by FSD 8 exposure, indicating that neural control of airway smooth muscle was not affected.

*3.4.9. Vascular permeability in the lung in vivo*

Vascular permeability of the lung was not affected by FSD 8 exposure, as indicated by the lack of effect of the dust on permeability to Evans blue dye before and after the administration of capsaicin, which releases substance P from sensory nerves.

*3.4.10. Epithelial ion transport in tracheal segments in vitro*

Whereas 10 mg/m$^3$ FSD (4 d of exposure) had no effect, the inhibitory effect of amiloride on $Na^+$ transport in epithelium of tracheal segments removed from rats was decreased by 30 mg/m$^3$ FSD 8 (4 d of exposure) at all post-exposure time points. $Cl^-$ transport, on the other hand, was not affected. FSD 8 inhalation, therefore, disrupted epithelial ion transport *in vivo*.

*3.4.11. Epithelial ion transport in primary normal human bronchial epithelial cells (NHBE) cultured in air interface*

Incubation of NHBE with FSD 8 had no effect on epithelial $Na^+$ or $Cl^-$ transport *in vitro*.

*3.4.12. LDH and cytokine release from NHBE*

Incubation of NHBE with FSD 8 did not stimulate apical LDH release. FSD 8 did cause release of cytokines, but this was not robust.

*3.5. Study V. Pulmonary inflammatory, cytotoxic and oxidant effects (Sager et al., 2020)*

The purpose of this study was to ascertain whether inhalation of FSD 8 stimulated an inflammatory response in rat lungs. Analyses of bronchoalveolar lavage components, examination of histopathological changes in the lung, and changes in global gene expression profiles, were conducted.

*3.5.1. Detection of FSD 8 particles in alveolar macrophages*

Black/brown particles representing inhaled FSD 8 particles were detected in the AMs of lung samples collected from all the FSD-exposed rats at all the post-exposure time intervals analyzed.

*3.5.2. Bronchoalveolar lavage (BAL) parameters of pulmonary toxicity*

Elevation in LDH levels in recovered BAL fluid following FSD 8 exposure was not observed.

The number of total BAL cells was not affected by FSD 8 exposure. A small increase in the number of PMNs in the BAL fluid occurred at some post-exposure periods.

Cytokine levels in the BAL fluid overall were not affected by FSD 8 inhalation.

There were no effects in the PMA- or zymosan-stimulated generation of chemiluminescence by the phagocytes present in the BAL fluid after FSD 8 exposure.

*3.5.3. Lung histopathology*

Following exposure to 10 mg/m$^3$ of FSD 8, alveolar macrophage numbers increased at post-exposure days 1 and 7. By day 27, there were no changes in the lung associated with injury or inflammation. Thus, macrophage responses were modest.

*3.5.4. Gene expression profiles in lungs*

No notable changes in global gene expression profiles were detected in the lungs of the majority of the FSD 8-exposed rats. This is in contrast to statistically significant changes in hundreds of genes reported in the lungs of rats in response to inhalation exposure to MIN-U-SIL (Sellamuthu et al., 2013).

*3.6. Study VI. Cardiovascular effects (Krajnak et al., 2020)*

The purpose of this study was to assess the effects of FSD 8 inhalation on factors and tissues that affect cardiovascular function *in vivo* and peripheral vascular function *in vitro*.

*3.6.1. Microvessel reactivity to phenylephrine and acetylcholine*

In ventral tail arteries *in vitro*, sensitivity to phenylephrine, a vascular

smooth muscle contractile agonist, was increased 1 and 7 d post-exposure to FSD 8, with 10 mg/m$^3$ FSD having a greater effect than 30 mg/m$^3$ FSD; the effect abated by 27 d. Endothelial-derived, nitric oxide-mediated relaxation responses to acetylcholine were unaffected by FSD exposure.

#### 3.6.2. Reactive oxygen species ($N_{ox}$ and $H_2O_2$)

In the heart $N_{ox}$ concentrations were not affected after exposure to 10 mg/m$^3$ FSD 8. However, $H_2O_2$ concentrations were reduced 7 and 27 d after exposure.

FSD 8 did not affect $N_{ox}$ or $H_2O_2$ concentrations in the kidneys following inhalation of 10 mg/m$^3$, but $N_{ox}$ levels were lowered after 30 mg/m$^3$ FSD 8 inhalation. $H_2O_2$ concentrations in the kidneys were unaffected by 30 mg/m$^3$ FSD.

#### 3.6.3. Cardiac function (telemetric and in vivo measurements)

Telemetric measurements indicated that the high frequency heart rate variability was increased transiently by 10 mg/m$^3$ FSD 8 inhalation 1 d post-exposure.

Diastolic but not systolic blood pressure was elevated 7 d after 4 d exposure to 30 mg/m$^3$ FSD 8.

Responses to dobutamine ($\beta_1$-adrenoreceptor agonist)-induced increases in work/stroke, cardiac output and stroke volume were reduced 1 d after exposure to 10 mg/m$^3$ FSD 8.

One day after exposure, norepinephrine (α- and β-adrenoceptor agonist)-induced increases in systolic and diastolic blood pressures were reduced by inhalation of 10 mg/m$^3$ FSD 8.

#### 3.6.4. Transcript levels

FSD 8 (10 mg/m$^3$) increased *Il-1β* and *Il-6* levels in the heart 1 d after exposure and *Il-6* levels 27 d after 30 mg/m$^3$ FSD 8 exposure.

#### 3.6.5. Protein array analysis

FSD 8 (30 mg/m$^3$) exposure did not alter the concentrations of measured proteins in the heart at any post-exposure time point. There were reductions in OPN, IP10 and VEGF in the kidneys of rats exposed to 30 mg/m$^3$ FSD 8, 7 d after the exposure, and a reduction in GST 27 d after FSD 8 exposure.

### 3.7. Study VII. Neuroinflammation and altered synaptic protein expression (Sriram et al., 2020)

The purpose of this investigation was to evaluate the potential neurotoxicological effects of inhaled FSD 8.

#### 3.7.1. Brain-region specific neuroinflammation

Neuroinflammatory responses in the brain were examined using low-density inflammatory gene expression assays for proinflammatory mediators. Brain region-specific responses were observed. The low dose of FSD 8 (10 mg/m$^3$) caused an early induction of *Alox5*, *Ptgs1* and *Ptgs2* mRNAs, key intermediaries in the arachidonic acid pathway, in the olfactory bulb (OB), hippocampus (HIP) and cerebellum (CER) by 1 or 7 d post-exposure. On the other hand, the expression of *Il6*, *Tnfa* or *Nos2* mRNAs in these regions exhibited a delayed response, increasing only at 27 d after FSD 8 (10 mg/m$^3$) exposure. In general, neuroinflammation was elicited by the lower dose of FSD 8, while at the higher dose (30 mg/m$^3$) there was lack of an inflammatory response and, in some cases, even an inhibition/down-regulation of the response.

#### 3.7.2. Expression of blood-brain barrier (BBB)-associated markers

Expression of markers associated with the BBB were examined using low-density inflammatory gene expression assays for proinflammatory mediators. Brain region-specific changes in the mRNA expression of *Mmp9*, *Cldn1* and *Cldn3*, markers of BBB integrity and vascular blood flow, were observed. In the HIP, only the low dose of FSD 8 (10 mg/m$^3$) caused up-regulation of *Mmp9* and *Cldn1* by 27 d post-exposure, while down-regulating *Cldn3* at 7 d and 27 d post-exposure. In the CER, both 10 mg/m$^3$ and 30 mg/m$^3$ FSD 8 caused down-regulation of *Cldn1* by 1 d after cessation of exposure. Up- or down-regulation of such markers are linked to disruption of the BBB.

#### 3.7.3. Biogenic amine neurotransmitters

Following FSD 8 exposure, brain region-specific changes in the neurotransmitters norepinephrine (NE), epinephrine (EPI), dopamine (DA) and serotonin (5-HT) were observed. Levels of NE, EPI, DA and 5-HT were increased in HIP and striatum (STR), 7 d following exposure to 10 mg/m$^3$ FSD 8 but not 30 mg/m$^3$ FSD 8. Abnormality or dysregulation of the physiological levels of neurotransmitters are linked to neuronal dysfunction.

#### 3.7.4. Expression of neuronal synaptic proteins

Neuronal synaptic proteins are involved in transport and release of neurotransmitters. Following exposure to the high dose of FSD 8 (30 mg/m$^3$), a persistent reduction of synaptophysin 1 (SYP) and synaptotagmin 1 (SYT1) proteins occurred in the CER at 7, 27 and 90 d post-exposure, suggestive of synaptic disruption and/or injury.

#### 3.7.5. Astroglial activation

Changes in the expression of the astroglial marker, glial fibrillary acidic protein (GFAP), was assessed. The high dose of FSD 8 (30 mg/m$^3$) caused a robust increase in GFAP in the CER by 7 d post-exposure, indicative of neuronal perturbation or injury.

#### 3.7.6. Expression of the oligodendroglia marker, myelin basic protein

Alterations in the expression of the oligodendroglial marker, myelin basic protein (MBP), was evaluated. The high dose of FSD 8 (30 mg/m$^3$) caused up-regulation of MBP protein in the HIP by 7 d post-exposure, while reducing MBP levels in the CER and OB by 7 or 27 d post-exposure, suggesting the association of oligodendroglia with neuronal injury.

### 3.8. Study VIII. Immunotoxicity (Anderson et al., 2020)

The immunotoxicity of FSD 8 following inhalation exposure was investigated.

#### 3.8.1. Immunophenotyping

The immune cell subsets present in local lymph node (LLN), bronchoalveolar lavage, and spleen, along with total cellularity, were characterized using flow cytometry. Inhalation of 10 mg/m$^3$ FSD 8 caused a decrease in LLN cellularity at 7 d post-exposure and a decrease in total B-cells, CD4+ T-cells, CD8+ T-cells and total NK cells at 7 d post-exposure. Increases in LLN lymph node cellularity and increases in total CD4+ and CD8+ T-cells occurred following 30 mg/m$^3$ FSD 8 exposure at 1 d post-exposure. No change in total cellularity of the BAL fluid was observed; however, increases in the frequency and number of CD4+ T-cells and NK cells were observed at 7 d post-exposure (10 mg/m$^3$) along with an increase in total CD4+ T-cells, CD11b+ cells, and NK cells 1 d after exposure to 30 mg/m$^3$ FSD 8. Increases in the numbers of B-cells and CD8+ T-cells were observed in the spleen but only 1 day after 30 mg/m$^3$ FSD 8 exposure.

#### 3.8.2. Natural killer cells

Exposure to FSD 8 suppressed NK cell function 1 d (30 mg/m$^3$) and 27 d post-exposure (10 mg/m$^3$).

#### 3.8.3. Hematology and serum chemistries

No significant changes in serum chemistries or hematology were identified in any of the post-exposure time points at either FSD 8 inhalation dose.

*3.8.4. IgM response to sheep red blood cells (SRBC)*

The IgM response to SRBC was examined to evaluate whether exposure to FSD 8 was immunosuppressive. No statistically significant reductions in the PFC/spleen or specific (PFC/$10^6$ cells) IgM antibody activity against SRBC were observed.

## 4. FSD ≠ MIN-U-SIL

Several of the *in vivo* and *in vitro* experiments performed in this series of experiments to examine the effects of intratracheally-administered FSDs and FSD 8 inhalation have not been performed previously using MIN-U-SIL. Although it was not the purpose of these studies to compare side-by-side the effects of the two dusts, some of the effects of FSD observed in the present studies can be compared with the effects of MIN-U-SIL in the literature.

First, the chemical compositions of MIN-U-SIL and FSD dusts differ in subtle ways that affect their bioactivity. X-ray crystallography indicated that FSDs are predominantly crystalline silica as α-quartz; appreciable amounts of several other "impurities" were identified as comprising the remaining constituents. In contrast, MIN-U-SIL, often regarded as pure, crystalline silica, contains Fe, Al, Mg, Na, K, Ca and Ti in trace amounts (1.7% total). These elements, except for Ti, were identified in FSD using EDS. The small amounts of non-silica impurities in MIN-U-SIL have been generally regarded as without biological consequence. However, their presence has been shown to be sufficient to affect the bioactivity of MIN-U-SIL, as their removal with hydrofluoric acid decreased its hemolytic activity (Pavan et al., 2017). In contrast, washing MIN-U-SIL with hydrochloric acid removes surface iron contaminants and greatly increases silica toxicity as reflected in lung weight, phospholipidosis, and changes in lung protein concentration (Miles et al., 1994).

Second, an appreciable difference exists in the rates of clearance of FSD 8 and MIN-U-SIL from the lungs. Whereas FSD was cleared with a $t_{1/2}$ of ~11.3 d after a single inhalation exposure, the $t_{1/2}$ for MIN-U-SIL particles was markedly longer, *i.e.*, ~82 d, after a 20-d inhalation exposure (Porter et al., 2004). It cannot be ruled out with present data that the slower clearance of MIN-U-SIL particles is due to the longer exposure period, but this is unlikely, as lung burden overload did not occur under these exposure conditions (Porter et al., 2001). Rather, it would appear that FSD is cleared faster from the lung than MIN-U-SIL because of the chemical properties conferred on the particles attributable to the presence of greater quantities of impurities. The impurities in FSD 8 may render the dust with advantageous toxicokinetic characteristics that favor more rapid clearance to the lymph nodes compared to MIN-U-SIL particles. A major route of particle clearance from the lung is to the lymph nodes (Harmsen et al., 1985; Choi et al., 2010). Redistribution to the lymph nodes is important in workplace silica exposures. In multiple worker autopsy studies, silicosis of the lymph nodes precedes silicosis in the lung parenchyma (Cox-Ganser et al., 2009; Honma et al., 2007; Taeger et al., 2011). This lymph node damage is hypothesized to increase the risk of silicosis in the lung parenchyma by impairing silica clearance (Cox-Ganser et al., 2009). In addition, lymph node deposition of FSD may contribute to immune stimulation and potential further particle redistribution *via* the lymphatics. The potential role of lymphatic clearance in the extrapulmonary effects of FSD is an important area for future research.

Third, in a rat model of silicosis employing MIN-U-SIL, inflammatory changes in the lung increased at a rapid rate during the 36 to 116 d post-exposure interval following 40 or 60 but not 20 d of silica dust exposure (Porter et al., 2002; Langley et al., 2004). Therefore, in the present study, whether inflammatory changes were also evident in the lung 90 d following FSD inhalation was evaluated. No comparable changes were detected. In comparison to MIN-U-SIL, the greater abundance of impurities in FSD may have mitigated the inflammatory responses. However, inhalation exposure to FSD 8 was limited to 4 d.

Fourth, surface properties (Warheit et al., 2007) and the presence of surface metals in association with silica particles affect its bioactivity. After inhalation exposure of rats to FSD, no evidence of an inflammatory response was found. It is very likely that the greater abundance of metals in FSD compared to MIN-U-SIL plays a critical role in the pulmonary responses. Inasmuch as several minerals bind to FSD 8 and to MIN-U-SIL, and both dusts contain a nearly identical metal content profile (although metal percentages differ), it is not possible to ascribe one or more metals or cellular pathways to the pathways responsible for these differences. The presence of aluminum blunts the lung inflammatory response to silica (Nolan et al., 1981; Begin et al., 1986, 1987; Donaldson et al., 2001; Duffin et al., 2001). In contrast, the presence of iron has been associated with increased and decreased effects of silica particles (Schins et al., 2002; Ghiazza et al., 2011; Pavan and Fubini, 2017). In a study in which minerals in MIN-U-SIL were extracted with hydrofluoric acid to reduce metal impurities and the hemolytic activity of neat and acid-treated silica on erythrocytes were compared, the hemolytic activity of the dust was decreased (Pavan et al., 2017). Binding of metal salts or the polymer, polyvinyl-pyridine-N-oxide, to the surface of MIN-U-SIL decreased its hemolytic activity (Nolan et al., 1981). Similarly, MIN-U-SIL that was washed in HCl to remove surface iron had greatly enhanced toxicity relative to unwashed MIN-U-SIL (Miles et al., 1994). As both aluminum and iron are present in FSD, the preponderance of the overall pulmonary effects of FSD must reflect the influence and interplay of the effects of the metals. Inasmuch as the other metals in FSD have not been reported to influence toxicity appreciably in studies using MIN-U-SIL, it seems possible, at least tentatively, that effects of these two metals determine the pulmonary response to FSD. Alternatively, pathways stimulated by the other metals in FSD may participate in blunting FSDs' relative toxic effects in ways that have not yet been described. In addition, quartz with an occluded surface is less toxic than DQ12, a reference material that is 87% quartz and 13% amorphous silica but has an unoccluded surface (Creutzenberg et al., 2008; International Programme on Chemical Safety, 2000). It is also unknown how the metals in FSD influence the toxicity profile of the dust on the cardiovascular and immune systems, brain and kidney, especially in comparison with MIN-U-SIL, the effects of which on these organ systems have not been investigated.

Given the differences in the characteristics and bioactivities of FSD and MIN-U-SIL discussed above, the question arises as to whether FSD 8 is unique in its toxicological profile among other silica-containing dusts inhaled in the workplace. Chen et al. (2005) compared the risk of silicosis among tin miners, tungsten miners, and pottery workers in relation to inhaled cumulative total dust and cumulative respirable silica dust. The risk of silicosis in pottery workers was less than that of the miners, even though they experienced a higher cumulative total dust exposure; moreover, the latency for silicosis was longer in pottery workers than in the other two cohorts. The authors concluded both the characteristics of the silica dust and the cumulative exposure to respirable silica influence silicosis risk (Chen et al., 2005). A likely hypothesis to explain these findings was set forth from the investigation of Harrison et al. (2005), who proposed that the degree of surface occlusion by alumino-silicate affects the bioactivity of silica dusts. The investigators proposed that the extent of surface coating of silica particles influences toxicity. Using energy-dispersive X-ray spectroscopy (EDS) to map the extent of surface occlusion by clay coating, the dusts inhaled by pottery workers and tin and tungsten miners were found to be 45%, 18% and 13%, respectively. These findings buttress the view of these investigators that the differences in susceptibility to silicosis in the three worker cohorts may be attributable to the degree of surface occlusion by clay. EDS was performed on FSD particles, and the results indicated that surface "coating" of particles was heterogeneous, both between individual FDS particle types and within individual particles in each sample (the degree of surface occlusion was not quantified in this study). This was in contrast to MIN-U-SIL particles, which gave a single EDS spectrum irrespective of the location of the x-ray beam (although trace minerals are present), indicating that the dust particles were not surface-occluded.

There are several limitations of this study. Sands utilized for fracking

purposes are derived from many locations and they are not geologically equivalent (Benson and Wilson, 2015). Processing of the sand for use, *e.g.*, washing with water, and environmental conditions will add a variety of exogenous factors to the product that could affect its bioactivity, depending on locale. The amount and chemical nature of the "impurities," both exogenous and endogenous, would vary among sands obtained from different vendors. The findings and conclusions of this study are, therefore, not applicable to fracking sands from all sources, as the bioactivity of FSD could be linked to its geographical source. Unconventional gas extraction workers are not breathing "pure" crystalline silica dust (MIN-U-SIL) in the industrial setting, and, in light of the differences observed in this study, potential lung disease in these workers would be best modeled using sand dusts obtained at the drilling sites.

The FSDs used in this study may have some similarities to some other workplace respirable quartz samples. A previous study demonstrated that two workplace quartz samples from sandstone milling and sand feeder stock were less inflammatory, less cytotoxic and had less surface reactivity than DQ12 silica (Clouter et al., 2001). Comparing the physicochemical properties of other types of industrial sand dusts with the physiochemical properties of fracking sand dust is important because workers exposed to industrial sand have increased risk for silicosis and lung cancer (Hughes et al., 2001; McDonald et al., 2005; Steenland and Sanderson, 2001; Rando et al., 2018; Vacek et al., 2019). Recently, industrial sand workers exposed to low concentrations of industrial sand for long time periods were found to have a greater risk for silicosis than workers with comparable cumulative exposures received at a high dose over a shorter time period (Vacek et al., 2019). It is certainly possible that the physico-chemical properties of FSD and similar sand dusts change the timing of disease onset relative to MIN-U-SIL. Workers are not exposed to MIN-U-SIL and most cases of silicosis in workers develop over decades, a process that cannot be replicated in short-lived rodents. However, a more rapidly progressive form of pneumoconiosis has recently been reported under some mining conditions and crystalline silica is implicated in this process (Cohen et al., 2016). In short, humans get silicosis from many different workplace silica exposures and it is important to study factors that enhance the risk or facilitate rapid disease progression. Our study may provide clues regarding some of those potential factors.

Another limitation of these studies is the fact that the FSD used in these experiments was "aged." By this it is meant that silica that has undergone fracturing (*i.e.*, "freshly fractured"), such as during preparation of hydraulic fracturing fluid, gives rise to free radicals that exacerbate its toxicity in the lungs compared to silica that is "aged" with the passing of time (Vallyathan et al., 1988, 1995; Dalal et al., 1990; Shoemaker et al., 1995; Castranova et al., 1996). The FSDs that were utilized in the present study were collected at gas well drilling sites and were stored until animal exposures were conducted several months later. During the interval between collection and experimentation the FSD became aged. On the other hand, workers exposed to recently generated FSD on site would have inspired freshly-fractured FSD particles, not aged particles. The significance of this difference is that this study did not completely emulate workplace exposures. Most investigations of MIN-U-SIL toxicity also utilize aged dust, and it is against this background that FSD *vs.* MIN-U-SIL comparisons are being made. Additional study of the bioactivity of fresh *vs.* aged FSD is warranted.

Silicosis and lung cancer are risks in industrial sand workers (Hughes et al., 2001; Vacek et al., 2019; Rando et al., 2018), and our findings can inform investigations into etiology of these diseases in this cohort, especially from the standpoint of the differences our studies revealed between FSDs and MIN-U-SIL in terms of their physico-chemical properties. The dusts generated during sand quarrying and processing have not, to our knowledge, been characterized to the extent done in the present investigations. However, there are clear differences in the chemical compositions of the sand dusts arising at fracking sites, which reflect to a certain degree the origins of the fracking sands. Assuming that the dusts generated during quarrying also contain minerals other than silica, the presence of the minerals could influence the onset of silicosis in workers.

A number of investigations are suggested by the present studies to address questions and gaps that arose during these experiments. The abrogated pulmonary fibrotic response to FSD treatments in comparison to MIN-U-SIL treatment does not necessarily suggest that a fibrotic response to FSD would not have developed in time. It is possible that a fibrotic response is delayed in onset but that it would eventually occur following a longer exposure period to FSD. Side-by-side exposures to MIN-U-SIL and FSD in animal models could reveal time-course differences in the progression of inflammation.

Exposures of workers at drilling sites are not continuous and, perhaps, exposure "bursts" in the animals over extended periods of time might better mimic work site conditions experienced by the itinerant workers at drilling rigs.

Whereas the particles of FSD 8 were respirable when generated for animal inhalation exposures, the particle sizes of the nine neat FSDs given intratracheally were heterogeneous and contained non-respirable sized particles. Doses were based on dust weight. Using inhalation delivery of respirable particles generated from the nine FSDs could provide a more accurate assessment and possible detection of differences in the toxic potencies of the dusts, allowing additional evaluation of the roles of silica and metal contents in determining bioactivity.

It is intuitive that inhalation delivery of FSD 8, even on a sub-chronic basis, might be expected to induce changes in lung performance, but marked alterations were not observed. Effects in the cardiovascular system, immune system, kidneys, and brain were, however, observed. Whether other organs, such as liver and gut, are affected is worthy of consideration. As well, the mechanisms by which FSD 8 provoked responses in organs distant from the lung are not apparent at present.

The presence of minerals associated with silica dust (Pavan and Fubini, 2017; Pavan et al., 2017) and FSDs (Fedan et al., 2020) affects their toxicity. It might be predicted that removal of minerals from FSDs using chemical means (HCl and HF treatment) might change the toxicity profile from what was observed in the present studies to that described for crystalline silica.

In summary, a comprehensive analysis of the biological effects of FSD on several organ systems has been made using a rat intratracheal instillation and inhalation exposure model and *in vivo* and *in vitro* models. Two major findings made were that the bioactivities of the FSD have differences compared with MIN-U-SIL following short-term testing in rats. In addition, the toxicities of FSD extended to non-pulmonary organ systems, which, for the most part, were greater than those seen in the lungs. These findings are relevant to the understanding of the potential risk of cases of silicosis in the unconventional gas extraction and industrial sand industries.

## Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention. Mention of brand name does not constitute product endorsement.

## Declaration of Competing Interest

The authors declare that they have no conflicts of interest in relation to this publication.

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## Appendix A

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