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A water-soluble, organosilane-based coating blocked acute and sub-chronic respirable crystalline silica-induced lung toxicity and systemic inflammation in an animal model

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ABSTRACT

Inhalation of respirable crystalline silica can cause pulmonary fibrosis and inflammation. Silica particles have a highly reactive surface that generates cell-damaging reactive oxygen species when fractured. The objective was to evaluate lung toxicity after exposure to silica coated with and without a water-soluble, organosilane-based coating (SIVO160). Male Sprague-Dawley rats were intratracheally instilled with silica (1 mg/rat), silica coated with SIVO160, SIVO160 alone, or saline (vehicle control). At 3, 10, 45, and 90 d after exposure, bronchoalveolar lavage (BAL) and histopathology were performed to assess lung toxicity. Whole blood was collected to evaluate systemic inflammation by differentiating circulating white blood cells. Also, samples of uncoated and coated silica were analyzed [(1) RapiFlex MALDI-ToF/ToF mass spectrometry; (2) digestion in phagolysosomal simulant fluid (PSF) and serum ultrafiltrate (SUF)] to confirm the SIVO160 coating on the surface of the silica particles after incubation in biological media. At each time point, silica significantly increased BAL fluid lactate dehydrogenase (lung injury) and the number of recovered lung macrophages and neutrophils (lung inflammation). These silica-induced elevations in lung toxicity were completely blocked at each time point when silica was coated with SIVO160 before exposure. At 45 d after exposure to uncoated silica, circulating total white blood cells, neutrophils, and lymphocytes were significantly elevated in the blood compared to the other groups. Lung exposure to silica pretreated with SIVO160 did not cause a significant elevation in any of the peripheral blood cell types at any time point when compared to the saline and SIVO160 alone. As assessed by mass spectrometry, multiple unique spectral peaks were detected on the surface of the silica+SIVO160 particle samples after an overnight incubation in saline. The peaks were absent in the uncoated silica sample spectra, confirming the coating's presence on the particles. The time it took for removal by digestion of the SIVO160 coating on the silica was reflected by a short-term delay after incubation in both PSF and SUF, suggesting protection could be conveyed to lung cells by the coating during phagocytosis and particle deposition on lung tissue structures. Organosilane materials may be used as a possible mitigation strategy to

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potentially protect large numbers of workers exposed to respirable crystalline silica in multiple industries is important.

1. Introduction

Pulmonary exposure to respirable crystalline silica puts thousands of workers from multiple industries at risk for pulmonary disease. Inhalation of crystalline silica dust has been shown to cause silicosis (pulmonary fibrosis and inflammation), chronic obstructive lung disease (COPD), kidney disease, autoimmune disease, lung cancer, increased susceptibility to tuberculosis [8], DNA damage, telomere dysfunction and shelterin dysregulation [17–19]. Importantly, once crystalline silica deposits in the lungs of exposed workers, it generally is too late to prevent the development of debilitating lung disease because of a lack of proven and effective treatment options. A mitigation procedure is needed that would reduce the toxicity of the respirable crystalline silica dust before it was inhaled.

Studies have observed that freshly cut silica is more toxic than aged or uncut silica [15,21,22]. The surface of freshly fractured silica generates cell-damaging reactive oxygen species (*e.g.*, hydrogen peroxide, hydroxyl radical, superoxide anion, peroxynitrite). It has been proposed that non-toxic, water-soluble organosilane materials could effectively coat the surface of silica particles and reduce their bioreactivity [15,25]. The effectiveness of organosilane coating of silica has been found to be stable for at least one week, but studies examining longer periods of time have not been performed [25]. Specific organosilanes also have been shown to reduce *in vitro* silica-induced toxicity of lung macrophages, the lung cell primary involved in removal and de-toxification of inhaled material [25].

Organosilanes have been safely and extensively used in different industries to provide a finishing coat on metal, glass, and ceramic surfaces. There have been no reported adverse health effects of workers exposed to materials during coating with Dynasylan® SIVO160. The hydrophilic Dynasylan® SIVO160 material has been tailored for optimized wetting behavior and cross-linking activities, leading to the formation of homogeneous cross-linked surface layer substrates [25]. Due to optimized wetting properties, high reactivity, and high cross-linking activity/density through amino functional groups of Dynasylan® SIVO160, water penetration through the resulting tight coating layer is prevented, leading to unavailability of potentially free silica surface silanol groups. In addition, Dynasylan® SIVO160 does not need a curing step to produce a tight inorganic–organic network without defects and thereby effectively modifies the silica surface charge by rendering the surface isoelectric or even positive, with subsequent blunting of biological activity [25].

The primary goal of the study was to examine whether the coating of respirable crystalline silica particles with an organosilane-based material (Dynasylan® SIVO160) would prevent the development of acute and sub-chronic lung inflammation and injury in an animal model. Male Sprague-Dawley rats were intratracheally instilled with silica (1.0 mg/rat), silica coated with the Dynasylan® SIVO160, Dynasylan® SIVO160 alone, and saline (vehicle control). Different markers of pulmonary inflammation and injury as well as lung histopathology were assessed at 3, 10, 45, and 90 d after exposure. In addition, the coating effectiveness and persistence of Dynasylan® SIVO160 on the surface of respirable crystalline silica particles was assessed in biological media using *in vitro* models up to 28 d after treatment.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley (Hla: SD CVF) rats were acquired from Hilltop Lab Animals (Scottdale, PA), weighing 250–300 g, and free of viral pathogens, parasites, mycoplasmas, *Helicobacter*, and *CAR Bacillus*. Rats were fed irradiated Teklad 2918 regular rodent diet (Envigo Teklad Diets, Madison, WI) and water *ad libitum*. After arrival, the animals were acclimated for 1 wk in an animal facility that is specific pathogen-free, environmentally controlled, and accredited by AAALAC, International (Frederick, MD). All animal procedures used during the study were reviewed and approved by the CDC—NIOSH Morgantown Institutional Animal Care and Use Committee and performed in accordance with the relevant guidelines and regulations by CDC—NIOSH and AAALAC International.

2.2. Silica and SIVO160 coating

Min-U-Sil 5 silica (U.S. Silica, Berkeley Springs, WV) was used in the study and had a respirable mass median aerodynamic diameter of 1.6 μm. To remove any possible endotoxin contaminants, silica samples were heated at 400 °C for 24 h before animal treatment. For some animal treatments, silica then was coated with the hydrophilic, organosilane Dynasylan® SIVO160 (oligomeric, amino-modified siloxane; Evonik Corporation USA, Parsippany, NJ). The silica coating procedure was modified as previously described by Ziemann et al. [25]. Briefly, SIVO160 (0.2 % w/w of silica) was incubated with silica for 2 hr. After the incubation, the silica+SIVO160 suspension was centrifuged three times at 500 x g for 10 min. The pellet was washed each time and resuspended in sterile PBS. Uncoated silica particles were processed similarly but without the SIVO160 treatment.

2.3. MALDI mass spectrometry

To confirm the presence of the SIVO160 coating on the surface of the silica particles, suspensions of uncoated silica and silica coated

with SIVO160 were analyzed after incubation for 24 h in PBS using a RapiFlex MALDI-ToF/ToF mass spectrometer (MALDI-MS; Bruker, Bremen, Germany). A total of 1.5 μL of each suspension was spotted onto a polished stainless-steel target plate. Prior to sample drying, 1.5 μL of 2,5-dihydroxybenzoic acid (DHB) (Sigma Aldrich, Darmstadt, Germany) in 70 % acetonitrile (Thermo Scientific, San Jose, *CA*) at a concentration of 30 mg/ml was co-spotted with each suspension. The laser was operated at 1 khz with 1000 shots collected per location in positive reflector mode. The mass range for the analysis was set at 100–1500 Da. Prior to analysis the mass spectrometer was calibrated, and laser energy was adjusted to optimize signal intensity for the selected mass range. The mass range for the imaging experiments was set at 500–1500 Da. After a 24 h incubation, multiple unique spectral peaks were detected on the surface of the silica+SIVO160 particle samples (Fig. 1). These unique peaks were absent in the uncoated silica sample spectra, confirming the presence of the coating on the particles.

2.4. Hydrodynamic size and zeta potential

The hydrodynamic size of the agglomerated uncoated and coated silica particulate samples in aqueous suspensions (water and PBS) was determined by dynamic light scattering (DLS). Malvern Zetasizer Nano ZS90 (Worcestershire, UK) equipped with a 633 nm laser at a 90° scattering angle was used to estimate the agglomerate charge (zeta potential) and hydrodynamic size. Particles were prepared in stock solution of water or PBS at 5 mg/ml. All measurements were performed at a particle concentration of 25 μg/ml in water and PBS, while maintaining a constant temperature of 25 °C. Samples were equilibrated inside the instrument for two min, and five measurements, each consisting of at least three runs, were recorded. Hydrodynamic size was not statistically different when comparing the uncoated silica sample with the silica samples coated with SIVO160 as determined by DLS (Table 1). However, the SIVO160 coating charged the surface of the silica particle sample from a negative charge to a positive charge.

2.5. Animal treatment

Before animal treatment, the uncoated silica and the SIVO160-coated silica samples were suspended in sterile phosphate-buffered saline (PBS) and briefly sonicated to disperse the particulates. Rats were lightly anesthetized by an intraperitoneal injection of 25 mg/kg body weight of methohexital sodium (Brevital 500 mg; JHP Pharmaceuticals, LLC, Rochester, MI) and intratracheally instilled with 1.0 mg/rat in 300 μl of sterile PBS. Vehicle control animals were intratracheally instilled with 300 μL of either sterile PBS or 0.2 % (w/w) SIVO160. A dose of 1 mg/rat was chosen based on earlier studies with Min-U-Sil 5 that showed that the critical dose of Min-U-Sil 5 for inducing inflammation is 0.20 mg, and 1 mg was enough to induce injury, inflammation and pathological alterations [5,6].

2.6. Blood collection and phenotypic differentiation of circulating immune cell populations

At 3, 10, 45, and 90 days (d) after intratracheal instillation, animals were euthanized with an intraperitoneal injection of sodium

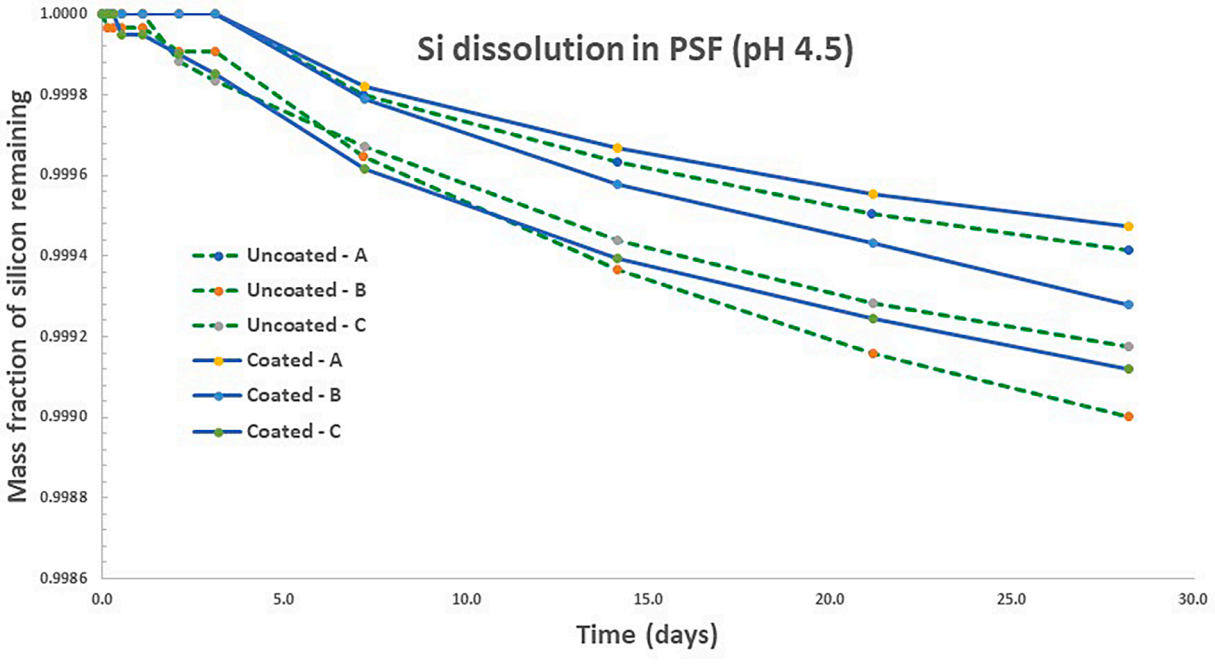

Fig. 1. Representative MALDI-ToF mass spectra of (A) Silica+SIVO160 and (B) uncoated silica. Putative peaks of silica with SIVO160 additive were observed at *m/z* 256.081 and 300.069 and were absent in the raw uncoated silica spectra.

Table 1
Particle size and surface charge of uncoated silica and silica coated with SIVO160.

	Hydrodynamic Size – Cumulative Mean Diameter (d.nm ± STD)		Zeta Potential [mV (range)]	
Sample	Water	PBS	Water	PBS
Silica	2182 ± 712	2440 ± 174	−38 (−35, −38)	−20 (−18, −23)
Silica coated with SIVO60	2326 ± 311	1659 ± 198	14 (8, 19)	2.3 (−2.6, 11)

Note. Z-Average values are means of diameter (nm) + standard deviation (STD; $n = 3$).

pentobarbital (>100 mg/kg body weight, IP; Fatal-Plus Solution, Vortech Pharmaceutical, Inc., Dearborn, MI). Whole blood was directly drawn from the abdominal aorta using an 18-gauge needle following euthanasia. A 500 μL aliquot was separated from the total volume and collected into EDTA-coated vacutainers (Becton, Dickinson, and Co., Franklin Lakes, NJ) to prevent clotting of whole blood and were agitated continuously until analysis. Total leukocyte number was determined using an IDEXX Procyte Dx Hematology Analyzer (IDEXX Laboratories; Westbrook, ME). White blood cells were also differentiated to determine absolute number and percentage of circulating monocytes, neutrophils, eosinophils, lymphocytes, and basophils.

2.7. Bronchoalveolar lavage and lung histopathology

At 3, 10, 45, and 90 d after intratracheal instillation, the left bronchus was clamped, and bronchoalveolar lavage (BAL) was performed on the right lung of each animal to assess lung injury and inflammation. The right lungs were first lavaged with a 1 mL/100 g body weight aliquot of calcium- and magnesium-free PBS, pH 7.4. The first fraction of recovered bronchoalveolar lavage fluid (BALF) was centrifuged at 500 x g for 10 min, and the resultant cell-free supernatant was analyzed for lactate dehydrogenase (LDH) as a marker for lung cell damage. The right lungs were further lavaged with 6-mL aliquots of PBS until 30 mL were collected. These samples also were centrifuged for 10 min at 500 x g, and the cell-free BALF was discarded. The cell pellets from all washes for each rat were combined, washed, and re-suspended in 1 mL of PBS buffer to be counted and differentiated. Non-lavaged left lungs from rats from each group were preserved with 10 % neutral buffered formalin by airway fixation at total lung capacity. Portions of fixed left lungs were stained with hematoxylin and eosin (H&E), embedded in paraffin, and sectioned for light microscopy and histopathological analyses.

2.8. Lung injury and inflammation

Total lung cell number recovered by BAL was determined using a Coulter Multisizer II and AccuComp software (Coulter Electronics, Hialeah, FL). Cells were differentiated using a Cytospin 3 centrifuge (Shandon Life Sciences International, Cheshire, England). Cell suspensions (5×10^4 cells) were spun for 5 min at 800 rpm and pelleted onto a slide. Cells (200/rat) were identified after labeling with Leukostat stain (Fisher Scientific, Pittsburgh, PA) as lung macrophages (*i.e.* alveolar macrophages or AMs), neutrophils, lymphocytes, and eosinophils. Using the acellular first fraction of BALF, LDH activity was determined by measuring the oxidation of lactate to pyruvate coupled with the formation of NADH at 340 nm. Measurements were performed using a COBAS MIRA auto-analyzer (Roche Diagnostic Systems, Montclair, NJ, USA) and expressed as units/liter (U/L).

2.9. Lung histopathology

Fixed, H&E-stained lung tissue samples were examined microscopically by a contract board-certified veterinary pathologist to determine any lesions associated with exposure to uncoated silica and silica coated with SIVO160. Histopathologic lesions were classified using standard published International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) terminology [16] to the extent possible.

Histopathologic findings in the lung tissue samples were assessed using the following as derived from Mann et al. [12]: *unremarkable or within normal limits*: tissue considered to be normal, under the conditions of the study and the strain of the rat; *minimal*: the amount of change present barely exceeds that which is considered within normal limits; *mild*: in general, the lesion is easily identified but of limited severity; *moderate*: the lesion is prominent, but there is significant potential for increased severity; *marked*: the degree of change is as complete as possible (*i.e.*, occupies much of the organ).

2.10. Persistence of SIVO160 surface coating in simulated biological media

To determine if there was a qualitative difference in the persistence of the coated *versus* uncoated silica particles in lung phagocytes after exposure, transmitted light microscopy was performed on cytospin-stained slides of recovered BAL cells from the different treatment groups using a CytoViva energy dispersive microscope (EDM; CytoViva, Auburn, AL; as described in Mercer et al., [13] Toxicol Pathol 46:28–46, 2018).

In addition, the persistence of the SIVO160 coating on silica samples was assessed *in vitro* using serum ultrafiltrate (SUF), a model of extracellular lung airway lining fluid that had a pH of 7.3 and phagolysosomal simulant fluid (PSF), a model of alveolar macrophage phagolysosomes, the site of degradation for engulfed foreign material, that had an acidic pH of 4.5 [20]. Samples of SIVO160-coated

silica or uncoated silica prepared for the *in vivo* experiments were weighed into separate centrifuge tubes and suspended in SUF or PSF. Controls consisting of SUF or PSF without particles were prepared in the same manner. To mimic the short-term residence time of particles in the conducting airways, aliquots of SUF were collected at 3, 6, 12, and 24 h and 1, 2, 3, and 7 d after silica particle exposure. To mimic the long-term residence time of particles in alveolar macrophages, aliquots of PSF were collected at 3, 6, 12, and 24 h and at 1, 2, 3, 7, 14, 21, and 28 d after particle exposure. The collected samples were analyzed for dissolved silicon concentration using inductively coupled plasma-mass spectrometry (*i.e.*, dissolution of SIVO160-coated silica particles was hypothesized to be lower than uncoated silica particles if the coating remained intact).

2.11. Statistical analysis

Continuous variables were analyzed using two-way (Treatment by Day) analyses of variance (ANOVA). Post-hoc pairwise comparisons for each day were derived from the full model using the Student's *t*-test and the pooled variance estimate. Data were analyzed using JMP statistical software (SAS Institute, Cary, NC). Differences were considered significant at $p < 0.05$.

3. Results

3.1. Systemic inflammation

Neutrophils were increased in the blood as early as 3 d after lung exposure to uncoated silica when compared to the saline and SIVO160 alone control groups but not the SIVO160-coated silica group (Table 2). However, by 10 d after exposure to uncoated silica, blood neutrophils were significantly elevated compared to all groups and continued to be elevated at 45 d post-exposure. Also, at 45 d after exposure, total blood WBCs and lymphocytes were significantly increased in the uncoated silica group compared to all other groups. By 90 d after exposure to uncoated silica, blood neutrophils and monocytes were significantly elevated compared to the saline vehicle group. Importantly, lung exposure to silica pretreated with SIVO160 did not cause a significant elevation in any of the peripheral blood cell types at any time point when compared to the saline and SIVO alone control groups.

3.2. Lung inflammation and injury

Intratracheal instillation exposure to uncoated silica caused a significant increase in both lung injury (BAL LDH activity) and inflammation (total recovered BAL cells) at all time points compared to the saline and SIVO160 alone controls and the group exposed to the silica coated with SIVO160 (Fig. 2). A slight but significant increase in LDH and total recovered BAL cells was observed in the group exposed to silica coated with SIVO160 at 3 d, but not at the other time points when compared to the saline and SIVO160 alone control groups. Importantly, lung exposure to SIVO160 alone produced no increase in lung injury and inflammation. After differentiating the recovered BAL cells, it was observed that coating the silica particles with SIVO160 before exposure prevented the increase in

Table 2
Peripheral blood cell phenotypes.

Treatment Groups	Total WBCs (K/uL)	Neutrophils (K/uL)	Lymphocytes (K/uL)	Monocytes (K/uL)	Eosinophils (K/uL)	Basophils (K/uL)
Day 3						
Saline	4.51 ± 0.50	0.61 ± 0.09	3.61 ± 0.45	0.22 ± 0.03	0.06 ± 0.01	0.01 ± 0.00
SIVO160	4.92 ± 0.53	0.58 ± 0.06	4.02 ± 0.51	0.25 ± 0.05	0.07 ± 0.01	0.01 ± 0.00
Silica	4.88 ± 0.34	0.84 ± 0.08^	3.71 ± 0.29	0.26 ± 0.03	0.07 ± 0.01	0.01 ± 0.00
Silica+SIVO160	5.30 ± 0.36	0.76 ± 0.36	4.18 ± 0.38	0.25 ± 0.04	0.10 ± 0.01	0.01 ± 0.00
Day 10						
Saline	4.83 ± 0.41	0.54 ± 0.05	4.04 ± 0.37	0.19 ± 0.01	0.06 ± 0.02	0.01 ± 0.00
SIVO160	5.19 ± 0.59	0.67 ± 0.07	4.01 ± 0.49	0.31 ± 0.07	0.06 ± 0.01	0.01 ± 0.00
Silica	4.70 ± 0.34	0.92 ± 0.07*	3.51 ± 0.31	0.21 ± 0.02	0.05 ± 0.01	0.01 ± 0.00
Silica+SIVO160	5.42 ± 0.39	0.71 ± 0.07	4.36 ± 0.32	0.28 ± 0.06	0.07 ± 0.01	0.01 ± 0.00
Day 45						
Saline	4.82 ± 0.56	0.52 ± 0.09	4.05 ± 0.52	0.18 ± 0.05	0.06 ± 0.01	0.01 ± 0.00
SIVO160	4.91 ± 0.46	0.72 ± 0.08	3.72 ± 0.38	0.13 ± 0.03	0.08 ± 0.02	0.01 ± 0.00
Silica	6.45 ± 0.94*	0.97 ± 0.14*	5.13 ± 0.73*	0.23 ± 0.08	0.11 ± 0.02	0.01 ± 0.00
Silica+SIVO160	4.86 ± 0.41	0.55 ± 0.07	3.95 ± 0.32	0.28 ± 0.08	0.07 ± 0.01	0.01 ± 0.00
Day 90						
Saline	4.21 ± 1.88	0.48 ± 0.22	3.55 ± 1.59	0.08 ± 0.04	0.08 ± 0.04	0.01 ± 0.00
SIVO160	4.80 ± 2.39	0.78 ± 0.35	4.33 ± 0.14	0.14 ± 0.06	0.09 ± 0.04	0.01 ± 0.00
Silica	4.90 ± 0.82	0.91 ± 0.14#	3.54 ± 0.62	0.38 ± 0.08#	0.07 ± 0.01	0.01 ± 0.00
Silica+SIVO160	4.67 ± 0.28	0.60 ± 0.17	3.81 ± 0.14	0.16 ± 0.05	0.09 ± 0.03	0.01 ± 0.00

Note. Peripheral blood phenotypes. Values are means ± standard error (n = 5–6).
* Significantly different from all other groups within a time point.
# Significantly different from saline group within a time point.
^ Significantly different from saline and SIVO160 groups within a time point ($p < 0.05$).

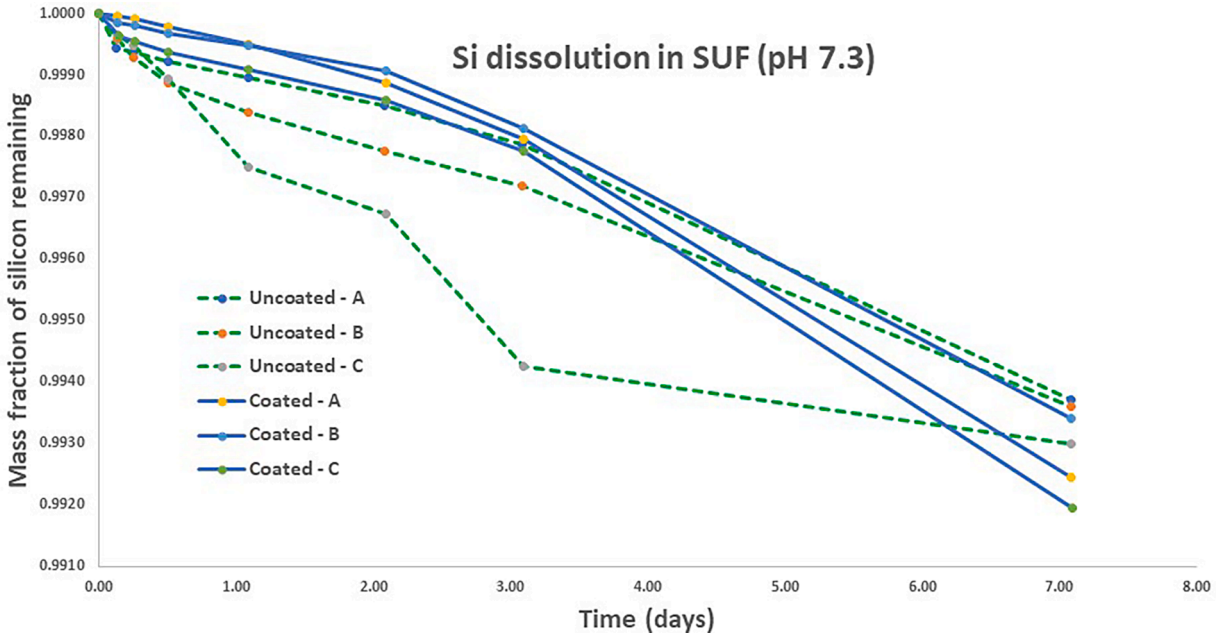

Fig. 2. (A) BALF LDH and (B) total BAL recovered cells at 3, 10, 45, and 90 d after intratracheal instillation of 1 mg/rat of silica, silica+SIVO160, SIVO160 alone, and saline (vehicle control). Values are means ± standard error ($n = 6$ rats/group); *Significantly different from all other group within a time point; #significantly different from SIVO160 and saline control groups ($p < 0.05$).

both AMs and neutrophils after exposure to uncoated silica at all time points (Fig. 3).

As depicted in cytospin-stained images of the recovered BAL cells, increased numbers of lung neutrophils were observed to persist in the lungs of the animals exposed to uncoated silica throughout the 90-d post-exposure period (Fig. 4). A minimal number of neutrophils were observed at each time point in the group exposed to silica coated with SIVO160 (arrowheads), indicating that the SIVO160 coating protected the exposed animals from lung inflammation induced by uncoated silica. Interestingly, numerous large foamy alveolar macrophages (F) were observed in the lungs at 45 and 90 d after exposure to uncoated silica but not in the lungs of the rats exposed to the silica coated with SIVO160.

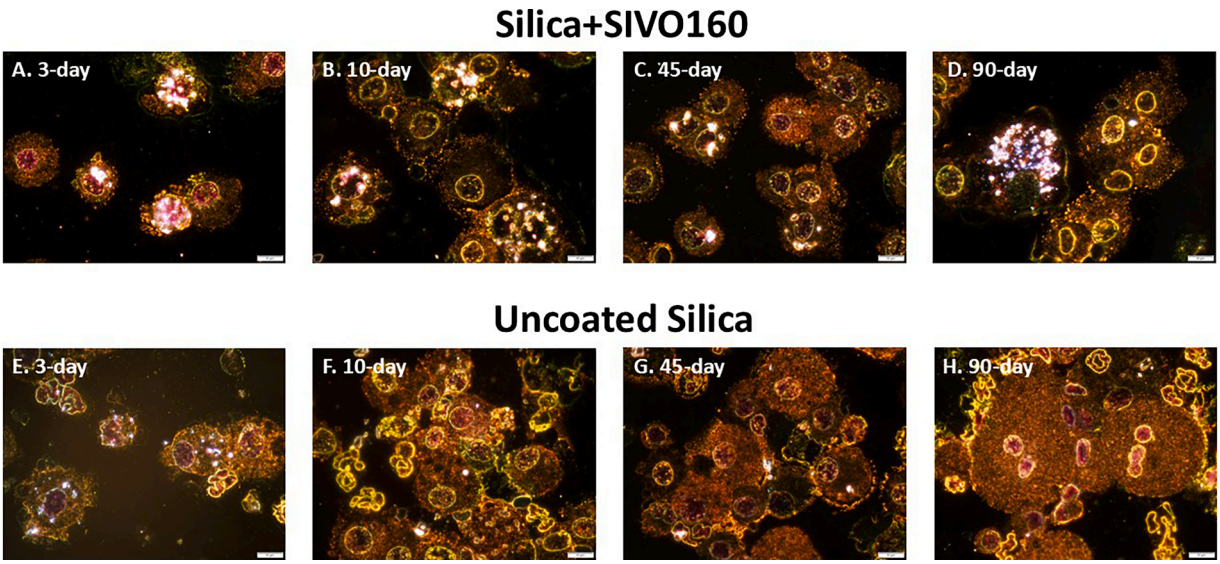

Fig. 3. (A) BAL alveolar macrophages and (B) BAL neutrophils recovered at 3, 10, 45, and 90 d after intratracheal instillation of 1 mg/rat of silica, silica+SIVO160, SIVO160 alone, and saline (vehicle control). Values are means ± standard error ($n = 6$ rats/group); *Significantly different from all other group within a time point ($p < 0.05$).

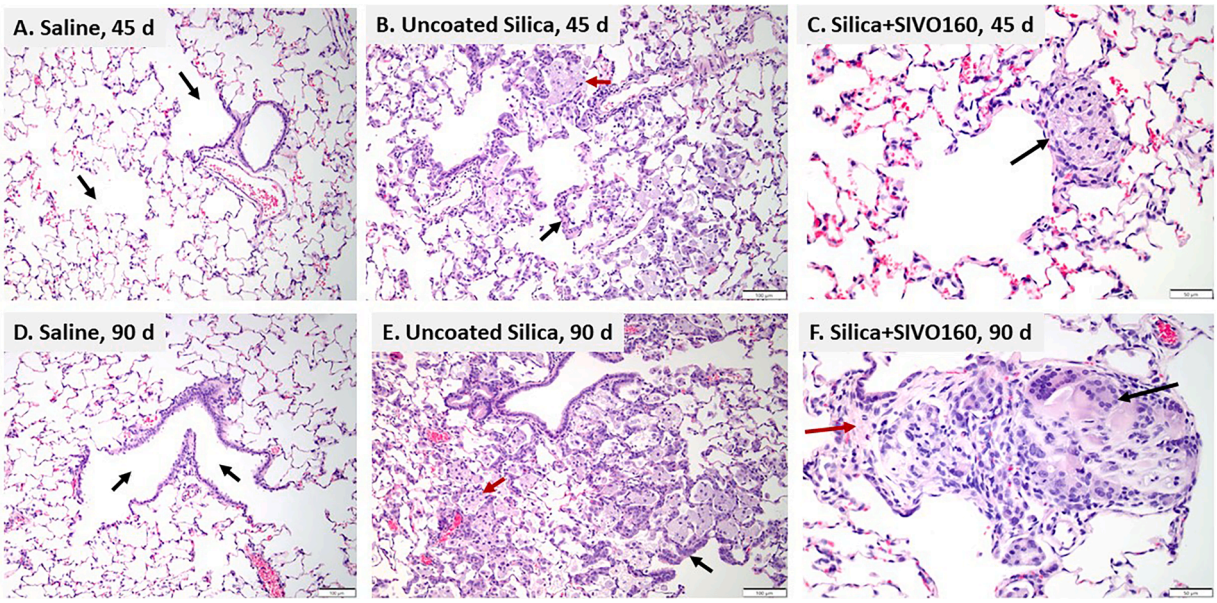

Fig. 4. Representative cytospin-stained photomicrographs of recovered BAL cells at (A) 3, (B) 10, (C) 45, and (D) 90 d after intratracheal instillation of 1 mg/rat of silica or silica+SIVO160. Arrowheads = examples of neutrophils; *F* = examples of foamy cells. Magnification is 40x.

3.3. *Lung histopathology*

Lung histopathology was assessed for all groups (Fig. 5). Lungs appeared normal for the saline (Fig. 5A&D) and SIVO160 alone (data not shown) groups at both 45 and 90 d post-exposure as the terminal bronchioles and surrounding alveoli were clear. Intratracheal instillation exposure to uncoated silica was associated with several lung lesions at 45 d post-exposure (Fig. 5B), including

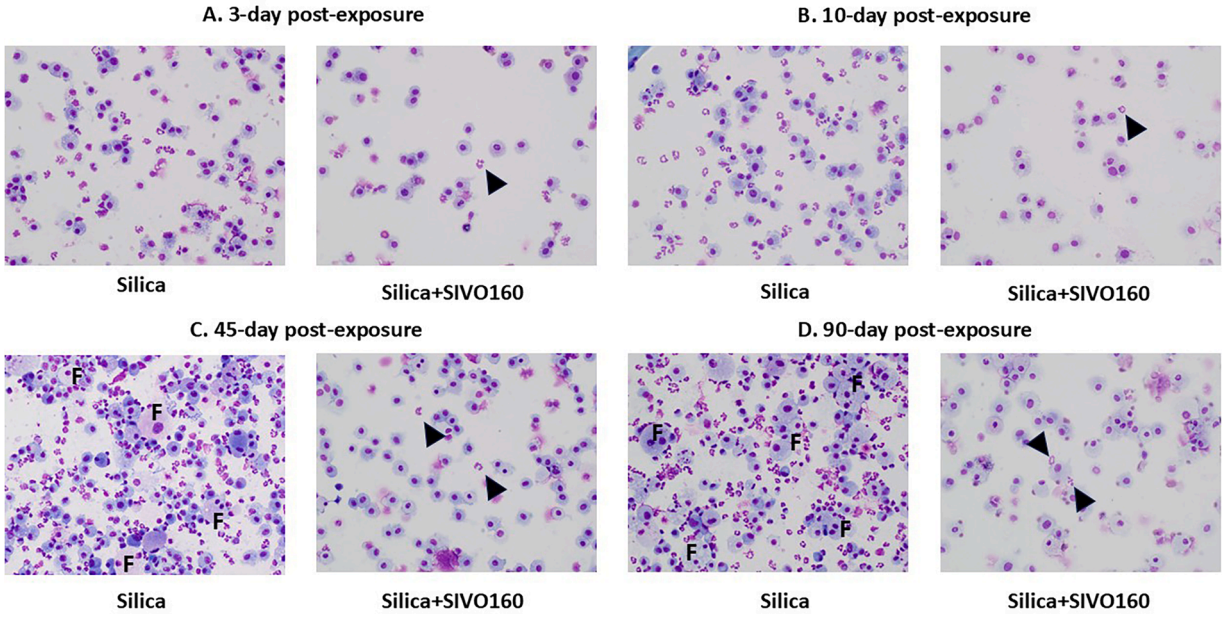

Fig. 5. (A) Saline control, 45-d post-exposure. Normal lung. Terminal bronchioles (arrows) and surrounding alveoli are clear, 10X magnification; (B) Uncoated silica, 45-d post-exposure. Bronchiolo-alveolar hyperplasia (black arrow) and bronchiolo-alveolar histiocytic infiltration (red arrow), 10X magnification; (C) SIVO160-Coated Silica, 45-d post-exposure. Focal aggregate of histiocytes within an alveolus adjacent to a terminal bronchiole (arrow), 20X magnification; (D) Saline control, 90-d post-exposure. Normal lung. Terminal bronchioles (arrows) and surrounding alveoli are clear, 10X magnification; (E) Uncoated silica, 90-d post-exposure. Bronchiolo-alveolar epithelial hyperplasia (black arrow) and bronchiolo-alveolar histiocytic infiltrates (red arrow), 10X magnification; (F) SIVO160-Coated silica, 90-d post-exposure. Bronchiolo-alveolar granulomatous inflammation (black arrow) and bronchiolo-alveolar pulmonary fibrosis (red arrow), 20X magnification.

bronchiolo-alveolar epithelial hyperplasia, bronchiolo-alveolar chronic-active inflammation, bronchiolo-alveolar histiocytic infiltration, and perivascular lymphoplasmacytic infiltration. Bronchiolo-alveolar epithelial hyperplasia, bronchiolo-alveolar chronic-active inflammation, and bronchiolo-alveolar histiocytic infiltration of predominantly mild severity were present in all rats at 45 d (6/6) post-exposure to uncoated silica. Perivascular lymphoplasmacytic infiltration of minimal severity was observed in two rats (2/6) at 45 d post-exposure to uncoated silica.

Bronchiolo-alveolar hyperplasia was characterized by plump cuboidal epithelial cells segmentally lining alveoli surrounding terminal bronchioles. There was occasional piling up of the epithelial cells and/or the presence of cilia. Bronchiolo-alveolar chronic-active inflammation was characterized by variable numbers of neutrophils with fewer lymphocytes and plasma cells located within alveoli surrounding terminal bronchioles. Frequently, chronic-active inflammation was associated with karyorrhectic and cellular debris indicative of localized cellular necrosis. Bronchiolo-alveolar histiocytic infiltration was characterized by variable numbers of plump histiocytes with abundant eosinophilic granular material to finely vacuolated cytoplasm located within alveoli surrounding terminal bronchioles. These histiocytes were often associated with clumps of finely beaded short linear eosinophilic material (presumptive fibrin). Perivascular lymphoplasmacytic infiltration was characterized by variable numbers of lymphocytes and plasma cells surrounding multifocal pulmonary blood vessels. These findings were graded based upon the extent of lung involvement as follows: minimal = <10 % lung affected, mild = 10–40 % lung affected, and moderate = 41–75 % lung affected.

Intratracheal instillation exposure to SIVO160-coated silica at 45 d post-exposure was associated with similar but fewer and less severe lung lesions to the uncoated silica group at 45 d post-exposure (Fig. 5C), including bronchiolo-alveolar epithelial hyperplasia, bronchiolo-alveolar chronic-active inflammation, and bronchiolo-alveolar histiocytic infiltration, all minimal severity. Bronchiolo-alveolar hyperplasia was observed in one rat (1/6) at 45 d post-exposure to SIVO160-coated silica. Bronchiolo-alveolar chronic-active inflammation was observed in one rat (1/6) at 45 d post-exposure to SIVO160-coated silica. Bronchiolo-alveolar histiocytic infiltration was observed in four rats (4/6) at 45 d post-exposure to SIVO160-coated silica. These findings were similar in description to those observed in the uncoated silica group at 45 d post-exposure. These findings were graded based upon the extent of lung involvement as follows: minimal = <10 % lung affected.

Lung exposure of uncoated silica at 90 d post-exposure was associated with similar lung lesions to those seen at 45 d post-exposure (Fig. 5E), including bronchiolo-alveolar epithelial hyperplasia (of mild to moderate severity), bronchiolo-alveolar chronic-active inflammation (of minimal to mild severity), bronchiolo-alveolar histiocytic infiltration (of mild to moderate severity), and perivascular lymphoplasmacytic infiltration (of minimal severity). Bronchiolo-alveolar epithelial hyperplasia, bronchiolo-alveolar chronic-active inflammation, and bronchiolo-alveolar histiocytic infiltration were observed in six rats (6/6) at 90 d post-exposure to uncoated silica. These findings were similar in description to that observed in the 45 d post-exposure group, although the severity of the chronic-active inflammation was less in 4/6 rats. Perivascular lymphoplasmacytic infiltration was observed in one rat (1/6) at 90 d post-exposure to uncoated silica and was similar in description to that observed in the 45 d post-exposure group. These findings were graded based upon the extent of lung involvement as follows: minimal = <10 % lung affected, mild = 10–40 % lung affected, and moderate = 41–75 % lung affected.

Intratracheal instillation of SIVO160-coated silica at 90 d post-exposure was associated with several lung lesions including bronchiolo-alveolar granulomatous inflammation, bronchiolo-alveolar pulmonary fibrosis, and bronchiolo-alveolar histiocytic infiltration. Bronchiolo-alveolar granulomatous inflammation of minimal and mild severity was observed in two rats (2/6) at 90 d post-exposure to SIVO160-coated silica. This lesion was characterized by accumulation of multinucleated macrophages that frequently formed aggregates within alveoli surrounding terminal bronchioles. Interestingly, bronchiolo-alveolar pulmonary fibrosis of minimal severity was associated with granulomatous inflammation and observed in the same two rats (2/6) at 90 d post-exposure to SIVO160-coated silica (Fig. 5F). Bronchiolo-alveolar pulmonary fibrosis was characterized by a few, focal areas within terminal bronchioles

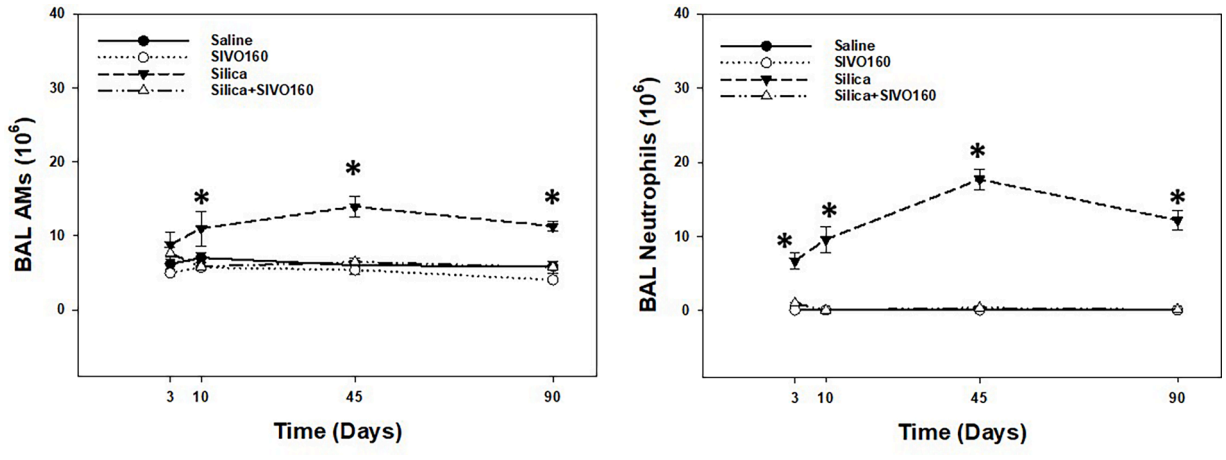

Fig. 6. Representative micrographs of recovered BAL cells from rats treated with Silica+SIVO160 (A-D) or uncoated silica (E-H) at 3, 10, 45, and 90 d after exposure (1 mg/rat) using a transmitted light microscope CytoViva EDM. Bar = 10 μm.

and/or surrounding alveolar septa expanded by collagen and fibroblasts. Bronchiolo-alveolar histiocytic infiltration was observed in one rat (1/6) at 90 d post-exposure to SIVO160-coated silica. Bronchiolo-alveolar histiocytic infiltration was similar in description to that observed in the 45 d post-exposure study and was of minimal severity. These findings were graded based upon the extent of lung involvement as follows: minimal = <10 % lung affected and mild = 10–40 % lung affected.

3.4. *Persistence of SIVO160 surface coating in simulated biological media*

EDM was used to qualitatively examine the persistence of uncoated and SIVO160-coated silica particles in phagocytic cells recovered by BAL at different time points post-exposure (Fig. 6). Silica particles that were coated with SIVO160 were easily observed intracellularly at all time points after intratracheal instillation, indicating that significant numbers of these particles persisted in the recovered lung phagocytes up to 90 d post-exposure (Fig. 6A-D). Interestingly, uncoated silica particles did not appear to persist in the lung phagocytes for the entire post-exposure time (Fig. 6E-H). Uncoated silica particles were easily observed within phagocytic cells at 3 d post-exposure but not as much at the later time points. Minimal numbers of silica particles from the uncoated group were observed by EDM at 45 and 90 d post-exposure.

There was measurable silicon in the SUF beginning at 3 h for both the SIVO160-coated and uncoated materials, though masses were approximately an order of magnitude higher for uncoated silica particles for all time points up to 24 h (Fig. 7). Thus, it appeared the SIVO160 coating had a slight protective effect for approximately 24 h in SUF. There was higher measurable mass of silicon from uncoated particles during the first 7 d in PSF, thereafter the mass of silicon dissolved from the SIVO160-coated and uncoated particles was more similar, indicating the SIV0160 coating appeared to have a slight protective effect for 7 d (Fig. 8). In comparing total silicon dissolution in the SUF and PSF over the time after particle exposure, it was observed that the % total fraction of silicon dissolved was higher from the materials exposed to SUF (0.698 % ± 0.007) compared with PSF (0.080 % ± 0.012).

4. Discussion

Inhalation of respirable crystalline silica dust has been shown to cause pulmonary fibrosis and inflammation, COPD, and lung cancer. Currently, there are no proven treatment options to effectively treat or prevent the development of these serious and potentially life-threatening adverse lung conditions once silica is inhaled into the lungs. A mitigation strategy is needed that could potentially "de-toxify" the crystalline silica dust before it becomes inhaled. Numerous substances, such as surfactant, phospholipids, metal chelators, and polymers have been used in previous animal and cell-based studies to coat or treat crystalline silica particles and reduce the associated toxicity [2,10,24]. Studies also have observed that different organosilanes can coat the surface of silica particles and reduce their cytotoxic properties [15,25]. The protective mechanism is due to a block of damaging reactive oxygen species by the coating and a change in the charge from negative to positive of the surface of silica particles [23].

During fracking operations for example, sand coated with organosilane materials may be used as a possible exposure mitigation method at oil and gas extraction sites. Hydraulic fracturing (*e.g.*, fracking) is a process in which underground natural gas-laden rock is fractured under pressure to enhance retrieval of gas. Sand is present in the fracking fluid pumped down the well bore to stabilize the fissures and maintain their patency and facilitate gas flow. The manipulation of sand at the well site creates respirable dust to which workers are exposed. Esswein et al. [9] measured exposures to dusts at gas well drilling sites where the mechanical handling of sand to prepare fracking fluid generates significant levels of respirable dust. Respirable crystalline silica during fracking has been identified as a significant exposure hazard [14]. It has been reported that nearly 80 % of oil and gas extraction workers sampled were exposed to levels of respirable crystalline silica greater than the NIOSH Recommended Exposure Limit (REL) of 0.05 mg/m^3 [14].

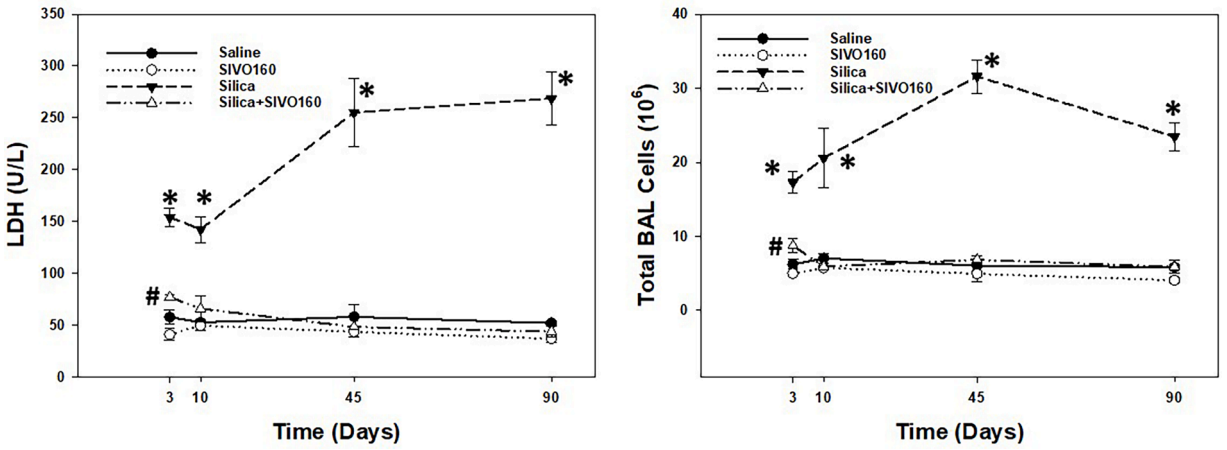

Fig. 7. Digestion of uncoated silica and silica coated with SIVO160 as measured by mass fraction of silicon remiaing in SUF (pH 7.3) at 3, 6, and 12 h and at 1, 2, 3, and 7 d after particle exposure (n = 3/group). Total silicon % dissolution for the 7-d time course was 0.698 % ± 0.007 %.

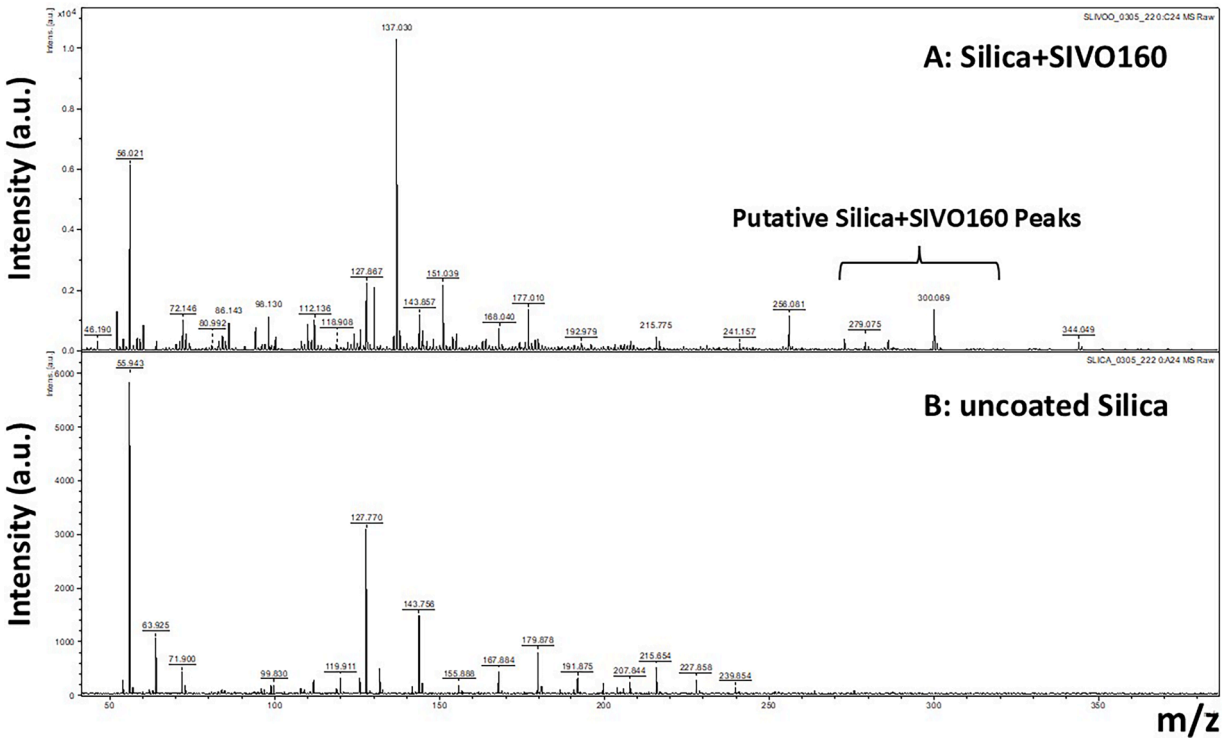

Fig. 8. Digestion of uncoated silica and silica coated with SIVO160 as measured by mass fraction of silicon remiaing in PSF (pH 4.5) collected at 3, 6, and 12 h and 1, 2, 3, 7, 14, 21, and 28 d after particle exposure ($n = 3$/group). Total silicon % dissolution for the 28-d time course was 0.080 % $\pm$ 0.012.

In coal mining, progressive massive fibrosis (PMF), an aggressive form of workers' pneumoconiosis, has dramatically increased among miners in different areas of Appalachia [3,4]. PMF has been observed in young, healthy workers from multiple states in as little as five years. Allen and Werntz [1] have proposed that current mining operations are cutting into narrower seams of coal compared to seams mined years ago. Because of this, more rock or sandstone adjacent to the coal seam is removed, producing substantial amounts of highly toxic, respirable crystalline silica dust. It is believed that the rapid onset and progression of PMF in coal miners from the Appalachia region is due to inhalation of high concentrations of silica dust. Water sprays are often used in the cutting processes and on roadways leading into and out of the coal mines to reduce respirable dust levels at active mining sites [7]. However, this does not reduce the toxicity of the inhaled dust, and once the dust deposits in the lungs of exposed miners, it generally is too late to prevent the development of PMF. Thus, the spraying with an organosilane material (as opposed to or in combination with water) during cutting processes in mines has been proposed as a possible mitigation strategy to protect workers exposed to dusts generated in coal mines [23].

In the current animal study, both peripheral and pulmonary inflammation was completely blocked as assessed by BAL blood cell differentials for the entire 90-d post-exposure period when the silica particle samples were coated with SIVO160. Lung injury also was completely inhibited by coating the silica with SIVO160 over the 90-d period as determined by measurement of BALF LDH. These lung response observations were consistent with what has been reported in a previous animal study by Ziemann et al. [25]. The current study was different from the Ziemann et al. [25] study in that a more highly reactive silica sample (Min-U-Sil 5) was used that produced a significantly greater pneumotoxic response when uncoated in rats. The protective coating effect of SIVO160 on this more highly reactive form of crystalline silica further illustrates its promise in protecting large numbers of workers exposed to silica. In addition, the potential toxic effect of the SIVO160 coating alone was not assessed in the *in vitro* and *in vivo studies* performed by Ziemann et al. [25]. In the current study, it was observed that lung exposure to the SIVO160 coating alone did not produce an increase in lung injury and inflammation, further demonstrating its potential to be safely used in workers. Additional studies such as lung function tests, micro-CT, and measurement of lung extracellular matrix (ECM) proteins could be helpful to further understand the beneficial effect of utilizing SIVO160 as a possible mitigation strategy for silica induced occupational toxicity.

Also unique to the current study, lung histopathology was performed. Pulmonary changes were observed to be the most prevalent and severe at 45 and 90 d post-exposure to uncoated silica. Similar changes were observed at 45 d post-exposure to SIVO160-coated silica but at fewer incidences and lesser severity. This pattern was the same as that observed with the BAL analysis where SIVO160-coated silica induced a less severe reaction than uncoated silica in the lungs of treated rats. In addition, the incidence, severity, and characterization of the findings observed in rats at 45 d post-exposure to uncoated silica persisted until 90 d, which was not observed in the SIVO160-coated silica-treated rats.

Interestingly, the changes of bronchiolo-alveolar granulomatous inflammation and bronchiolo-alveolar pulmonary fibrosis observed in a few, but not all, of the rats at 90 d post-exposure to SIVO160-coated silica suggested a chronicity not observed in the uncoated silica-treated rats. This may be explained by the observation of significant numbers of silica particles that had been pre-treated with SIVO160 within recovered lung phagocytes up to 90 d post-exposure, whereas uncoated silica particles were not. Uncoated silica particles were easily observed within phagocytic cells at 3 d post-exposure but not at the later time points. The shortened retention time of the uncoated silica particles in the lung phagocytes was likely due to their highly cytotoxic properties and their subsequent extracellular release into the interstitium as has been previously well-documented [22,23]. Moreover, the SIVO160 coating limited the interaction of the phagocyte with the highly reactive and toxic surface of the engulfed silica particles, thus increasing their intracellular retention time.

To further assess the coating of the SIVO160 persistence on the silica particles, several unique spectral peaks were detected using MALDI-MS on the surface of the silica samples coated with SIVO160 but not the uncoated silica after a 24-hr incubation, thus confirming the presence of the coating on the particles. In addition, the persistence of the SIVO160 coating on the particles was assessed in SUF and PSF over time. For SUF, there was measurable silicon beginning at 3 h for coated and uncoated materials, though mass of silicon appeared higher for uncoated particles for all time points up to 24 h, indicating the SIVO160 coating had a slight protective effect for 24 h. But once the coating was removed, the particle surface was exposed, likely resulting in a burst of silicon dissolution from the particles. For PSF, there was higher measurable mass of silicon from uncoated particles during the first 7 d but not after, indicating that the coating had a slight protective effect for 7 days, and thereafter silicon dissolution became similar for the SIVO160-coated and uncoated particles. This observation was confirmed in a previous study that showed the organosilane coating of silica was stable for at least a week on the particle surface [25].

It was expected that more of the SIVO160 coating would dissolve from the particle surface at an acidic pH (PSF; 4.5) compared with a neutral pH (SUF; 7.3). Interestingly, when comparing silicon dissolution in the two simulated lung fluids over the time after particle exposure, it was observed that the % total fraction of silicon dissolved was higher from the materials exposed to SUF (0.698 % $\pm$ 0.007) compared with PSF (0.080 % $\pm$ 0.012). In agreement, Larson et al. [11] examined the dissolution of crystalline silica particles in artificial airway lining fluid with varied pHs that ranged from approximately 6 to 7.5 and reported that dissolution was increased as pH increased, which was consistent with our observations. One explanation is that this lag may be a detection limit issue. The same amount of material (and therefore surface area) relative to the solvent volume was used for both types of particles and fluids. The detection limit for silicon in SUF was 0.007 ug/L and in PSF it was 0.01 ug/L (which were nearly the same). With the higher dissolution rate at higher pH, it is possible to measure silicon in SUF at a shorter exposure duration, and in PSF (with a slower dissolution rate), it took a longer exposure duration in the solvent to build up enough dissolved silicon to be measurable.

5. Summary and conclusions

The SIVO160 organosilane coating completely blocked crystalline silica-induced lung toxicity as assessed by BAL analysis for up to 90 d after exposure. At 45 d after exposure to uncoated silica, circulating total white blood cells, neutrophils, and lymphocytes were significantly elevated in the blood compared to the other groups. Coating the silica with SIVO160 before exposure prevented the observed changes in blood cell profiles at all time points. Lung histopathology confirmed the observations of the BAL analysis as the SIVO160-coated silica particles induced a less severe lung response than what was observed after exposure to the uncoated silica. Importantly, both granulomatous inflammation and fibrosis were observed in the lungs of some of the rats at 90 d post-exposure to SIVO160-coated silica but not in the uncoated silica-treated rats. Based on microscopic analysis, this may be due to an increased persistence of the coated silica particles in lung phagocytes as opposed to the shorter intracellular retention time of the more cytotoxic uncoated silica particles. After incubation in different stimulated biological media, the SIVO160 coating was observed to persist for up to 7 days in PSF, suggesting some protection, at least initially after exposure, could be conveyed to lung cells by the coating during phagocytosis and particle deposition on lung tissue structures. More studies are important to further assess the persistence of the organosilane coating on the surface of crystalline silica particles in lung tissues and cells. Based on the results of this *in vivo* lung toxicity study, organosilanes may be used as a possible mitigation strategy to potentially protect large numbers of workers exposed to respirable crystalline silica in multiple industries (*i.e.*, fracking, mining) would be beneficial.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention/Agency for Toxic Substances and Disease Registry. Mention of brand name does not constitute product endorsement.

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Data availability

Data will be made available on request.

Author agreement statement

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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