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Development of experimental silicosis in inbred and outbred mice depends on instillation volume

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There is considerable variation in methods to induce experimental silicosis with the effects of dose and route of exposure being well documented. However, to what extent the volume of silica suspension alters the dispersion and severity of silicosis has not been adequately investigated. In this study, the optimal volume of a crystalline silica suspension required to obtain uniform distribution and greatest incidence and severity of silicosis was determined in inbred and outbred mice. Silica dispersal, detected by co-inspiration with India ink and polarized light microscopy, was highly dependent upon volume. Furthermore, although peribronchitis, perivascularitis, and increases in bronchoalveolar lavage fluid cell numbers were detected at lower doses and volumes, significant alveolitis required exposure to 5 mg of silica in 50 μ l. This dose and volume of transoral instillation led to a greater penetrance of silicosis in the genetically heterogeneous Diversity Outbred strain as well as greater alveolar inflammation typical of the silicosis in human disease. These findings underscore the critical importance of instillation volume on the induction, severity, and type of inflammatory pathology in experimental silicosis.

Silica exposure is a well-known occupational hazard for individuals working in the dusty trades and can result in the fibrotic lung disease silicosis^{1–3}. Inhalation of silica containing dust leads to chronic inflammation of the lung, with development of silicosis being dependent upon the cumulative dose of silica exposure^{4–8}. This is supported by animal model studies, which have shown a direct correlation between dose and severity of lung disease^{9,10}. The presentation and severity of disease also depends on the genetic predisposition with DBA/2, MRL/MpJ, NZB, NZM2410, (NZBxNZW)F1, and C57BL/6 being more sensitive than C3H/HeN, BALB/c, and CBA/J^{9–13}. Experimental induction of silicosis also depends on the form of silica^{1,14} and the route of exposure^{14–16}.

There is currently no accepted standard experimental protocol for the exposure of mice to silica or the induction of silicosis and pathological sequelae such as autoimmunity. A wide range of approaches have been reported including inhalation of aerosolized silica using either whole body or head/nose-only exposures³, or instillation of silica suspension via intranasal (IN)^{16–18}, intratracheal (IT)^{15,16,19–23}, or transoral/oropharyngeal (TO) routes^{11,14–16,24–26}. Inhalation of aerosolized particles most closely model human exposure³, however these procedures require specialized equipment, use large amounts of potentially valuable material, and require repeated exposures over lengthy time periods^{3,14,15}. Instillation of silica in a liquid carrier, most often phosphate buffered saline (PBS), can be less wasteful of valuable reagents, requires less costly apparatus, but can also be technically challenging. IN instillation is the easiest method to administer particulate material such as crystalline silica, however it produces less severe disease than either IT or TO routes^{16,27}. IT and TO instillation also allows reproducible delivery of material to the lungs in a short time period and avoids exposure to skin or pelt which is an issue with whole body inhalation²⁸. However, the IT technique is technically challenging and invasive as it requires instilling silica directly into the lumen of the trachea either by use of a catheter or needle, or by surgically exposing the trachea (also called transtracheal instillation)²⁸. The non-surgical catheter approach does not have the potential issues of recovery from surgery and the complication of unwanted inflammation¹⁵ but is best suited to species where the mouth can be opened enough to view the vocal cords²⁸. The TO route has been shown to distribute silica evenly and more uniformly than the IT surgical technique, with less variability and more pronounced alveolitis¹⁵. Furthermore, the TO route was superior for inducing other respiratory diseases such as asthma²⁹ and bleomycin induced fibrosis³⁰. Moreover, a greater percentage of radiolabeled submicrometer colloidal suspension was

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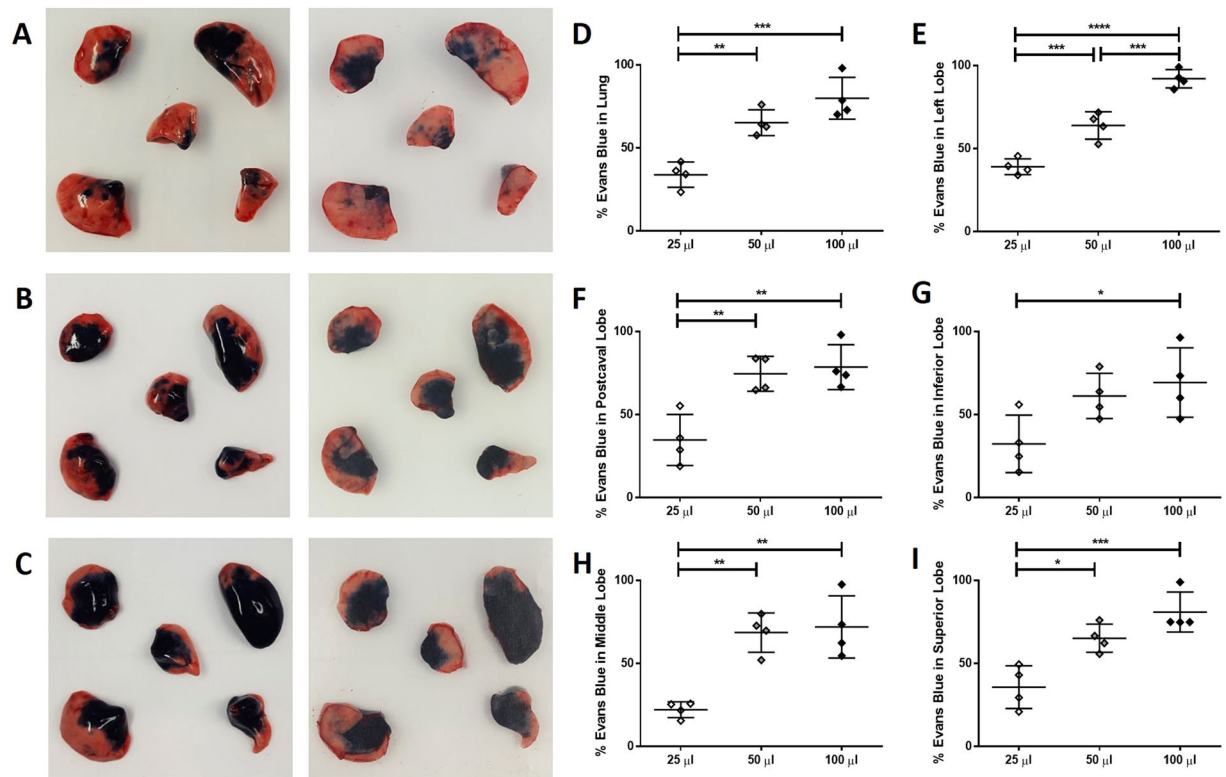


Figure 1. Effect of instillation volume on dispersal of Evans Blue dye in the lung. Male and female 8 to 12-week-old C57BL/6J mice were given one TO instillation of 25 ($n = 4$), 50 ($n = 4$) or 100 ($n = 4$) μl of PBS containing 0.15 mg of Evans Blue dye. Lungs were removed and photographed after 45 minutes. (A, B, C) Representative top (left column) and bottom (right column) views of the five lobes. In each image superior lobe (top left), left lobe (top right), postcaval lobe (bottom right) inferior lobe (bottom left), and middle lobe (center), are shown for (A) 25 μl , (B) 50 μl , and (C) 100 μl instillations. The percent area of each lung lobe stained with dye was calculated for both the top and bottom view using ImageJ and averaged as shown for (D) whole lung, (E) left lobe, (F) postcaval lobe, (G) inferior lobe, (H) superior lobe, and (I) middle lobe. Results were expressed as mean \pm SEM. A one-way ANOVA was used to determine statistical significance between instillation volumes, 25 μl (white diamond), 50 μl (grey diamond), and 100 μl (black diamond) which are represented as (**** $p < 0.0001$), (*** $p < 0.001$), (** $p < 0.01$), and (* $p < 0.05$).

detected in the lung after IT (77%) or TO (62–81%, 25–50 μl s respectively) routes compared with aerosolized (32%) delivery, with the percentage in the lung following TO instillation found to be volume dependent³¹. Thus, the TO approach has several advantages over other methods of inducing silicosis.

Although previous studies have noted the role of dose, time, mouse strain and instillation route on the severity of experimental silicosis^{9–11,15,16}, little attention has been given to the volume of material instilled. While several volumes, notably 1–2 ml/kg (50–100 μl s/20 gram mouse)²⁸ and 3 μl /gram of body weight³², have been recommended for IT instillation of particulate material, these were not based on studies using the TO route or crystalline silica for the induction of silicosis.

Initial experiments using TO instillation of Evans Blue dye showed that dye dispersal was dependent upon volume and lung lobe size. We hypothesized that larger volumes of a crystalline silica containing solution would similarly enhance distribution of particles to the periphery of the lungs leading to more widespread inflammation particularly of the alveoli. To test this, we used the TO route to instill different doses and volumes of crystalline silica and assessed subsequent lung pathology in inbred and outbred mice. We determined that the volume of administration affects the severity of silicosis and that mice receiving the same dose in a larger volume have more diffuse inflammation and more severe pathology. This was especially true for genetically heterogeneous Diversity Outbred (DO) mice where the larger volume led to greater disease incidence. We conclude that TO instillation of silica requires an appropriate volume for the induction of diffuse inflammation and severe experimental silicosis, particularly in a genetically heterogeneous population.

Results

Effect of instillation volume on pulmonary dispersion of instilled fluid. The effect of different volumes on the distribution of TO instilled material in the lungs was initially investigated using Evans Blue dye. The distribution of dye in the lung and all lobes of B6 mice was significantly greater for 50 and 100 μl instillations compared to the 25 μl volume (Fig. 1A–H). In contrast, although comparison of 50 and 100 μl instillations also revealed a significant difference, this effect was limited to the left lung lobe only (Fig. 1D–I). There were no

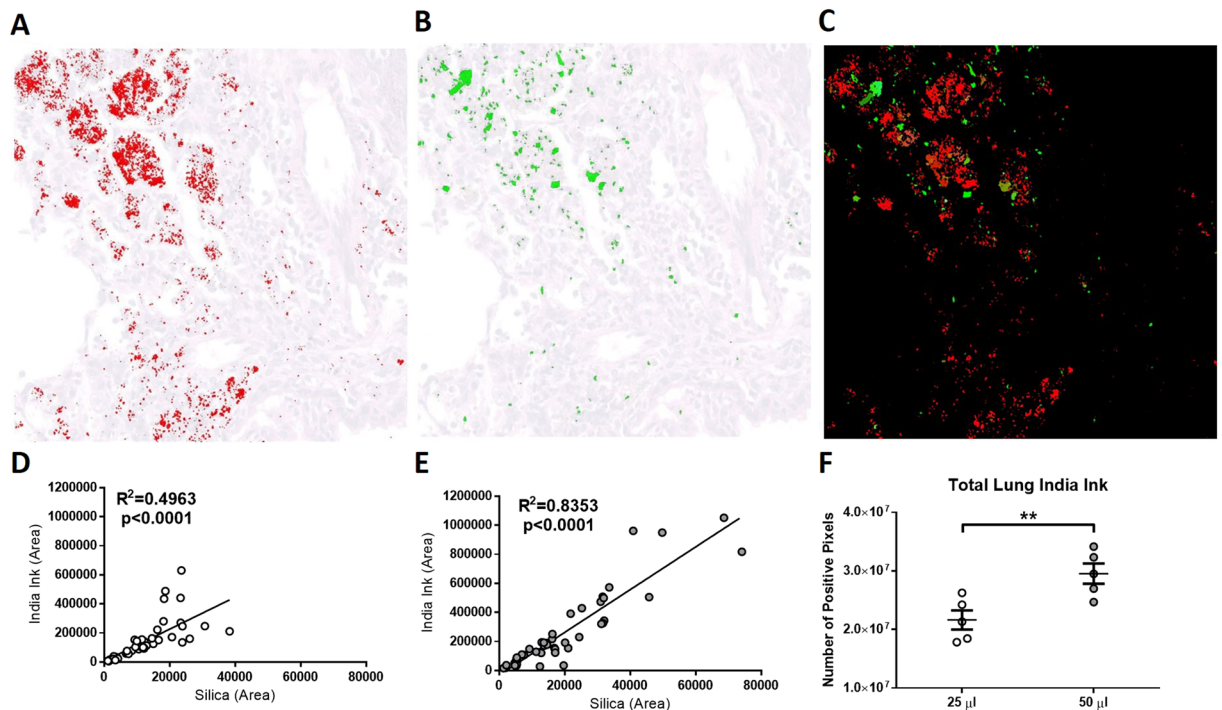


Figure 2. Effect of volume on silica and India ink dispersal in the lung. Female 8 to 12-week-old C57BL/6J mice were given one TO instillation of 10 mg of crystalline silica in a 20% India ink solution of 25 (n = 5) or 50 µl (n = 5). Images of the dispersed (A) India ink (red) and (B) silica (green) were overlaid on H&E stained sections. (C) Merged images of the India ink (red) and silica (green). Nine images per animal were taken to determine the correlation between India ink and silica in (D) 25 µl and (E) 50 µl volumes. (F) The amount of India ink in the entire lung at 40X was quantified. Because the 50 µl group received twice the amount of India ink, their values were divided by two. Results were expressed as mean ± SEM. Statistical significance between 25 µl (white circle) and 50 µl (grey circle) instillations are represented as (**p < 0.01).

differences in dispersal between males and females within each instillation volume (data not shown). This suggests that dispersal of the largest (i.e. 100 µl) volume is only effective in the largest lung lobe.

Pulmonary dispersion of silica in 25 vs 50 µl instillations. The increased dispersal of Evans Blue within the lung following 50 µl instillation suggested that larger volumes allowed greater dispersal of particulate material, therefore, subsequent experiments using crystalline silica compared 25 and 50 µl volumes. To test this hypothesis, 10 mg of silica was administered in 25, 50, or 100 µl of 20% India ink to visualize the dispersion, however 80% (4/5) of the mice receiving the 100 µl instillation survived less than 2 hours following exposure and were not included in this study. After one week, the total amount of India ink quantified in the lung was found to be significantly higher in the 50 µl group (Fig. 2F). Importantly, India ink and silica could be seen to clearly localize to areas of inflammation (Fig. 2A–C). Indeed, a positive correlation between the amount of silica and the amount of India ink was found with the 25 µl group (Fig. 2D) but was particularly strong in the 50 µl group (Fig. 2E). Taken together, the data show that the total amount of silica in the lung of mice receiving 50 µl is higher than the mice receiving 25 µl.

Effect of instillation volume on silica-induced lung pathology. To determine whether the volume of transoral instillation has any effect on the development of silicosis, we exposed B6 mice to silica in either 25 or 50 µl and compared their lung pathology and inflammatory response. As expected, greater lung pathology was seen with higher silica doses for both instillation volumes (Fig. 3A–D, Supplemental Table S1, Supplemental Figs S1–2). However, instillation volume had a greater effect on total lung score, alveolitis, perivascularitis, and bronchoalveolar lavage fluid (BALF) cell number particularly at the higher exposures (Fig. 3A–D; p < 0.0001). Notably, there was a marked increase in severity between the 1 to 10 mg exposures with the 50 µl volume that was much less apparent with 25 µl. However, the 10 mg dose in a 50 µl instillation proved toxic with 20% (2/10) mortality within the first 4 days of exposure.

To quantify the amount of silica-induced fibrosis, we measured total lung hydroxyproline content as a measure of collagen deposition. We observed no differences in the amount of hydroxyproline in mice receiving the 25 µl instillation, while mice receiving the 50 µl instillation had significantly higher hydroxyproline at 10 mg (Fig. 3E). Furthermore, there was a significant difference in the amount of total lung hydroxyproline in mice receiving 10 mg of silica between the 25 µl and 50 µl instillations (Fig. 3E). Thus, the volume of instillation plays a major factor in the induction and severity of silicosis pathology.

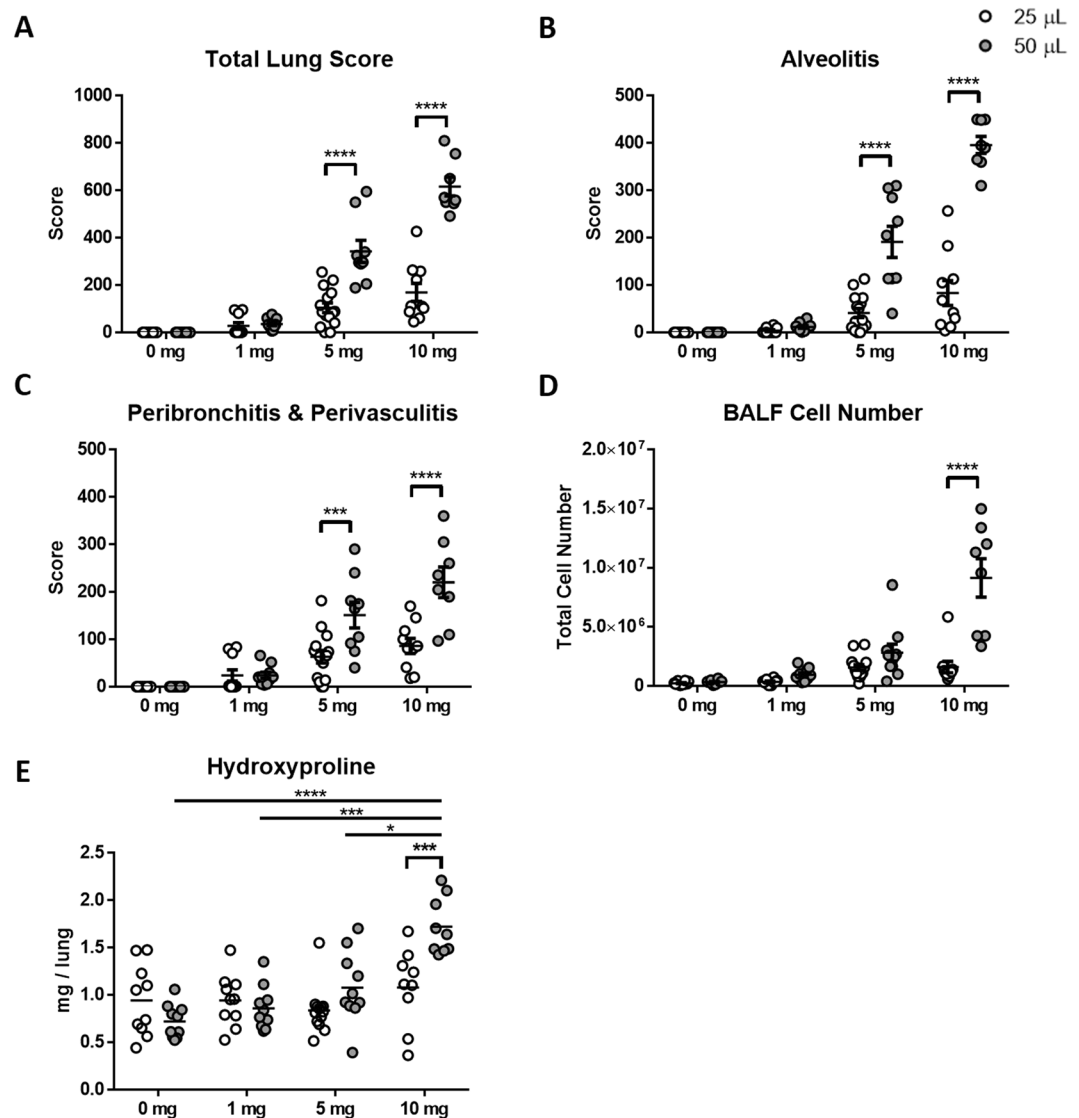


Figure 3. The effects of dose and volume of silica suspension on the severity of silicosis. Female 8 to 12-week-old C57BL/6J mice were given one TO instillation of 25 μ L of PBS alone ($n = 10$) and with 1 ($n = 10$), 5 ($n = 15$), and 10 ($n = 10$) mg, or 50 μ L of PBS alone ($n = 10$) and with 1 ($n = 10$), 5 ($n = 10$), and 10 ($n = 8$) mg crystalline silica for 4 weeks. Lungs were removed, and histology was scored for (A) total lung score, (B) alveolitis, and (C) peribronchitis and perivasculitis. Bronchoalveolar lavage fluid (BALF) was collected and cells were counted for (D) BALF cell numbers. Lung hydrolysates were used to measure (E) total lung hydroxyproline. A two-way ANOVA was used to determine statistical significance between dose and volume, 25 μ L (white circle) and 50 μ L (grey circle) instillations which are represented as (**** $p < 0.0001$) and (***) $p < 0.001$.

Sex Effects in the development of silicosis. We¹¹ and others^{33–35} have found differences in the responses of male and female mice to silica. To determine if silicosis following silica administration in a 50 μ L volume exhibits sex effects male and female B6 mice were exposed to 1, 5, or 10 mg of crystalline silica in a volume of 50 μ L of PBS for 4 weeks; controls were given PBS alone. Similar to the females, 10% (1/10) of males receiving 10 mg in 50 μ L died within 10 days following instillation. We found a significant dose ($p < 0.0001$) effect for total lung score, alveolitis, perivasculitis, and BALF cell number for both sexes (Supplemental Table S1). Total lung score and perivasculitis was significantly higher in females compared to males at the 10 mg dose. In contrast, male mice showed a significant increase in alveolitis at 5 mg (Fig. 4A–C, Supplemental Fig. S2) and BALF cell numbers were significantly increased in males compared to females at both 5 and 10 mg exposures (Fig. 4D). We observed no significant differences in total lung hydroxyproline between males and females (Fig. 4E), however while females showed significantly higher hydroxyproline at 10 mg compared to 0 mg, 1 mg, and 5 mg (Fig. 3E), males showed significantly higher hydroxyproline at both 5 mg and 10 mg compared to 0 mg and 1 mg (Fig. 4E). Furthermore, when males and females are combined, there is no significant difference in the amount of hydroxyproline between 5 mg and 10 mg (Fig. 4F). Thus the more pronounced severity of silicosis produced using a 50 μ L volume is associated with sex differences in response.

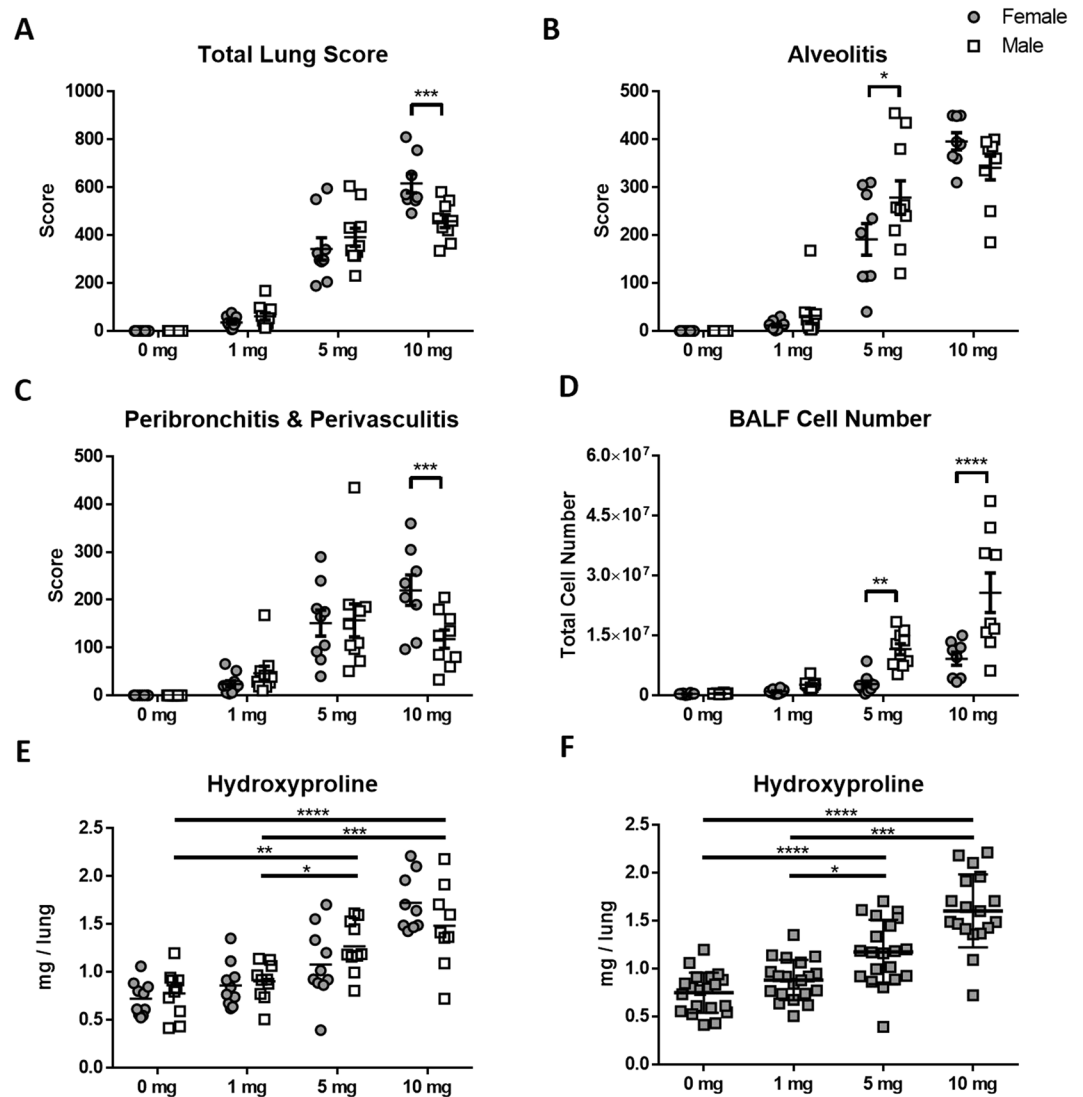


Figure 4. Sex effects in the development of silicosis. Female and male 6–8-week-old C57BL/6J mice were given one transoral instillation of 50 μ l of PBS alone ($n = 10$) and with 1 ($n = 10$), 5 ($n = 10$), and 10 ($n = 8–9$) mg crystalline silica for 4 weeks. Lungs were removed, and histology was scored for (A) total lung score, (B) alveolitis, and (C) peribronchitis and perivasculitis. Bronchoalveolar lavage fluid (BALF) was collected and cells were counted for (D) BALF cell numbers. Lung hydrolysates were used to measure (E) total lung hydroxyproline with males and females separate and (F) together. Results were expressed as mean \pm SEM. A two-way ANOVA was used to determine statistical significance between females (grey circle) and males (white square) which are represented as (**** $p < 0.0001$), (***) $p < 0.001$, and (* $p < 0.05$).

Effects of instillation volume on silicosis in outbred mice. The inbred B6 was selected for initial experiments because it is susceptible to silicosis and is the most common strain studied (Supplementary Table S1)⁹. However, susceptibility to silicosis is significantly influenced by genetic background^{9–11}. In previous studies using the DO mouse as a model of silicosis, mice were exposed to silica for 12 weeks following one 5 mg of silica in 25 μ l transoral instillation¹¹. To assess how the volume of instillation affects the severity of silicosis in mice of diverse genetic backgrounds, we compared silicosis in DO mice that received 5 mg of crystalline silica in 25 μ l with those receiving 50 μ l by TO instillation. The DO mice were exposed to silica for 12 weeks rather than the 4-week exposure in the B6 mice. Silicosis is a chronic inflammatory fibrotic disease and while the pathology, total lung score, alveolitis, perivasculitis and peribronchitis, and BALF cell numbers were all higher at 12 weeks as compared to 4 weeks (data not shown), all measurements of pathology (Fig. 5A–D) were significantly increased with the larger instillation volume. Importantly, the larger volume resulted in development of some degree of silicosis in essentially all mice, while a significant number of the animals given silica in the smaller volume failed to develop histological evidence of inflammation.

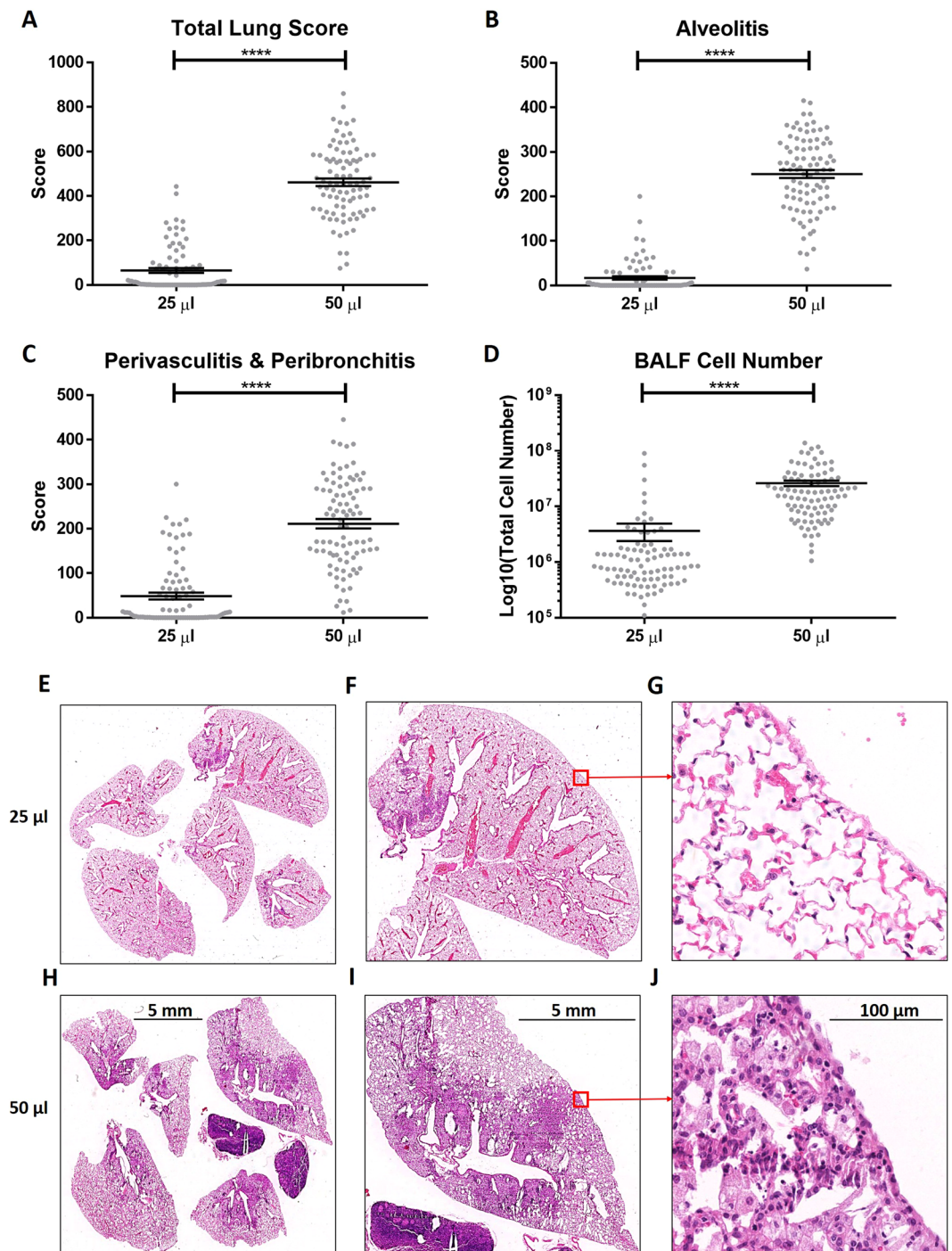


Figure 5. Effect of instillation volume on silicosis in outbred mice. Female and male 8 to 12-week-old DO mice were given one TO instillation of 5 mg crystalline silica in 25 (n = 86) or 50 µl (n = 89) of PBS. After 12 weeks lungs were removed, and histology was scored for (A) total lung score, (B) alveolitis, and (C) peribronchitis and perivasculitis. Bronchoalveolar lavage fluid (BALF) was collected and cells were counted for (D) BALF cell numbers. H&E images of the the whole lung for (E) 25 µl and (H) 50 µl at 0.5X, left lobe for (F) 25 µl and (I) 50 µl at 1X, and at 40X for (G) 25 µl and (J) 50 µl. Results expressed as mean ± SEM. A two-way ANOVA was used to determine statistical significance which are represented as (****p < 0.0001).

Discussion

The observations made in this study are significant because there is no standardized method for crystalline silica exposure in experimental animals. A survey of the literature using TO, IT, or IN instillation methods (Supplemental Table S2) shows widely differing doses, volumes and exposure periods used in mouse models of silicosis. A recent study has even argued that the well-established intratracheal instillation method has yet to be standardized³⁶. Studies have demonstrated that the severity of silica-induced inflammation and silicosis depend

on the type and amount of silica instilled, the method of instillation, the length of exposure, and the mouse strain used. In this study we further establish the critical importance of volume in TO aspiration and its impact on the severity of alveolitis, perivascularitis and peribronchitis, and numbers of inflammatory infiltrating BALF cells. Instillation of Evans Blue dye showed this is due to greater dispersion of instilled material throughout the lung. This was confirmed by comparison of dispersal of India ink and crystalline silica. Significantly, the importance of instillation volume on disease severity was also demonstrated in genetically heterogeneous DO mice. These studies argue that optimizing instillation volume allows better distribution of crystalline silica and greater incidence of silicosis independent of genetic background.

Previous studies of instillation volume of soluble and particulate materials into the lung have focused on dispersal of soluble dyes, inks, and radiolabeled compounds. Pellikan black ink was used to determine the effect of instillation volume on lung distribution in hamsters³⁷ while India ink was used to examine distribution in rats by IT administration under a variety of conditions³⁶. Evans Blue dye has been used to visualize distribution in the lung by IT instillation and TO aspiration of crystalline silica¹⁵ and to ensure distribution of soluble recombinant antibodies into the lung in a model of mucosal allergic inflammation³⁸. Radiolabeled sulfur colloid has been used to compare aspiration and instillation methods³¹ as well as different volumes³⁹. In the current study, we investigated the dispersion of silica with two different instillation volumes of silica in an India ink solution. The amount of silica and India ink from different areas within the left lobe had a better correlation in the 50 μ l volume compared to the 25 μ l volume. Digital images of unstained sections were then used to quantify the amount of India ink dispersed across all lobes within that section. Similarly, to the Evans Blue dispersal, the amount of India ink quantified in the 50 μ l instillation was higher than the 25 μ l instillation. This raises the question of where the ink or silica goes in the 25 μ l instillation if not in the lung. No staining was observed in the gut, therefore, we assume that a large portion of the silica and India ink does not reach the lung and remains largely within the upper airway and is eventually cleared as previously reported⁴⁰. This is further supported by data obtained in the India ink quantification in the left lobe where we observe a much larger portion in the perihilar region in the 50 μ l instillations compared to the 25 μ l instillations (Supplemental Fig. S3). The efficient dispersal of Evans Blue in all lung lobes, and the better correlation between silica and India ink dispersal, shows that a single, appropriate volume results in effective dispersal of soluble and particulate material.

In addition to dispersal, several studies have also examined the effects of instillation volumes on consequent lung pathology with other agents and in other species. Introduction of silica into the lungs of experimental animals is achieved by constant inhalation or one or a few aspirations by IN, IT, or TO instillation¹⁴. TO aspiration was chosen for this study primarily because it was shown to distribute silica more evenly and uniformly with greater severity than the more frequently used IT instillation^{14,15}. Similarly, instillation volume is known to impact experimental observations, but in reference to different species that vary in size²⁸. We are unaware of any studies that examine the effect of the volume of the vehicle containing silica on its dispersal within the lung, development of lung inflammation, and silicosis, especially in outbred mice. Previous recommendations for instillation volumes were also for substances other than silica^{28,32,37}. Notably, the IN instillation of radiolabeled sulfide colloid (0.2–0.8 μ m) showed that volumes of $\geq 35 \mu$ l gave optimal delivery to the lower respiratory tract³⁹. Comparison of 25 or 50 μ l volumes of radiolabeled submicrometer sulfide colloid suggested that the larger volume delivered a greater amount to the lung, however, this was not formally documented³¹. In another study, a greater portion of radiolabeled 7 μ m diameter microspheres, instilled IN into mice, entered the lungs when given in a 50 μ l volume, whereas more particles remained in the nasal passages when a 10 μ l volume was given⁴⁰. With instillation of fluorescent Cy5.5 conjugated to dextran, 3 μ l per gram of weight (approximately 30–75 μ l/mouse) was the optimal volume with IT instillation³². Our findings show that instillation volume not only affects the distribution of micrometer sized crystalline silica but also the subsequent incidence and severity of inflammation.

In this study we found that both the dose and volume of silica administered affect the severity of disease. As expected, we found the severity of silicosis to be dose dependent, however 1 mg was insufficient to induce silicosis at either volume of instillation. This could be due to insufficient amounts of silica in the lung since a previous study found that approximately 20% of silica is retained in the lung following transoral instillation⁴¹. There were few differences found between controls and 1 or 5 mg using the 25 μ l instillations. Conversely, using the same dose but a higher instillation volume of 50 μ l, significant differences were found between controls and 5 and 10 mg in total lung pathology as well as alveolitis and perivascularitis and peribronchitis. A hydroxyproline assay was used to measure the amount of collagen in the lung. No differences were found in the amount of hydroxyproline between doses at the 25 μ l instillations, however elevated levels were found in the higher instillation volume, with significantly higher levels at the 50 μ l instillations in females receiving 10 mg and males receiving 5 and 10 mg. While no differences were found between the sexes, volume of instillation played a role in the amount of hydroxyproline in the lung with significantly higher levels found in females receiving 10 mg in 50 μ l compared to 25 μ l instillations. We found a significant sex affect in the number of BALF cells, with males accumulating much higher numbers of cells in the lung than females. However, at high doses females developed worse pathology than males. This supports our previous finding in the diversity outbred mouse, where we showed the males had higher BALF cell numbers than females¹¹. One other study found gender effects in BALF cell numbers and hydroxyproline levels inconsistent with this study, however differences in the method of instillation and length of exposure might explain these discrepancies. Using a higher volume of instillation allows us to use a lower dose of silica and achieve the same, if not more severity of silicosis in our mouse models.

The severity of silica-induced pulmonary inflammation differs among inbred mouse strains^{9,10}, suggesting genetic regulation. Using inbred C57BL/6 mice our findings show that optimizing the volume used to administer silica significantly increases the incidence and severity of lung pathology in both male and female mice. To examine if the administered volume affects disease severity in genetically diverse animals we compared responses in DO mice given 5 mg of crystalline silica in 25¹¹ or 50 μ l (this study). As found for the B6, the larger volume led to more severe pathology with essentially all mice developing disease to some extent, unlike mice that received 25

μls . Based on our results comparing Evans blue, India ink, and silica deposition, the difference between 25 and 50 μl is insufficient distribution of the silica into the lung parenchyma. Thus, the use of the appropriate instillation volume is critical for obtaining consistent silicosis in mice using the TO method and is essential for studying the influence of genetic variations such as in the DO mouse.

In this study we establish the critical importance of volume of transoral instillation for induction of experimental silicosis. Instilled volume had significant effects on the severity of alveolitis, perivascularitis and peribronchitis, and numbers of inflammatory infiltrating BALF cells. Thus, the appropriate instillation volume is necessary for obtaining consistent silicosis in mice using the TO method and is essential for studying the influence of genetic variations such as DO mouse studies.

Methods

Mice. C57BL/6Scr (B6) mice were obtained from The Scripps Research Institute (TSRI) Animal Colony. Diversity Outbred (DO) mice were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were maintained under specific pathogen-free conditions. Animal rooms were kept at 68–72 °F and 60–70% humidity, with a 12 hr/12 hr light-dark cycle, and sterilized cages were replaced each week with fresh water and food (autoclaved standard grain diet 7012, Teklad, Envigo, Madison, WI, USA) to which the mice had access ad libitum. Experiments were initiated in 8–12-week-old animals. All animal procedures were carried out in accordance with the regulations and guidelines of the Institutional Animal Care and Use Committee at The Scripps Research Institute. The experimental protocols were approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute (protocol# 08-0150) and use of silica was approved by TSRI Department of Environmental Health and Safety.

Evans Blue instillation. To visualize the effect of volume on dispersal of aspirated fluid, 0.15 mg of Evans Blue dye (Fisher Scientific Company, Fair Lawn, New Jersey) in PBS was administered TO in 25, 50 and 100 μl volumes (0.6, 0.3, and 0.15% solutions) to male ($n = 2$) and female ($n = 2$) B6 mice. Lungs were recovered 45 minutes after instillation, separated into lobes and photographed (top and bottom view). ImageJ (<https://imagej.nih.gov/ij/>) was used to determine area/lung lobe and the portion stained with dye calculated as a percent of the total area/lobe and as a percent of the total area of all five lobes/mouse as an average of both the top and bottom view images.

Exposure to crystalline silica. Crystalline silica (Min-U-Sil-5, average particle size 1.5–2 μm ; U.S. Silica Company, Frederick, MD) was washed in 1 M HCl at 100 °C for 2 h, washed three times with sterile water, autoclaved for 1 h at 121 °C, and dried and reconstituted in PBS⁴². Immediately prior to use, silica was disbursed by sonication. Female and male mice were exposed to a single dose of 1, 5, or 10 mg by TO instillation in a volume of 25 μl or 50 μl in PBS as described^{16,43}. Briefly, mice were anesthetized with isoflurane and suspended on an angled board by a rubber band hooking the front teeth after which the tongue was pulled forward and sideways (to block the swallow reflex). Silica solution was then delivered by micropipette to the posterior pharynx where it was spontaneously aspirated into the lungs.

Bronchoalveolar lavage and lung histology. Mice were sacrificed at 4- or 12-weeks post-exposure and bronchoalveolar lavage fluid (BALF) was collected. Total cell counts were determined using a Countess II FL Automated Cell Counter (Thermo Fisher Scientific, Waltham, MA). Lungs were excised and fixed for 24 hours in zinc formalin. Hematoxylin and eosin paraffin sections (5 μm) were prepared by TSRI Histology Core and slides scanned by Digital Image Hub (Slidepath, Dublin, Ireland). Silicosis was scored under blinded conditions for the percent of each lobe affected by alveolitis or peribronchitis/perivascularitis (5 lobes, 500 maximum score each)¹¹. The two values were combined to give a total lung score.

Detection and quantitation of pulmonary silica and India ink. To visualize the dispersion of silica, a cohort of female B6 mice were exposed to 10 mg of silica in 25, 50, or 100 μl in 20% India ink solution for 1 week. To detect India ink and silica in the lung, unstained lung sections of identical areas of the left lobe were analyzed with both brightfield microscopy and polarizing lenses, respectively. Nine areas representing three each for perihilar, mid and distal lung regions were analyzed (Supplemental Fig. S3). Silica and India ink were detected and quantified by setting the threshold and region of interest (ROI) in ImageJ to detect birefringence (silica) or black color (India ink), respectively. The total amount of India ink in the lung section was determined by ImageScope at 40X using a positive pixel count algorithm. Both 25 and 50 μl instillations were composed of a 20% India ink solution for visualization, therefore the 50 μl group received twice as much India ink. This was corrected for by dividing the total amount of India ink by two for the 50 μl group in the comparison of total lung India ink.

Hydroxyproline analysis. Briefly, formalin fixed paraffin embedded whole lungs were acid hydrolyzed in 0.8 ml 6 N HCl at 95 °C for 20 h. Samples were centrifuged for 10 min at 13,000 rpm, and hydroxyproline residue quantification was performed on the resulting supernatant at a 1:20 dilution. Hydroxyproline was determined using the Quickzyme Sensitive Tissue Collagen Assay (QZBtiscol1, QuickZyme, Leiden, Netherlands) as described by the manufacturer.

Statistical analysis. Data are expressed as mean and standard error unless otherwise stated. Statistical analysis used GraphPad Software V6 (San Diego, CA). One- and two-way ANOVA were used for multiple comparisons and Pearson's correlation coefficient for linear relationships. $p < 0.05$ was considered significant.

Ethics approval and consent to participate. All animal procedures were approved by TSRI Institutional Animal Care and Use Committee (IACUC).

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

J.M.M., D.H.K., and K.M.P. contributed conception and design of the study. J.M.M. contributed to mouse maintenance, sample acquisition, execution of experiments, and data analysis. J.M.M., D.H.K., K.M.P. wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

Additional Information

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