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UNIVERSITY OF CALIFORNIA,
IRVINE

Cellular Responses and Mechanisms Relevant to Engineered Stone-Related
Silicosis in an *In Vitro* Respiratory Model

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Environmental Health Sciences

by

Elizabeth Hok Yee Choy

Dissertation Committee:
Professor Andrea De Vizcaya-Ruiz, Chair
Adjunct Professor Michael T. Kleinman
Professor Ulrike Luderer

2026

DEDICATION

To

my parents and sister

in recognition of their endless support and love

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LIST OF ABBREVIATIONS

ALI	Air liquid interface
AT2	Alveolar type II cells
CTGF	Connective tissue growth factor
DLS	Dynamic light scattering
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial mesenchymal transition
ES	Engineered stone
FBS	Fetal bovine serum
HUVEC	Human umbilical vein endothelial cells
JNK	c-Jun NH2-terminal kinase
LDH	Lactate dehydrogenase
PAH	Polycyclic aromatic hydrocarbons
PDI	Polydispersity index
PEL	Permissible exposure limit
PET	Polyester
SEM	Scanning electron microscopy
TEER	Transepithelial electrical resistance
Th1	T helper type 1
Th2	T helper type 2
TWA	Time-weighted average
VOC	Volatile organic compounds

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- Yang, Y., Arnold, M. L., Lange, C. M., Sun, L.-H., Broussalian, M., Doroodian, S., Ebata, H., **Choy, E. H.**, Poon, K., Moreno, T. M., Singh, A., Driscoll, M., Kumsta, C., & Hansen, M. (2024). Autophagy protein ATG-16.2 and its WD40 domain mediate the beneficial effects of inhibiting early-acting autophagy genes in *C. elegans* neurons.
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ABSTRACT

Cellular Responses and Mechanisms Relevant to Engineered Stone-Related

Silicosis in an *In Vitro* Respiratory Model

by

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Doctor of Philosophy in Environmental Health Sciences

University of California, Irvine, 2026

Professor Andrea De Vizcaya-Ruiz, Chair

Engineered stone (ES) is a composite material consisting of crystalline silica, pigment, and binding resins, and has become one of the most common materials used in kitchen and bathroom countertops, alongside natural stones such as marble and granite. The rise in popularity of ES in countertops is due to its low maintenance and customizable design; however, fabricating ES countertops generates respirable particles that pose an occupational inhalation hazard to workers. ES particle exposure has been linked to silicosis, a progressive and irreversible fibrotic lung disease resulting from inhalation of crystalline silica. When silica reaches the alveoli, macrophages attempt to clear the particles through phagocytosis while initiating inflammatory responses. Persistent inflammation and cellular injury can promote fibroblasts activation and collagen production, contributing to lung fibrosis. Few studies examine how silica content in ES influences the development of silicosis. This study aims to address this gap by utilizing an *in vitro* respiratory model to examine cellular responses after exposure to ES particles with varying silica content and to investigate mechanisms relevant to ES-related silicosis.

An *in vitro* co-culture system consisting of three rat cell lines, REL-6TN alveolar type II (AT2) cells, NR8383 alveolar macrophages, and RFL-6 alveolar fibroblasts, were used in this study to mimic aspects of the lung microenvironment. This model was assembled in a Transwell system, with AT2 cells and macrophages in the apical compartment and fibroblasts in the basolateral compartment. Particles generated from four commercially available ES slabs with low (< 40%; sample A and B) or high (> 40%; samples C and D) silica content, along with pure silica (Min-U-Sil 5), were used for exposure. AT2 cells and macrophages were exposed at 0, 1, 2.5, and 5 $\mu\text{g}/\text{cm}^2$ via an air-liquid interface (ALI) system. Particle size distribution, z-average, and polydispersity index (PDI) in suspension were assessed by dynamic light scattering (DLS). Cells and cell culture media were collected at 24, 48, and 72 hours after exposure for subsequent analyses of cytotoxicity, inflammatory responses, and fibroblast activation. Barrier integrity of AT2 cells was also assessed through 96 hours post-exposure.

At the concentration range used in this study, ES samples exhibited minimal cytotoxicity. At the highest concentration tested (5 $\mu\text{g}/\text{cm}^2$), AT2 cells showed a significant decline in barrier integrity after exposure to samples A and D. For the inflammatory profile, samples B and C predominantly induced innate pro-inflammatory cytokines, while sample D induced a distinct cytokine profile that included mediators associated with type 2 immune responses. Additionally, sample D triggered the strongest effect in fibroblast activation. Sample C and Min-U-Sil 5 elicited moderate activation, while samples A and B induced minimal effects. In conclusion, the results demonstrated sample-specific patterns of cellular responses that were not consistently associated with the silica content of the ES samples. These findings imply that silica content alone may not predict the biological activity of ES particles and that other particle characteristics

or constituents, together with silica, may contribute to cellular responses relevant to ES-related silicosis.

CHAPTER 1: Introduction

Over the past ten to fifteen years, engineered stone (ES) or artificial stone has become one of the most commonly used materials in kitchen and bathroom countertops (Fazio et al., 2025; Heinzerling et al., 2025; Hua et al., 2023). Consumers prefer ES over natural stones such as marble or granite because of its customizable color and pattern in addition to lower maintenance. While ES does not pose a direct risk during normal use, it has become a major occupational hazard during its fabrication and handling. ES is a composite material made of ground quartz, pigments, and polymer resin, which binds the components together; some ES products contain up to 90% crystalline silica (Ramkissoon et al., 2022). By comparison, silica accounts for only about 4-30% in natural stones like marble and granite, with the remaining materials being metals (29-37%) and other minerals (Ramkissoon et al., 2022). Studies have assessed the composition of resin in ES compared with silica, and Leso et al. suggests that resin could increase the toxicity of ES particles (2019).

Respirable crystalline silica generated from fabrication of ES countertops such as cutting, grinding, drilling, and polishing has become a hazardous occupational inhalation exposure to workers. ES-related silicosis, a progressive fibrotic lung disease caused by inhalation of respirable crystalline silica, has emerged as a significant occupational health concern, with numerous reported cases documented among exposed workers (Garcia & Harter, 2026; Garza, 2023; Greenfieldboyce, 2026). Since 2010, an increased number of workers in California have been diagnosed with ES-related silicosis (Flattery et al., 2025); among the 52 cases reported in Fazio et al. (2023), with ten were fatal, and the majority of affected individuals were immigrants from Latin and Central America, who may face barriers to timely healthcare access. ES-related silicosis is a phenomenon not unique to California; there are also reports from other states such

as Colorado, Texas, and Washington, and other countries including Israel, Spain, Italy, China, and Australia (Hoy et al., 2018; Kramer et al., 2012; Paolucci et al., 2015; Pérez-Alonso et al., 2014; Quan et al., 2022; Rose, 2019). In 2024, Australia became the first country to ban the use of ES due to concerns of silicosis and other ES-related lung diseases (*Australia's New Engineered Stone Ban to Begin in July*, n.d.; R. Hoy et al., 2025). Damage to the lung caused by crystalline silica is irreversible and there is no cure to the disease. Lung transplants can prolong life in those with silicosis, but it is not a cure.

In response to the outbreak of silicosis from ES fabrication in California, the state Division of Occupational Safety and Health (Cal/OSHA) enacted an emergency standard for respirable crystalline silica at 50 $\mu\text{g}/\text{m}^3$ of air for an eight-hour time-weighted average (TWA-8h) exposure, in 2023. The agency also set an action level at 25 $\mu\text{g}/\text{m}^3$ TWA-8h for medical surveillance and exposure monitoring (*OSHA's Respirable Crystalline Silica Standard for General Industry and Maritime*, 2023). This emergency standard was later adopted as permanent in December 2024. Studies from DeV Vaughn et al. and Surasi et al. reported that the median TWA-8h concentration was about 90 $\mu\text{g}/\text{m}^3$ at the ES workshops sampled (DeV Vaughn et al., 2025; Surasi et al., 2022), while one other study reported the geometric mean to be at 34 $\mu\text{g}/\text{m}^3$ (Weller et al., 2024). Besides the level reported by Weller et al. (geometric mean = 34 $\mu\text{g}/\text{m}^3$), the median concentration (90 $\mu\text{g}/\text{m}^3$) was almost two-fold of the PEL set by Cal/OSHA, suggesting that some workers are still exposed to respirable crystalline silica at a level that may cause adverse health effects.

The outbreak of ES-related silicosis cases indicated the urgent need to unfold the cellular responses and mechanisms leading to the disease, and many studies have focused on crystalline silica in ES. As the silica dust enters the alveoli, macrophages are activated and attempt to clear

the particles via phagocytosis. Inflammatory signals are then triggered due to tissue injury and to also promote wound healing. Since silica cannot be removed, inflammation sustains and subsequently stimulate fibroblast proliferation and collagen production. However, not many studies have explored how ES with varying silica content affects pathways related to silicosis. Moreover, most *in vitro* studies have examined the effects of ES exposure in a single cell type, such as macrophages or lung epithelial cells, where cell-cell interaction and communications are overlooked. Therefore, the objective of this study is to evaluate whether differences in silica content in ES induce differential silicosis-related cellular responses and to investigate the underlying mechanisms in an *in vitro* respiratory co-culture model, with particular focus on cytotoxicity, barrier integrity, inflammatory responses, and fibrotic cellular response.

This dissertation is structured into five interconnected chapters, each building upon the previous to provide a comprehensive evaluation of ES-induced respiratory toxicity. **Chapter 1** provides background on silicosis as a significant occupational health concern. **Chapter 2** characterizes the exposure-relevant size populations of ES particles and further assesses their size-distribution in suspension. This foundational characterization provides essential context for the *in vitro* respiratory co-culture studies in Chapters 3 and 4, as particle size influences suspension behavior, delivered dose, particle-cell interactions, and downstream biological responses. **Chapter 3** examines the effects of ES exposure on cytotoxicity and barrier cellular integrity, and **Chapter 4** investigates the inflammatory and fibrotic cellular response relevant to the pathogenesis of ES-related silicosis. **Chapter 5** summarizes the major findings of the dissertation, integrates the results across chapters, discusses their implications for occupational health, and highlights directions for future research. Collectively, these chapters provide an integrated toxicological framework for understanding how size-characterized ES particles with

distinct silica content contribute to adverse respiratory outcomes of occupational health relevance.

CHAPTER 2: Generation of Respiratory-Relevant Engineered Stone Particle

Size Populations

2.1 Introduction

As mentioned in Chapter 1, engineered stone (ES) is a composite material made of ground quartz containing crystalline silica, pigment, and polymer resin. Besides crystalline silica, which refers to the mineral phases quartz and cristobalite, ES also consists of other mineral phases such as rutile, albite, muscovite, and plagioclase, to name a few (Grytting et al., 2021; León-Jiménez et al., 2021; Ramkissoon et al., 2025). ES with different mineral phases induced cytotoxicity and inflammatory responses in a sample-specific manner. Grytting et al. reported that ES samples mainly consisting of quartz or plagioclase led to significant decrease in cell viability in monocyte THP-1 cell line and substantial increase in pro-inflammatory cytokines in human bronchial epithelial HBEC3-KT cell line (2021). Furthermore, majority of ES studies have focused on respirable crystalline silica, while polymer resin may also be a possible trigger of cytotoxicity (Leso et al., 2019). A study from Hall et al. reported that polyester-styrene was the most common resin type among ES tested (2022). Reports have also found that volatile organic compounds (VOC) such as phthalic anhydride, styrene, methyl tert-butyl ether, methyl methacrylate, and benzaldehyde were emitted during ES fabrication, as some of these VOC are considered lung irritants (Pavan et al., 2016; Ramkissoon et al., 2023). In addition to VOCs, León-Jiménez et al. found polycyclic aromatic hydrocarbons (PAH) such as naphthalene and phenanthrene were present in ES (2021).

ES is not a well-characterized material, as it increased its use for kitchen and bathroom countertops only about 15 years ago, and there is also considerable variation between ES samples depending on the manufacturer and particle-generation method. Thus, most studies have

included physicochemical characterization of the ES used, such as mineral phases, size distribution, and particle morphology (Carrieri et al., 2020; Pavan et al., 2016; Ramkissoon et al., 2022; Rishi et al., 2024). In the current study, relevant respiratory size fractions were generated, and size distribution, z-average, and polydispersity index (PDI) were analyzed. Particle size distribution shows the range of particle sizes and their relative abundance, and this property is important for determining whether particles exposed to cells are within a size range relevant to respiratory exposure. The respirable fraction includes particles capable of reaching the gas-exchange region of the lungs and is typically defined by an aerodynamic diameter with a 50% cut point of approximately 4 μm . *In vitro* ES exposure requires particles to be in suspended in a liquid medium; therefore, assessing the hydrodynamic diameter of particles and the heterogeneity of the suspension is necessary. The Z-average, which represents the intensity-weighted mean hydrodynamic diameter, and PDI can be determined using dynamic light scattering (DLS). Published ES studies have also characterized particle surface area and zeta potential (Ramkissoon et al., 2022, 2025).

2.2 Materials and methods

2.2.1 Engineered stone particles sample collection

Four commercially available engineered stone (ES) slabs with various silica content and color were obtained from Caesarstone (Figure 1). ES particles were generated using a drill press with a 6 mm burr inside a fume hood; the method was adapted from Leon-Jimenez et al. (2021). The particle samples were then stored at 4°C until size selection.

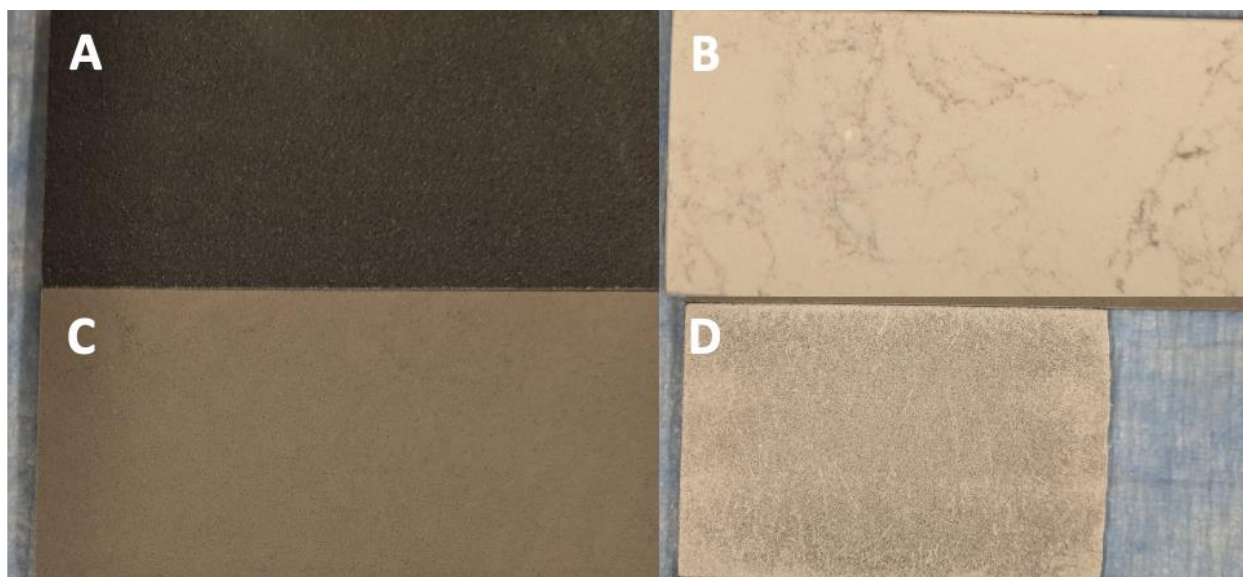


Figure 1. Engineered stone samples used in this study. Four ES slabs with various color and silica content were obtained from Caesarstone. Both samples A and C are dark gray in color, and samples B and D are light gray or mainly white with gray marble pattern. Samples A and B are low silica content ($Si < 40\%$) ES, while samples C and D are high silica content ($Si > 40\%$) ES.

2.2.2 Crystalline silica sample

Ground crystalline silica (Min-U-Sil 5) was obtained from U.S. Silica (Katy, TX) and used as an experimental control; it has a diameter of less than $5\ \mu\text{m}$. It has a 99.5% purity and a reported median diameter of $1.94\ \mu\text{m}$.

2.2.3 Size selection of engineered stone particles

Size selection of the particles was performed using an in-house built system with Sioutas personal cascade impactors (SKC Inc., Eighty Four, PA) (Figure 2). The impactor consists of four impaction stages, each with a collection plate. The particle-size cut points are 2.5, 1.0, 0.50, and $0.25\ \mu\text{m}$ for stages A through D, respectively. At a flow rate of $9\ \text{L/min}$, the particle-size ranges collected in stages A through D are > 2.5 , 1.0-2.5, 0.50-1.0, and $0.25\text{-}0.50\ \mu\text{m}$, respectively. To obtain ES particles within the respirable fraction, particles deposited on the

collection plates of stages B through D, corresponding to a nominal aerodynamic diameter range of 0.25-2.5 μm , were collected. Size-selected ES particles were stored at 4°C until *in vitro* exposures.

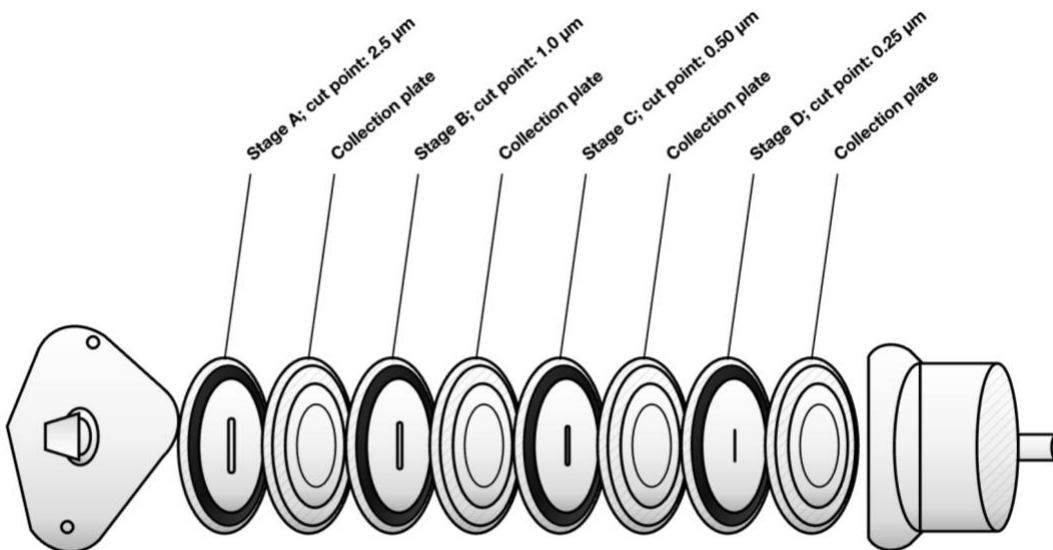


Figure 2. Diagram of Sioutas Personal Cascade Impactor. The cascade impactor has four stages A, B, C, and D with cut points at 2.5, 1.0, 0.50, and 0.25 μm respectively. Behind each stage is a collection plate to collect ES particles. Particles deposited on the collection of stages B through D were collected for this study. This diagram is created using AI (Model: Google Gemini 3.7 Flash).

2.2.4 Particle sizes and relative abundance in suspension

Particle size distribution and relative abundance of ES in suspension were determined using dynamic light scattering (DLS) with a Zetasizer (Malvern Panalytical, Westborough, MA). 2 mg of ES particles was resuspended in 2 mL of Milli-Q water in a glass vial. The suspension was sonicated continuously in a bath sonicator for at least 10 minutes to achieve uniform distribution of particles. 1 mL of suspension was transferred to a cuvette and insert into the Zetasizer to assess size distribution, z-average, and polydispersity index (PDI) using DLS.

2.2.5 Engineered stone exposure using air-liquid interface (ALI) system

About 10 mg of ES particles was resuspended in a mixture of phosphate-buffered saline (PBS) and sterilized Milli-Q water to prepare a 5 mg/mL stock suspension. The PBS suspension was sonicated continuously in a bath sonicator for 10 minutes to ensure the particles were evenly distributed. 100, 150, and 300 μL of suspension was nebulized using the nebulizer attached to a VITROCELL® Cloud Alpha 6 system (VITROCELL® Systems GmbH, Waldkirch, Germany) to achieve the concentrations of 1, 2.5, and 5 $\mu\text{g}/\text{cm}^2$ respectively, where 200 μL of PBS was nebulized for 0 $\mu\text{g}/\text{cm}^2$. The nebulizer pore size ranged from 4 to 6 μm . A quartz crystal microbalance was installed in the ALI system, and when the concentration of particles in the system was stabilized, the dose was recorded.

2.3 Results

2.3.1 Size selection of engineered stone particles

Four commercially available ES samples were obtained from Caesarstone, see Figure 1 with various color and silica content. The manufacturer claims that samples A and B have low silica content (< 40% silica), while samples C and D have high silica content (> 40% silica). Samples A and C are dark gray, and samples B and D are light gray or mainly white with a gray pattern that mimics marble. Z-average, particle size distribution, and polydispersity index (PDI) were determined before and after the ES samples were subjected to size selection via Sioutas personal cascade impactors. Size selection ensured the particles used in the study were within the respirable fraction, with nominal aerodynamic diameters ranging from 0.25 to 2.5 μm .

2.3.2 Hydrodynamic particles size and polydispersity index of engineered stone and reference silica particles

The Z-average, which represents the intensity-weighted mean hydrodynamic diameter of particles in suspension, was determined using the DLS feature in a Zetasizer. Results showed that ES samples were divided into two distinct groups after size selection, with samples A and C having strikingly larger hydrodynamic diameters than samples B and D. Before size selection, the z-averages of samples A through D were 1035, 620.1, 386.1, and 543.7 nm, respectively, while Min-U-Sil 5 showed a z-average of 408.1 nm (Table 1). After size selection, Z-average of ES samples appeared to separate into two groups: samples A and C showed z-averages of 1387 and 1500 nm, respectively, while samples B and D showed z-averages of 591.6 and 643.9 nm, respectively. Size selection also reduced the PDI of all ES samples, indicating more homogeneous particle-size distributions (Table 1).

Table 1. Z-average and polydispersity index (PDI) of engineered stone samples before and after size selection and of the reference silica Min-U-Sil 5.

	Z-average (nm)		Polydispersity index (PDI)	
	Before size selection	After size selection	Before size selection	After size selection
Sample A	1035	1387	0.815	0.278
Sample B	620.1	591.6	0.615	0.102
Sample C	386.1	1500	0.742	0.347
Sample D	543.7	643.9	0.699	0.141
Min-U-Sil 5	408.1	N/A	0.258	N/A

n = 3. Note: Min-U-Sil 5 was not subjected to size selection.

The intensity-weighted particle-size distributions showed that all ES samples had bimodal distributions before size selection (Figure 3). After size selection, a single predominant peak was observed for each ES sample (Figure 4). Min-U-Sil 5, which was not subjected to size selection, also showed a single predominant peak. The average diameter peak size ($\pm$ standard deviation) for the two populations were at 35.40 ± 2.578 and 337.0 ± 29.05 nm for sample A, 43.97 ± 4.181 and 567.6 ± 68.27 nm for sample B, 50.6 ± 5.120 and 1253 ± 206.8 nm for sample C, and 45.50 ± 3.976 and 868.2 ± 107.9 nm for sample D. Particle size distribution in suspension was assessed again after size selection, and a single peak was observed in all ES samples as well as the pure silica sample, Min-U-Sil 5 (Figure 4). The average peak size ($\pm$ standard deviation) for samples A to D was 972.4 ± 440.8 , 617.8 ± 115.9 , 1014 ± 357.7 , and 677.9 ± 126.6 nm, while the peak size of Min-U-Sil 5 was at 430 nm. The PDI for ES samples were at 0.815, 0.615, 0.742, and 0.699 without size selection, while the size selected ES samples had PDI at 0.278, 0.102, 0.374, and 0.141 respectively. Min-U-Sil 5 had a PDI of 0.258 (Table 1).

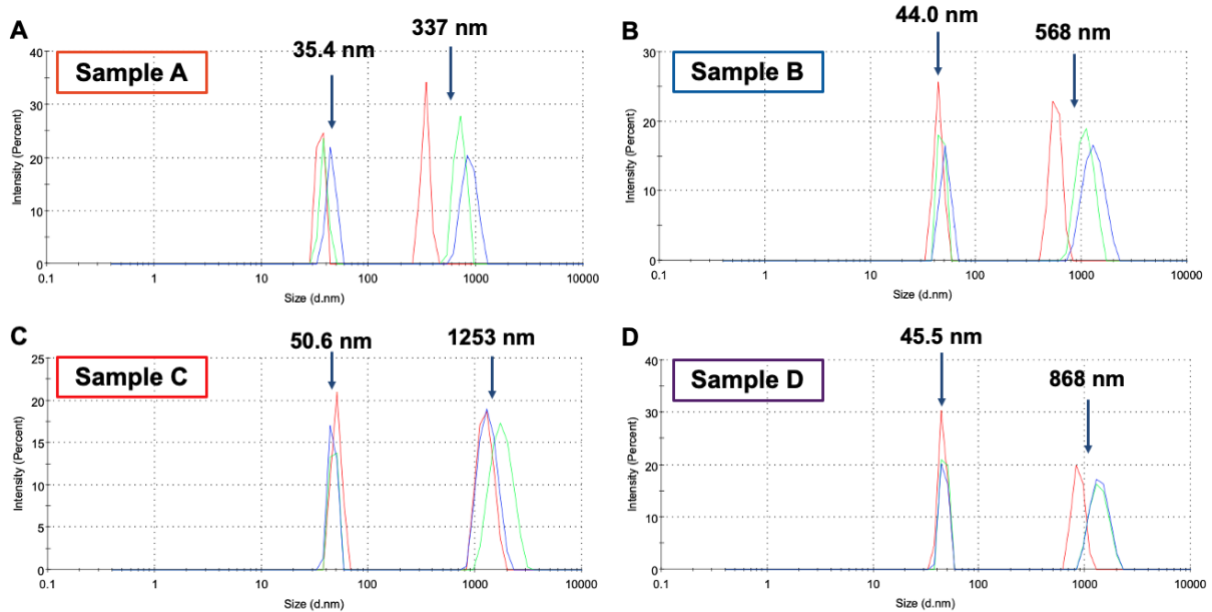


Figure 3. Size distribution of engineered stone particles in suspension. All the ES samples showed bimodal distribution which one peak size was at around 35 to 50 nm, and another peak size was at 337 nm, 568 nm, 1253 nm, and 868 nm for samples A-D (A-D) respectively. $n = 3$.

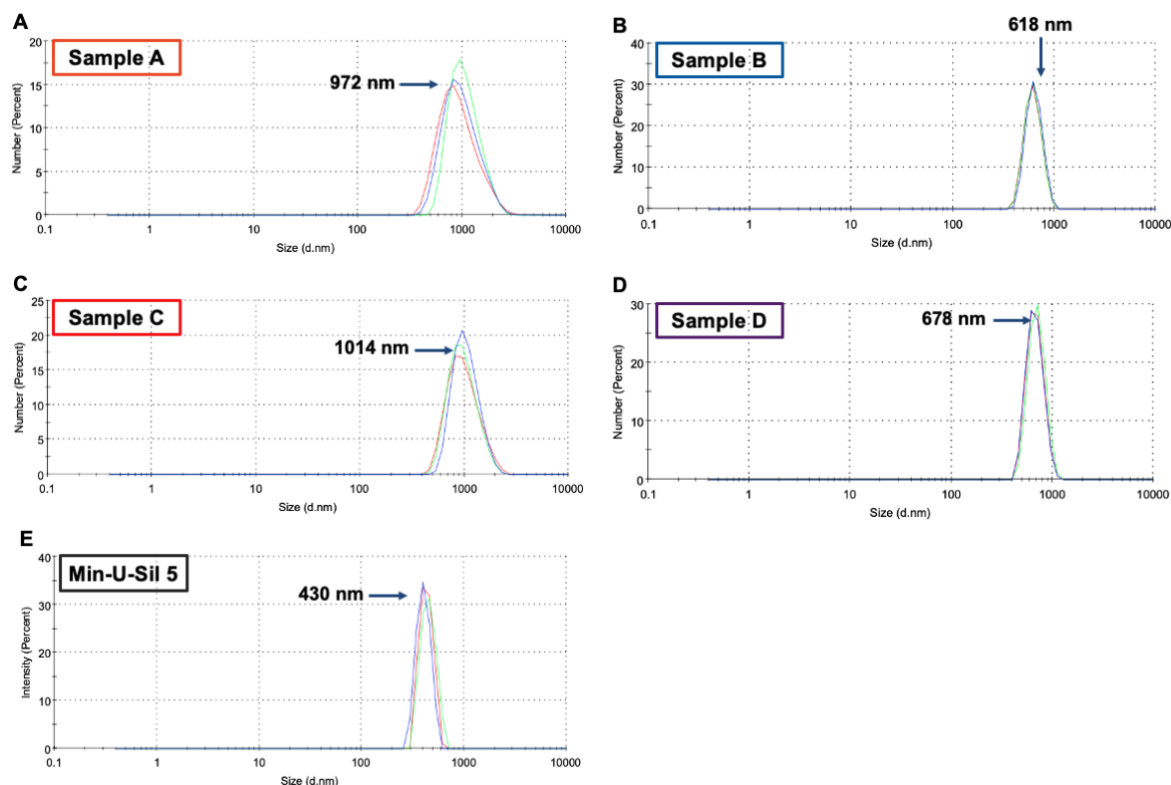


Figure 4. Size distribution of size-selected engineered stone particles in suspension. ES particles were size-selected using Sioutas personal cascade impactor, and a single peak in the size distribution graph was observed for all the ES samples. The average peak sizes were 972 nm, 618 nm, 1014 nm, and 678 nm for samples A-D (A-D), respectively. Min-U-Sil 5 had an average peak size of 430 nm (E). $n = 3$.

2.3.3 Particle aerosolization and deposition using an ALI system

A suspension of ES particles or Min-U-Sil 5 was prepared, and aerosolization of the suspension was conducted using the VITROCELL® Cloud Alpha 6 air-liquid interface (ALI) system. During the process, the suspension was nebulized, and concentrations of particles deposited onto the quartz crystal microbalance of the ALI were recorded.

Particle deposition generally approximated the target concentrations at the two lower doses but consistently exceeded the target concentration at the highest dose of 5 $\mu\text{g}/\text{cm}^2$, for all samples. Target surface concentrations for the *in vitro* exposure were set at 1, 2.5, and 5 $\mu\text{g}/\text{cm}^2$. Particle deposition during exposure was measured in a quartz crystal microbalance in the Cloud Alpha 6 ALI system after the deposition reading stabilized following nebulization (Table 2). At the target concentration of 1 $\mu\text{g}/\text{cm}^2$, the measured deposited doses were 1.48 ± 0.312 , 1.16 ± 0.385 , 1.47 ± 0.439 , 1.17 ± 0.359 , and 1.20 ± 0.377 $\mu\text{g}/\text{cm}^2$ of particles for samples A, B, C, D, and Min-U-Sil 5, respectively. At the target concentration of 2.5 $\mu\text{g}/\text{cm}^2$, the corresponding deposited doses were 2.58 ± 0.343 , 2.59 ± 0.502 , 2.78 ± 0.254 , 2.50 ± 0.527 , and 2.73 ± 0.494 $\mu\text{g}/\text{cm}^2$. At the target concentration of 5 $\mu\text{g}/\text{cm}^2$, the deposited doses were higher than the target for all samples, reaching 6.02 ± 0.822 , 6.06 ± 0.759 , 6.60 ± 0.385 , 6.80 ± 0.663 , and 6.97 ± 2.72 $\mu\text{g}/\text{cm}^2$ for all samples A through D and Min-U-Sil 5, respectively.

Table 2. Particle deposition from *in vitro* exposure via air-liquid interface (ALI) system.

Target concentration ($\mu\text{g}/\text{cm}^2$)	Particle deposition ($\mu\text{g}/\text{cm}^2$, Mean $\pm$ SD)				
	Sample A	Sample B	Sample C	Sample D	Min-U-Sil 5
1	1.48 ± 0.312	1.16 ± 0.385	1.47 ± 0.439	1.17 ± 0.359	1.20 ± 0.377
2.5	2.58 ± 0.343	2.59 ± 0.502	2.78 ± 0.254	2.50 ± 0.527	2.73 ± 0.494
5	6.02 ± 0.822	6.06 ± 0.759	6.60 ± 0.385	6.80 ± 0.663	6.97 ± 2.72

Values represent the mean $\pm$ SD of n = 4-6.

2.4 Discussion

The results in this chapter showed particle size selection was required for dust generated from commercially available ES samples to achieve respirable size, which is below 4 μm , and the intensity-weighted size distribution graphs before and after such process demonstrated that

size selection was necessary. PDI also showed less heterogeneity in the suspension after size selection. Z-average of ES particles narrowed from a broad range between 386 nm for sample C and 1035 nm for sample A to two groups at around 600 nm (for samples B and D) and 1450 nm (for samples A and C). Particle deposition results exhibited the exposure matched the target at lower concentrations (1 and 2.5 $\mu\text{g}/\text{cm}^2$) while the concentration exceeded the target for 5 $\mu\text{g}/\text{cm}^2$.

Before discussing the results, it is important to distinguish between the aerodynamic diameter used during size selection and the hydrodynamic diameter measured by DLS, where the particles were in two different media. During size selection, ES particles passed through the stages of Sioutas impactor in a stream of air at 9 L/min, and the particles were size selected according to their aerodynamic diameter. When the particles were in a suspension of PBS, the hydrodynamic diameter was measured by DLS. It is crucial to also assess particle properties in the suspension since *in vitro* exposure requires particles in a liquid medium. In addition, due to how the cut points are designed in a Sioutas cascade impactor, particles with nominal size between 4 and 2.5 μm were excluded in the study.

Z-average for ES samples before size selection going from lowest to highest was 386.1 nm for sample C, 543.7 nm for sample D, 620.1 nm for sample B, and 1035 nm for sample A. After size selection, the z-average divided into two clusters, 591.6 and 643.9 nm for samples B and D along with 1387 and 1500 nm for samples A and C, where this grouping does not follow the low vs. high silica content (samples A/B vs. samples C/D) classification. There are two possible factors contributing to this observation: 1) force applied on the drill press during particle generation and 2) particle size distribution before size selection skewed z-average. ES particles were generated using a drill press where the amount of force applied to the stone slab may affect

the size of particles being generated. From the process, samples A and C required more force applied on the drill press while samples B and D had to be handled with more care as they broke more easily. In addition, the bimodal size distribution before size selection skewed the result of z-average, where modality of the size distribution will be discussed later. Z-average assumes the suspension is monomodal and mostly homogenous, so the result before size selection was not very informative as size distribution was bimodal. Furthermore, Z-average is the intensity-weighted mean of hydrodynamic diameter, where larger particles carry more weight and can easily affect z-average compared to smaller particles because larger particles scatter more light. Z-average was higher before size selection for most of the ES samples suggests larger particles present in the suspension before size selection skewed the result. Few studies have measured ES hydrodynamic diameter using DLS, and one study from Ramkissoon et al. recorded ES particle size by DLS and found that ES samples were typically less than 1 μm in size, which was comparable to the results in this study (2022).

A bimodal particle-size distribution indicates there are two distinct populations of particles size in the suspension, and post-size selection, the two peaks merged to become a single predominant peak, which implies larger particles that might be above 2.5 μm were successfully removed via the process. A possible explanation to the substantial change in average peak size pre- and post-size selection, especially in sample A, is wall loss, meaning some of the particles were lost in the stages of the impactor during the process. It was also observed in studies on particulate matter (Misra et al., 2002), amorphous silicon dioxide (Berlinger et al., 2021), and polydisperse zinc nanoparticle aerosol (Durand et al., 2014) while using various types of impactors. Bimodality of size distribution of ES particles before size selection was consistent with other ES studies, where non-size selected ES particles had bimodal or trimodal size

distribution (Carrieri et al., 2020; Thompson & Qi, 2023). However, there are substantial differences in particle size of the primary peak. In Carrieri et al., the primary peak was around 200 nm while Thompson and Qi reported the primary peak at around 2.2 μm .

The result of PDI supports the notion that size selection reduced the heterogeneity of the suspension. PDI was above 0.6 for all ES samples pre-size selection while it was reduced to below 0.4 after. In general, $\text{PDI} < 0.1$ is considered monodisperse and > 0.4 is considered polydisperse (Marques et al., 2022). An assumption worth noting about PDI is that the measurement assumes the suspension is mostly homogenous and the particles are spherical in shape. Scanning electron microscopy (SEM) images from various studies on ES showed morphology and size difference in ES regardless of particle generation method, suggesting that non-size selected ES particles are highly polydisperse (Carrieri et al., 2020; Pavan et al., 2016; Ramkissoon et al., 2022). Therefore, PDI is relatively high for ES suspension due to the particle's non-spherical shape and consists of particles of various sizes. PDI decreased considerably from above 0.6 to below 0.4 for all ES samples after size selection, shows the suspension becomes less heterogenous. Sample C had a relatively higher PDI compared to the rest of ES samples and Min-U-Sil 5 even after size selection, which suggests that the suspension was slightly more heterogenous and may contribute to differences in biological responses post-exposure. When comparing to the study from Ramkissoon and colleagues, the suspensions from this study were less heterogenous (2022). From their study, they reported a high PDI (> 0.7) in the 12 ES samples tested in the study also measured using DLS.

Particle deposition closely matched the target concentrations for lower doses at 1 and 2.5 $\mu\text{g}/\text{cm}^2$, and deposition exceeded the target concentration for 5 $\mu\text{g}/\text{cm}^2$. The actual dose for 5 $\mu\text{g}/\text{cm}^2$ was higher than the target because this concentration was achieved by two rounds of

nebulization of the suspension. The highest volume used per nebulization was 200 μL , so that the particles could settle on the cells within a reasonable time, which the concentration was usually around 3 $\mu\text{g}/\text{cm}^2$. Therefore, a second round of nebulization with a volume between 80 and 150 μL was needed to achieve the target concentration of 5 $\mu\text{g}/\text{cm}^2$. Because the second round of nebulization involved extra handling of the suspension and counted as two aerosolization events, variability in the highest dose increased. The particle deposition was around 6 to 7 $\mu\text{g}/\text{cm}^2$ for a target concentration of 5 $\mu\text{g}/\text{cm}^2$, 5 $\mu\text{g}/\text{cm}^2$ will still be used throughout the study for consistency and simplicity purposes.

Overall, the results from this chapter demonstrated the necessity of particle size selection, so that the particle size was under the respirable range and the subsequent ES suspension was less heterogeneous. Additionally, particle deposition results confirmed that ES particles were deposited onto the cells via the ALI system and that the actual concentration matched the target dose.

CHAPTER 3: Cytotoxicity and Barrier Integrity

3.1 Introduction

Bronchial and lung epithelial cell lines such as BEAS-2B, CALU-3 and A549, along with macrophage models such as THP-1 and RAW 264.7, are commonly used in *in vitro* studies of ES or respirable crystalline silica to assess cytotoxicity and immune response (Friesen et al., 2022; Grytting et al., 2021; Maharjan et al., 2021; Mandler et al., 2026; Ophir et al., 2025; Pavan et al., 2016; Rafieepour et al., 2023; Ramkissoo et al., 2023; Skuland et al., 2020; Sun et al., 2026). Most of these studies have used monocultures, which do not account for crosstalk between cell types. Therefore, establishing a co-culture system that represents key components of the alveolar microenvironment can provide an informative approach for understanding the effects of ES exposure. The current study adopted an *in vitro* co-culture system for silicosis from Lv et al. (2022). This system consists of three rat cell lines: alveolar type II cells (RLE-6TN) (AT2 cells), alveolar macrophages (NR8383), and alveolar fibroblast cells (RFL-6), which represent cell types with important roles in the alveolar response to inhaled particles. The co-culture was established in a Transwell system, with the AT2 cells and macrophages in the apical compartment and the fibroblasts in the basolateral compartment. In the lung, macrophages and AT2 cells contribute to the immune responses, while AT2 cells also secrete pulmonary surfactant and participate in alveolar epithelial repair (Mason, 2006; Ruaro et al., 2021). Fibroblasts help maintain the structure of the alveoli and contribute to extracellular matrix production and remodeling (Tsukui et al., 2024). Therefore, this model incorporates epithelial, immune, and mesenchymal cell types involved in the initiation and progression of silicosis.

Cytotoxicity is a conventional endpoint in *in vitro* study used to evaluate the adverse effects of a substance on cells, and the lactate dehydrogenase (LDH) assay is commonly used for

this purpose. LDH is a cytosolic enzyme that is released into the cell culture following loss of plasma membrane integrity, in other words, membrane damage. An increase in extracellular LDH can be used as an indicator of membrane damage and cytotoxicity. Previous studies of ES and crystalline silica have used LDH release to assess cytotoxicity (Pavan et al., 2016; Ramkissoon et al., 2024).

Although LDH release can indicate loss of cellular membrane integrity, it evaluates cytotoxicity across a cell population and does not directly assess the integrity of an epithelial monolayer. Transepithelial electrical resistance (TEER) is a non-invasive and quantitative method used to evaluate barrier integrity of epithelial and endothelial cell monolayers by measuring their electrical resistance (Srinivasan et al., 2015). In practice, cells are grown on a permeable membrane in a two-compartment system, with electrodes placed in the apical and basolateral compartments. The EVOM instrument applies a low-frequency alternating current across the monolayer and measures the resulting resistance. In general, resistance is higher when the epithelial monolayer is intact and decreases when barrier integrity is compromised.

This chapter evaluated the effects of size-characterized ES particles on cytotoxicity and epithelial barrier integrity. Cytotoxicity was assessed by measuring LDH release from the co-culture system at 24, 48, and 72 hours after exposure, while the barrier integrity of RLE-6TN AT2 cells monolayer was evaluated by TEER measurements for up to 96 hours after exposure. It was hypothesized that ES exposure would increase cytotoxicity and reduce epithelial barrier integrity in a dose- and time-dependent manner.

3.2 Materials and Methods

3.2.1 Cell lines

Rat alveolar type II RLE-6TN cells (AT2 cells), alveolar macrophages, NR8383, and alveolar fibroblast RFL-6 cells were used in this study and were obtained from the American Type Culture Collection (ATCC, Manassas, VA). RLE-6TN cells were isolated from the lung of a 56-day-old male Fischer 344 rat, NR8383 cells were established from alveolar macrophages obtained by lung gavage from a wildtype rat, and RFL-6 cells were derived from the lung of a normal 18-day-gestation Sprague-Dawley rat fetus. The complete growth media of each cell line and the co-culture medium used are shown in Table 3. Cells were maintained at 37°C in a humidified incubator with 5% CO₂.

Table 3. Complete growth media of RLE-6TN, NR8383, RFL-6 cells and medium used for the co-culture system.

Cell line	Cell type and passage number range (PNR)	Complete medium
RLE-6TN	Alveolar type II cells (Passage 5-18)	F-12 (Ham's) medium, 10% FBS (fetal bovine serum), 1% penicillin-streptomycin, 2 mM L-glutamine, 10 µg/mL bovine pituitary extract, 5 ng/mL insulin, 2.5 ng/mL insulin-like growth factor (IGF-1), 1.25 ng/mL transferrin, 2.5 ng/mL epidermal growth factor (EGF)
NR8383	Alveolar macrophages (Passage 4-18)	F-12 (Ham's) medium, 15% FBS, 2 mM L-glutamine, 1% penicillin-streptomycin
RFL-6	Alveolar fibroblast cells (Passage 5-18)	F-12 (Ham's) medium, 20% FBS, 1% penicillin-streptomycin
Co-culture		F-12 (Ham's) medium, 15% FBS, 1% penicillin-streptomycin

Manufacturer:

F-12 (Ham's) medium, FBS, penicillin-streptomycin, L-glutamine: Gibco, Waltham, MA; **Bovine pituitary extract:** ScienCell, Carlsbad, CA; **Insulin:** MedChemExpress, Monmouth Junction, NJ; **IGF-1:** R&D Systems, Minneapolis, MN; **Transferrin, EGF:** Sigma-Aldrich, St. Louis, MO

3.2.2 Co-culture in the Transwell system

Twenty-four hours before *in vitro* exposure, RLE-6TN cells were seeded at 50,000/cm² in the apical compartment of a 6-well Transwell system (catalog number: 3450; Corning, Corning, NY) while RFL-6 were seeded at 21,000/cm² in a separate 6-well tissue culture plate. The transwell insert is made of polyester (PET) membrane with a 4.67 cm² growth surface area and 0.4 µm pore size. These seeding densities corresponded to approximately 233,500 RLE-6TN cells and 200,000 RFL-6 cells per well. Cells were kept in their corresponding growth media. Following *in vitro* exposure as described in section 2.2.5, growth media for individual cell lines were replaced with co-culture medium. NR8383 cells were seed into the apical compartment containing the exposed RLE-6TN cells at a NR8383/ RLE-6TN ratio of 1:5. Cells were kept at 37°C with 5% CO₂ in the incubator. At 24, 48, and 72 hours post-exposure, cells and/or cell culture media supernatant were collected for endpoint analyses. About 500 to 700 µL and 1500 µL of cell culture media supernatant was collected from the apical and basolateral compartment respectively.

3.2.3 *In vitro* exposure

On the day of exposure, an ES particle stock suspension of 5 mg/mL was prepared, and the exposure was done using the air liquid interface (ALI) system as described in section 2.2.5. In brief, the growth medium was removed from the apical compartment of the Transwell inserts containing RLE-6TN cells, and the inserts were transferred to the ALI exposure system. Particle suspensions were nebulized to achieve nominal target surface doses of 0, 1, 2.5, or 5 µg/cm². Following exposure, inserts were removed from the ALI system, NR8383 cells were added to the apical compartment, and the inserts were placed into 6-well tissue culture plates containing RFL-6 cells to establish the co-culture system. It is important to note that RLE-6TN cells were

exposed first and the macrophages and fibroblasts were incorporated afterward. The complete co-culture was therefore not directly exposed to the aerosol.

3.2.4 Cytotoxicity following *in vitro* engineered stone exposure

Cytotoxicity was assessed using LDH release assay (Roche, Mannheim, Germany). Culture-medium supernatant from the apical and basolateral compartments of the co-culture system were collected 24, 48, 72 hours after exposure. Cell-free culture medium was used as a background control, medium from untreated cells as a negative control, and medium from cells physically damaged by scraping with a pipette tip was used as a positive control. Supernatant was transferred to a 96-well plate and mixed with the reaction mixture containing the catalyst and the dye solutions. The plate was incubated in the dark for 30 minutes, and absorbance was measured at 490 nm with a reference wavelength of 620 nm using a microplate reader (Tecan Life Sciences, Männedorf, Switzerland). LDH release was calculated relative to the positive control according to the manufacturer's instructions:

$$\text{Cytotoxicity (\%)} = \frac{\text{Sample LDH} - \text{Low-control LDH}}{\text{High-control LDH} - \text{Low-control LDH}} \times 100$$

3.2.5 Transepithelial electrical resistance (TEER) of RLE-6TN cells

Twenty-four hours before *in vitro* exposure, RLE-6TN cells were seeded at 100,000 cells/cm² in the apical compartment of a 24-well Transwell system with a growth surface area of 0.33 cm², corresponding to approximately 33,000 cells per well. Baseline transepithelial electrical resistance (TEER) was measured using an EVOM™ Manual TEER Measuring System (World Precision Instruments, Sarasota, FL). 500 µL of cell medium at 37°C was added to the basolateral compartment of the measuring system and the transwell insert had 200 µL of cell medium. The first measurement taken each day was a cell-free reading where the system will

automatically subtract. During measurement, only the insert being measured was out of the incubator while the rest were kept in the incubator. Cells were then exposed to Min-U-Sil 5 or ES particles (samples A-D) at 5 $\mu\text{g}/\text{cm}^2$ using the ALI system. After exposure, cells were returned to the incubator, and TEER was measured at 24, 48, 72, and 96 hours post-exposure. Reported TEER was calculated by multiplying TEER reading by the surface area of the transwell insert (i.e., Reported TEER ($\Omega \cdot \text{cm}^2$) = TEER (Ω) x 0.33 cm^2). Reported TEER was then log transformed and change in reported TEER (Δ TEER) was the difference of log-transformed TEER between two consecutive time points.

3.2.6 Statistical analysis

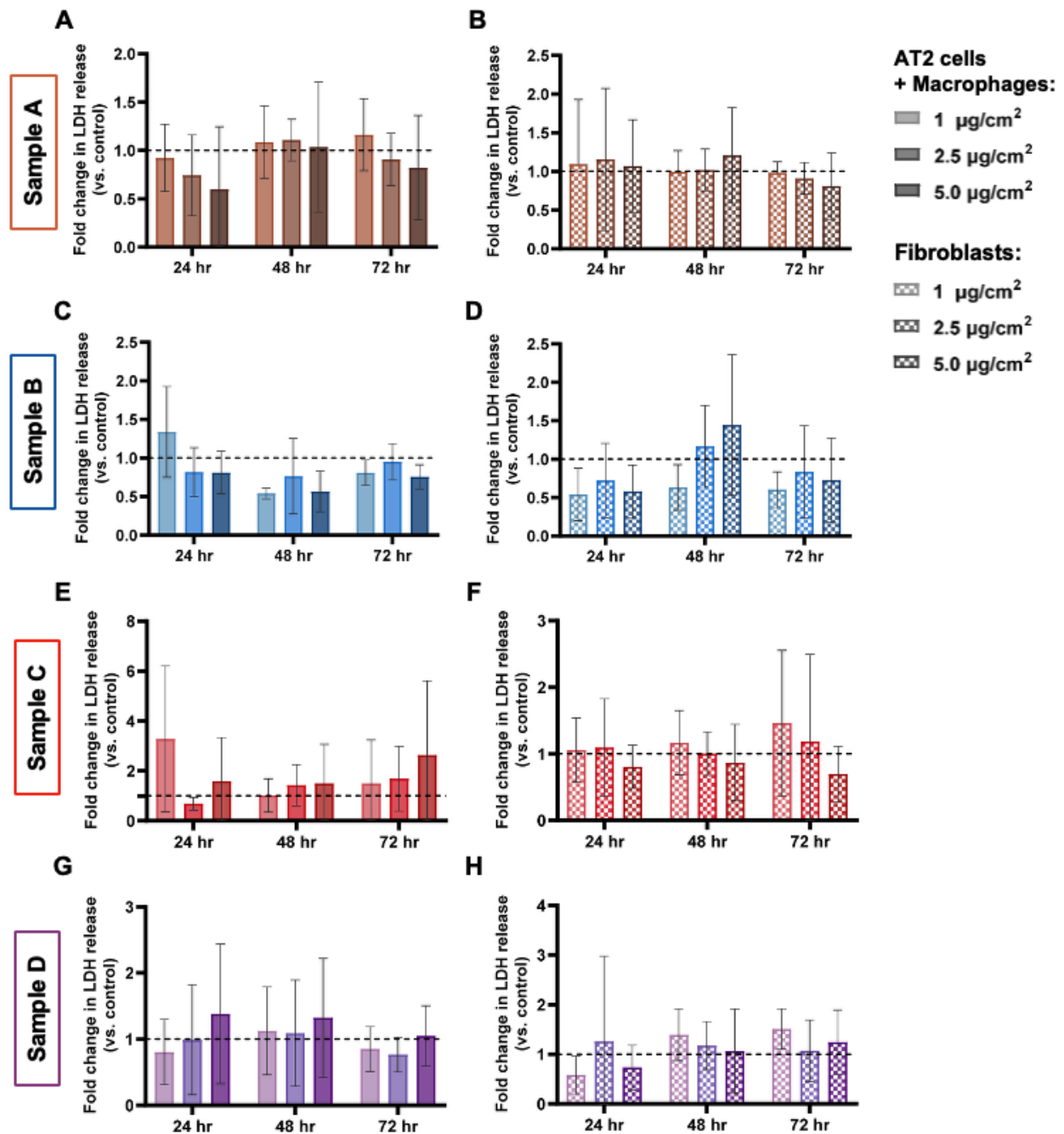
Data are presented as the mean $\pm$ standard deviation (SD), unless otherwise stated. Statistical analyses were performed using Prism GraphPad 11 (GraphPad Software, San Diego, CA). LDH release was analyzed using two-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons to compare each exposure group with the corresponding control. TEER was analyzed using sing two-way ANOVA with Dunnett's multiple comparisons to compare measurements across and within each time points. Statistical significance was defined as $p < 0.05$.

3.3 Results

3.3.1 Cytotoxicity following ES exposure

Cell medium was collected from both the apical and basolateral compartments of the Transwell system to assess LDH release from the co-culture system at 24, 48, and 72 hours after exposure (Figure 4). Exposure to ES samples A through D did not significantly increase LDH release at any of the concentrations or time points evaluated. In contrast, Min-U-Sil 5 significant

increased LD release at 48 hours following exposure to 2.5 and 5 $\mu\text{g}/\text{cm}^2$. This increase was observed in both the apical compartment containing AT2 cells and macrophages and the basolateral compartment containing fibroblasts. No significant increases were observed for Min-U-Sil 5 at 24 or 72 hours.



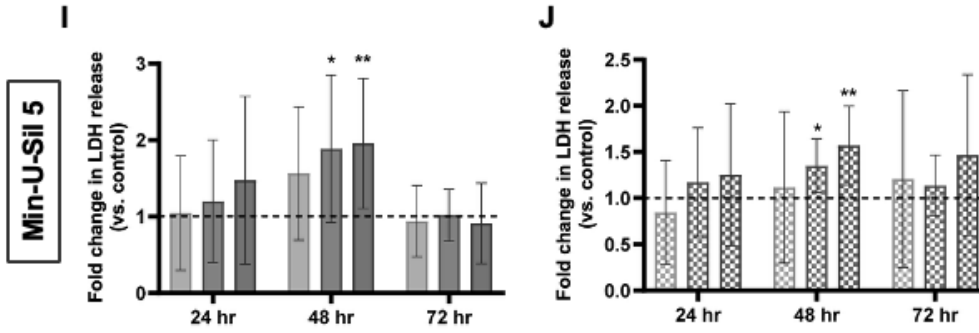


Figure 5. Cytotoxicity following exposure to ES and Min-U-Sil 5 U using LDH release assay. Cytotoxicity was evaluated by measuring the release of LDH in cell culture-medium supernatants collected from the apical and basolateral compartments at 24, 48, and 72 hours after exposure to ES samples A through D (A–H) or Min-U-Sil 5 (I–J). Results are represented as fold change relative to the corresponding control (dotted line). Data are mean $\pm$ SD, $n = 3-9$. Two-way ANOVA followed by Tukey’s multiple comparisons test. *, $p < 0.05$; **, $p < 0.01$ compared with the corresponding control.

3.3.2 Epithelial barrier integrity following ES exposure

Epithelial barrier integrity was evaluated in RLE-6TN AT2 cells monolayers exposed to ES or Min-U-Sil 5 at a nominal target dose of $5 \mu\text{g}/\text{cm}^2$. TEER was measured at 24, 48, 72, and 96 hours post-exposure, and change in log transformed reported TEER (Δ TEER) between time points was calculated (Figure 5). Δ TEER remained stable with no statistically significant change through 72 hours post-exposure for all samples. At 96 hours post-exposure a significant decline in TEER was observed in cells exposed to samples A and D by ($p = 0.0367$ and $p = 0.0052$, respectively). When compared to control at 96 hours post-exposure, sample D had a significant decrease in Δ TEER ($p = 0.0271$). Δ TEER for AT2 cells exposed to PBS remained stable throughout all the time points. Samples A, B, and D showed consistent TEER until the 72-hour time point, after which TEER decreased between 72 and 96 hours post-exposure, with samples A and D showing a statistically significant decrease. AT2 cells exposed to sample C showed a

steady decrease in TEER across time points while Min-U-Sil 5 showed an increase through 72 and 96 hours post-exposure, however, these changes were not statistically significant.

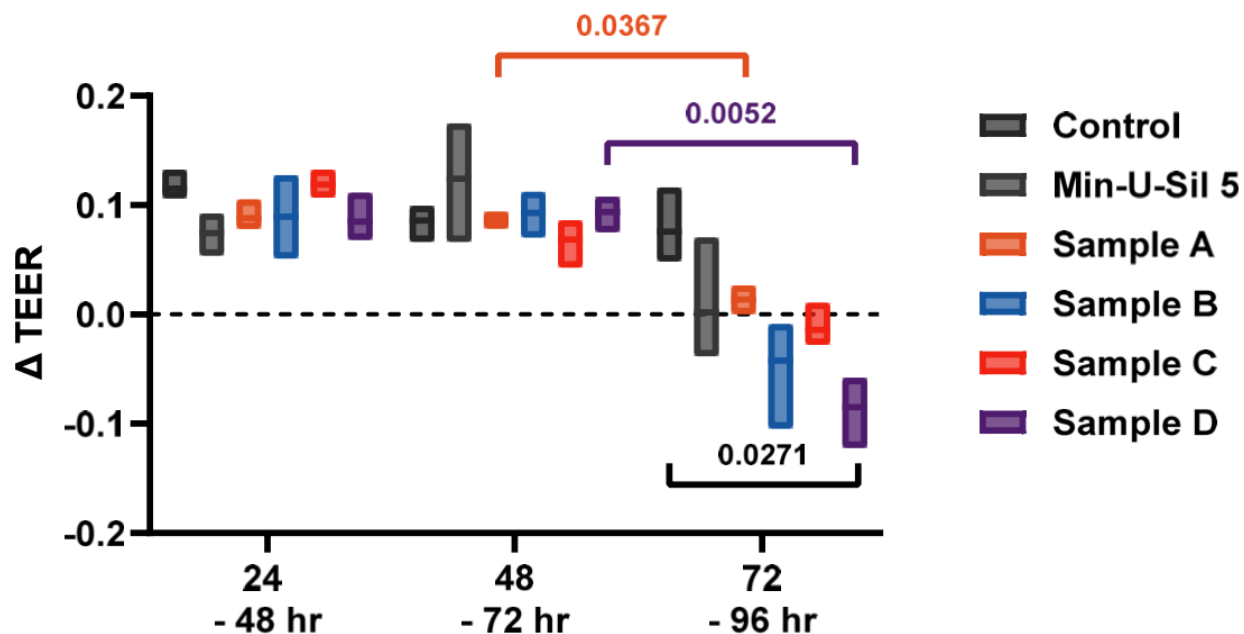


Figure 6. Epithelial barrier integrity following exposure to ES. Transepithelial electrical resistance TEER was measured in RLE-6TN (AT2) cells monolayers exposed to ES or Min-U-Sil 5 at a nominal target dose of $5 \mu\text{g}/\text{cm}^2$ at 24, 48, 72, and 96 hours post-exposure. The change in log-transformed TEER (ΔTEER) between time points was calculated. There was no statistically significant change in ΔTEER among all the samples until after 72 hours post-exposure. AT2 cells exposed to samples A and D showed a significant decrease ($p = 0.0367$ and $p = 0.0052$, respectively) in TEER between 72 and 96 hours post-exposure. At 96 hours post-exposure, sample D also showed significant decrease in TEER ($p = 0.0271$) when comparing to control. The line in the box represents the median; $n = 3$. p -value shown indicates significant differences compared to the previous time point among each sample using two-way ANOVA with Dunnett's multiple comparisons.

3.4 Discussion

This chapter assessed cytotoxicity and epithelial barrier integrity after ES exposure. Cytotoxicity was measured using an LDH release assay, which showed no significant increases following exposure to ES samples A through D, while Min-U-Sil 5 produced a significant

increase at 48 hours at 2.5 and 5 $\mu\text{g}/\text{cm}^2$. TEER was used to evaluate epithelial barrier integrity of AT2 cells. Cells exposed to ES samples and Min-U-Sil 5 demonstrated stable TEER measurements until 96 hours post-exposure, where samples A and D showed a significant decrease in TEER. Together, these findings suggest limited plasma membrane damage after ES exposure under the conditions evaluated, with delayed and sample-specific changes in epithelial barrier function.

In contrast to the significant increase in LDH release observed following Min-U-Sil 5 at 48 hours, ES exposure produced minimal non-significant changes in LDH release compared with the corresponding control. These findings suggest that ES particles did not cause detectable increases in plasma membrane damage under the concentrations and exposure conditions evaluated. Cells exposed to Min-U-Sil 5 showed a different response, with a significant increase in LDH release for Min-U-Sil 5 at 2.5 and 5 $\mu\text{g}/\text{cm}^2$ at 48 hours in both the apical compartment containing AT2 cells with macrophages and the basolateral compartment containing fibroblast. Min-U-Sil 5 produced a transient increase in LDH activity, which was not observed at 72 hours.

The z-average of Min-U-Sil 5 was 408.1 nm (Table 2), which was close to the 400-nm pore size of the Transwell membrane. Although a fraction of smaller silica particles may have crossed the membrane, the z-average represents an ensemble hydrodynamic diameter and does not demonstrate particle translocation. Direct contact between Min-U-Sil 5 particles and the fibroblasts therefore remains a possible explanation that would require confirmation by measuring particle movement across the membrane.

The lack of significant increase in LDH release following ES exposure in this study is consistent with some published findings but differs from studies reporting ES- or silica-induced cytotoxicity. Ramkissoon et al. (2024) observed increased LDH release in A549 and THP-1 cells

exposed under submerged conditions to 200 µg/mL of ES particles, while Rafieepour et al. (2023) reported reduced A549 cell viability primarily at higher silica concentrations. In contrast, Ophir et al. (2025), Skuland et al. (2020), and Kim et al. (2015) reported little or no change in cell viability or LDH release under their respective experimental conditions. Differences among these findings may reflect variation in cell models, exposure routes, particle doses, exposure duration, particle size and morphology, material composition, and the endpoints used to assess cytotoxicity. Ramkissoon et al. also associated selected metals in ES particles with THP-1 cytotoxicity, indicating that silica content alone may not account for the biological effects of ES dust. Collectively, the current findings indicate that ES exposure did not cause a detectable increase in plasma membrane damage at the doses and time points evaluated, but they do not exclude other sublethal cellular responses.

The TEER responses were sample-specific and did not correspond consistently with the silica-content classification. Samples A and D, representing low- and high-silica-content ES, respectively, showed significant decreases between 72 and 96 hours, while sample C showed a gradual but non-significant decrease. A reduction in TEER indicates decreased electrical resistance across the epithelial monolayer and may reflect changes in junctional organization, cell coverage, or cellular morphology; however, it does not directly demonstrate tight-junction disruption. The delayed response may involve cellular processes that developed after the initial exposure, including pro-inflammatory signaling and changes in epithelial junctional organization. ES and silica exposure can promote the release of cytokines such as TNF- α , IL-1 β , IL-6, and IL-8 (Ramkissoon et al., 2024), and several cytokines have been shown to affect junctional proteins and epithelial or endothelial permeability (Capaldo & Nusrat, 2009; Petecchia et al., 2012; Wittekindt, 2017). Epithelial mesenchymal transition (EMT), which involves loss of

epithelial cell-cell adhesion, may also contribute to reduced barrier integrity and subsequent tissue remodeling (Kyuno et al., 2021; Lamouille et al., 2014; Shi et al., 2025; Stone et al., 2016). However, tight-junction proteins, permeability, and EMT markers were not evaluated in this study; therefore, these mechanisms remain possible explanations rather than conclusions established by the TEER results.

Few studies have evaluated epithelial barrier integrity following ES exposure. Ophir et al. (2025) reported delayed decreases in TEER in Calu-3 cells following ES exposure, but not following Min-U-Sil 5 exposure, without a corresponding reduction in cell viability. This pattern is broadly consistent with the current findings, in which changes in barrier function occurred at later time points without marked LDH release. However, differences in cell models, exposure conditions, doses, and methods of assessing viability limit direct comparison between the studies.

Overall, ES exposure did not significantly increase LDH release under the conditions evaluated, suggesting limited plasma membrane damage. In contrast, samples A and D produced delayed decreases in TEER, indicating sample-specific changes in epithelial barrier function. These effects did not correspond consistently with silica-content classification and may involve other particle characteristics or delayed cellular responses. Chapter 4 evaluates inflammatory and fibrotic responses that may help explain the biological effects observed in the absence of marked acute toxicity.

CHAPTER 4: Inflammatory Profile and Fibrotic Cellular Response

4.1 Introduction

Silicosis is not a new disease, as it has long been recognized among workers exposed to respirable crystalline silica, including miners and sandblasters, who were diagnosed with silicosis due to inhalation of silica dust. Accordingly, numerous studies have investigated the potential mechanisms involved in disease development and progression (Kawasaki, 2015; She et al., 2021). As discussed in Chapter 1, lung macrophages attempt to clear silica particles through phagocytosis, however, silica uptake can activate macrophages and promote the release of inflammatory mediators, contributing to the persistent inflammation associated with silicosis (Hoy & Chambers, 2020). Therefore, evaluating the inflammatory response of cells exposed to ES particles is important for understanding their potential role in silicosis. In this study, a multiplex immunoassay was used to measure ten cytokines and chemokines representing innate inflammatory, regulatory, and T helper-associated responses.

The cytokines and chemokines included in the assay can be broadly categorized into four groups based on their principal immunological functions: innate pro-inflammatory, anti-inflammatory, T helper type 1 (Th1)-associated, and T helper type 2 (Th2)-associated mediators. The innate pro-inflammatory group includes TNF- α , IL-1 β , IL-6, and KC/GRO. These cytokines/chemokines are triggered when innate immunity is activated, starting with TNF- α and IL-1 β , which are early mediators of innate immune activation and can promote the subsequent production of IL-6 and chemokines such as KC/GRO. KC/GRO contribute to neutrophil recruitment, while IL-6 has broader roles in inflammation, immune regulation and tissue repair (Chen & Manning, 1996). IL-10, an anti-inflammatory cytokine included in the assay, is an

important regulator of inflammation that can suppress pro-inflammatory cytokine production and limit excessive inflammation (Ip et al., 2017).

IL-2 and IFN- γ are commonly associated with Th1 responses, which contribute to cell-mediated immunity against intracellular pathogens such as bacteria and viruses (Berger, 2000). IL-2 promotes T-cell proliferation and can support Th1 differentiation and IFN- γ production (Kasahara et al., 1983). The Th2-associated cytokines included in the assay are IL-4, IL-5, and IL-13. Although commonly associated with allergic and type 2 immune responses, they also participate in epithelial barrier regulation, airway remodeling, and pulmonary fibrosis (Akdis et al., 2020; Buchheit et al., 2024; Savin et al., 2023; She et al., 2021). Because inflammation is a central feature of silicosis, measuring this panel provides a broader assessment of the inflammatory and immune-related mediators released after AT2 cells and macrophages are exposed to ES particles.

As discussed in Chapter 3, lung fibroblasts are essential for maintaining alveolar structure and also participate in tissue repair (White, 2015). However, persistent inflammation or injury in the lung can promote fibroblast activation and their transition toward a myfibroblast phenotype through pathways involving TGF β , Wnt/ β -catenin, and NF- κ B signaling (Zhao & Su, 2025). The accumulation of activated myfibroblasts and excessive production of extracellular matrix (ECM) are important features of fibrosis progression (Ortiz-Zapater et al., 2022). Alpha-smooth muscle actin (α -SMA) is widely used as a marker of fibroblast activation and myfibroblast differentiation, although it is not specific to myfibroblasts. Its incorporation into stress fibers contributes to increased contractile activity characteristic of activated myfibroblasts (Gabbiani, 2021; Hinz et al., 2001). Therefore, α -SMA expression was evaluated in RFL-6 fibroblasts as an indicator of fibroblast activation following ES particle exposure.

Thus, this chapter evaluated inflammatory mediator release from ES-exposed AT2 cell-macrophage cultures and α -SMA expression in RFL-6 fibroblasts to investigate inflammatory and fibroblast responses relevant to the development of silicosis.

4.2 Methods

4.2.1 Multiplex immunoassay

Cell culture medium supernatant from the apical compartment of the Transwell system was collected 24 hours after exposure, and the supernatant was stored at -80°C until the day of the immunoassay. On the day of the assay, cell culture medium supernatant was centrifuged at 300 x g for five minutes to remove cell debris in supernatant, and it was diluted at a 1:4 ratio. Three biological replicates were used in the assay where each replicate was duplicated. Concentrations of ten cytokines and chemokines associated with inflammatory and immune responses, IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, KC/GRO (CXCL1), and TNF- α , were measured using the multiplex electrochemiluminescence immunoassay V-PLEX Proinflammatory Panel 1 Rat Kit (Meso Scale Diagnostics, Rockville, MD) according to manufacturer's instructions. Measurements below the lower limit of detection were excluded in the analysis.

4.2.2 Immunofluorescence staining of RFL-6 cells

At 72 hours after *in vitro* exposure, RFL-6 cells were fixed for ten minutes with 10% neutral buffered formalin and permeabilized for five minutes with 0.5% Triton X-100 in PBS. Nonspecific binding was blocked for ten minutes using 10% FBS in PBS. Cells were stained overnight at 4°C in the dark with Alexa Fluor 488-conjugated alpha-smooth muscle actin (α -SMA) antibody (catalog number: 53-9760-82; Invitrogen, Carlsbad, CA) and NucBlue™ Fixed

Cell ReadyProbes™ Reagent (DAPI) (catalog number: R37606; Invitrogen, Carlsbad, CA). The cells were mounted on microscope slides with VECTASHIELD® antifade mounting medium (Vector Labs, Newark, CA). Images were acquired using a Keyence BZ-X800 fluorescence microscope (Keyence, Itasca, IL), and microscope settings such as exposure time and magnification (20x) were held constant among all the groups. At least nine fields were imaged per sample, and α -SMA fluorescence was evaluated qualitatively.

4.2.3 Statistical analysis

Data are presented as the mean $\pm$ standard deviation (SD), unless otherwise stated. Statistical analyses were performed using Prism GraphPad 11 (GraphPad Software, San Diego, CA). Cytokine and chemokine concentrations measured using the multiplex immunoassay were analyzed by two-way ANOVA, followed by multiple-comparisons test comparing each exposure group with its corresponding control. Statistical significance was defined as $p < 0.05$.

4.3 Results

4.3.1 Inflammatory response of alveolar type II cells with macrophages after ES exposure

At 24 hours after ES exposure, concentrations of ten cytokines and chemokines were measured in medium collected from the apical compartment containing AT2 cells and macrophages. The response varied among ES samples, with samples B and C generally producing the strongest increases in pro-inflammatory mediators.

Exposure to samples B and C increased TNF- α release to approximately three times the control level at all tested concentrations ($p < 0.0001$), whereas TNF- α levels following exposure to samples A and D were comparable to the control (Figure 7A). Sample B at 1 $\mu\text{g}/\text{cm}^2$ produced a small but statistically significant decrease in IL-1 β ($p = 0.0102$). In contrast, sample C

increased IL-1 β release to approximately twice the control level at all concentrations ($p < 0.0001$; Figure 7B). IL-1 β levels following exposure to samples A and D were similar to the control. IL-6 release tended to be higher following exposure to samples A and C, but only the increase produced by sample C at 5 $\mu\text{g}/\text{cm}^2$ was statistically significant ($p = 0.0397$; Figure 7C). All four ES samples significantly increased KC/GRO release at each concentration tested (samples A-C: $p < 0.0001$; sample D: $p = 0.0036$ at 1 $\mu\text{g}/\text{cm}^2$, $p = 0.0023$ at 2.5 $\mu\text{g}/\text{cm}^2$, $p = 0.0008$ at 5 $\mu\text{g}/\text{cm}^2$; Figure 7D). Samples A, B, and C increased KC/GRO concentrations by approximately two- to three-fold, whereas the increases induced by sample D were smaller. Overall, the pro-inflammatory response was sample-dependent, with samples B and C producing the most consistent increases across the measured mediators.

No statistically significant difference was observed in Th1-associated cytokines IFN- γ and IL-2 following ES exposure. IFN- γ release was highest after exposure to sample D at 1 $\mu\text{g}/\text{cm}^2$ and decreased at higher concentrations, although these differences were not statistically significant (Figure 7E). IL-2 levels appeared higher following exposure to sample B but did not differ significantly from the control. Samples A, C, and D produced IL-2 levels similar to the control, and IL-2 level was below the lower limit of detection following exposure to sample D at 5 $\mu\text{g}/\text{cm}^2$ (Figure 7F). IL-10 release was generally lower following exposure to samples A, B and C. However, only sample C at 1 $\mu\text{g}/\text{cm}^2$ produced a statistically significant decrease relative to control ($p = 0.0352$; Figure 7G). IL-10 levels following sample D exposure were comparable to the control.

In summary, Th2-associated cytokines, IL-4, IL-5, and IL-13 were detected at relatively low levels. IL-4 release increased with concentration, and all four ES samples produced statistically significant increases at 5 $\mu\text{g}/\text{cm}^2$ ($p = 0.0444$; Figure 7H). IL-4 was below the lower

limit of detection following exposure to sample A at 1 $\mu\text{g}/\text{cm}^2$. IL-5 levels were generally higher after ES exposure, but only sample D at 2.5 $\mu\text{g}/\text{cm}^2$ produced a statistically significant increase ($p = 0.0188$) (Figure 7I). IL-13 was below the lower limit of detection in most samples. It was detected following exposure to sample D, but the measured levels were comparable to the control (Figure 7J).

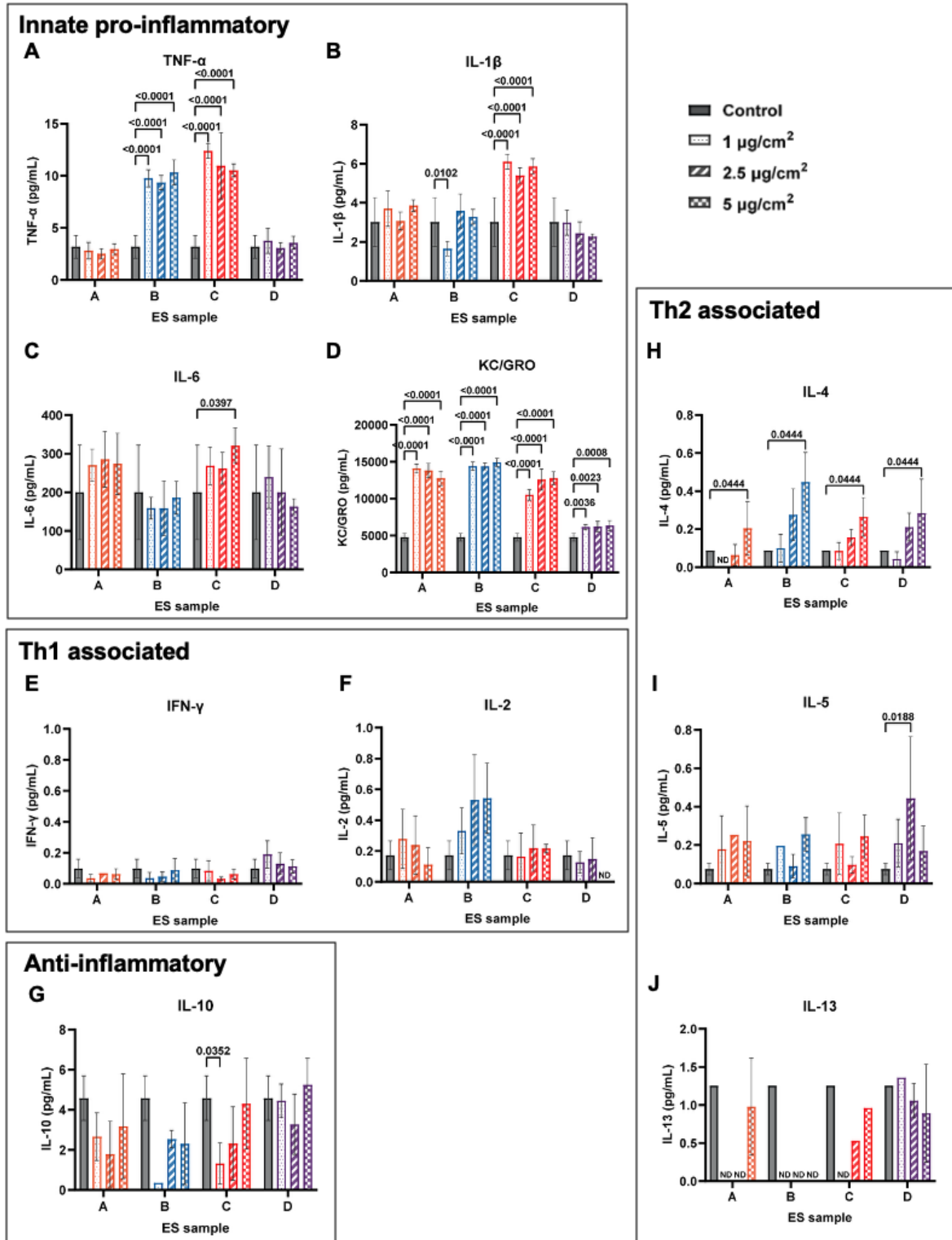


Figure 7. Inflammatory mediator release from AT2 cells and macrophages 24 hours after ES exposure. Medium from the apical compartment of the Transwell system was collected 24 hours after exposure to ES samples A-D at 0, 1, 2.5, and 5 $\mu\text{g}/\text{cm}^2$. Concentrations of ten cytokines and chemokines were measured using a multiplex immunoassay and grouped as follows: innate pro-

inflammatory mediators, TNF- α , IL-1 β , IL-6, and KC/GRO (A-D); Th1-associated cytokines, IFN- γ and IL-2 (E-F); IL-10 (G); Th2-associated cytokines IL-4, IL-5, and IL-13 (H-J). Samples B and C produced the most consistent increases in pro-inflammatory mediators. All ES samples increased KC/GRO release, and exposure to 5 $\mu\text{g}/\text{cm}^2$ increased IL-4 release. Data are presented as mean $\pm$ SD, $n = 6$. Statistical differences relative to the corresponding control were evaluated using two-way ANOVA followed by Tukey's multiple comparisons test for IL-2, IL-4, and IL-13, and Dunnett's multiple-comparisons test for the remaining mediators. Exact p -values are shown in the figure. ND, not detected or below the lower limit of detection.

4.3.2 Fibroblast activation

Alpha smooth muscle actin (α -SMA) staining showed differences among fibroblasts co-cultured with AT2 cells and macrophages exposed to the four ES samples. Representative images are shown in Figure 8, and α -SMA fluorescence was evaluated quantitatively. Minimal α -SMA fluorescence was observed following exposure to samples A and B across the tested concentrations. Fibroblasts associated with sample C and Min-U-Sil 5 exposure showed moderate increase in α -SMA fluorescence at 5 $\mu\text{g}/\text{cm}^2$. Sample D produced the most evident concentration-related pattern, with α -SMA fluorescence increasing across concentrations and the strongest signal observed at 5 $\mu\text{g}/\text{cm}^2$. These qualitative observations suggest that the fibroblasts response varied among the particle samples, with sample D producing the most pronounced α -SMA staining,

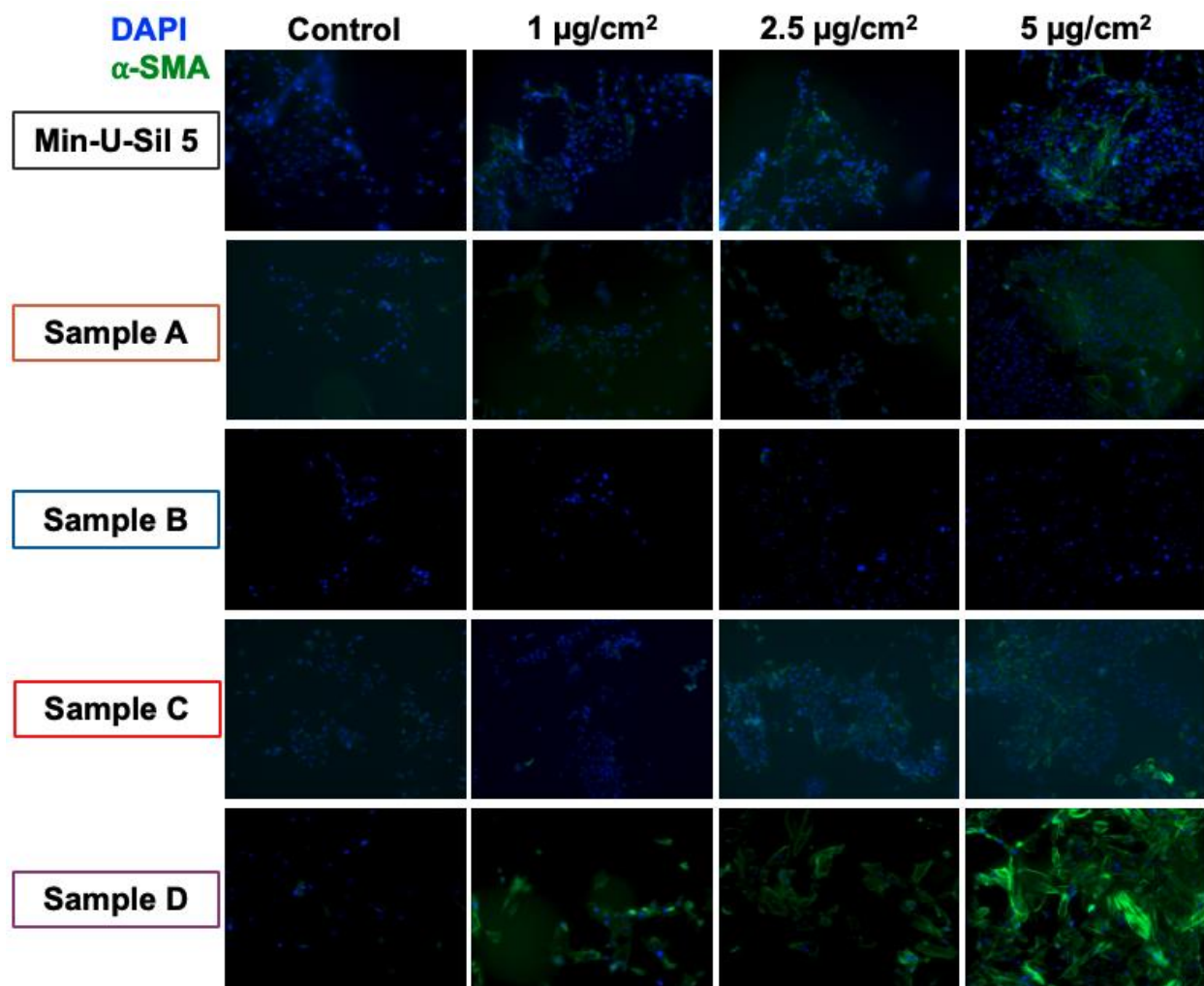


Figure 8. α -SMA expression in fibroblasts following ES exposure. RFL-6 fibroblasts in the basolateral compartment were stained for α -SMA 72 hours after ES exposure of AT2 cells and macrophages in the apical compartment to Min-U-Sil 5 or ES samples A-D at 0, 1, 2.5, or 5 $\mu\text{g}/\text{cm}^2$. Nuclei were stained with DAPI. Samples A and B produced minimal α -SMA fluorescence across the tested concentrations, whereas sample C and Min-U-Sil 5 produced moderately higher fluorescence at 5 $\mu\text{g}/\text{cm}^2$. Sample D produced an apparent concentration-related increase in α -SMA fluorescence. All images were acquired using the same exposure settings. Representative images from three independent experiments are shown. Images were evaluated qualitatively; α -SMA fluorescence was not quantified.

4.4 Discussion

This chapter evaluated inflammatory mediator release from AT2 cell-macrophage cultures and α -SMA fluorescence in fibroblast following exposure to four ES samples. The

inflammatory response varied among samples, with samples B and C producing the most consistent increases in innate pro-inflammatory mediators. KC/GRO was the only mediator increased by all four samples. Qualitative α -SMA staining also differed among samples, with the strongest concentration-related pattern observed following sample D exposure. Together, these findings indicate that the cellular responses to ES particles were sample dependent and were not explained by crystalline silica content alone.

The assessment of inflammatory response demonstrated that ES exposure mostly induces cytokines associated to innate immunity in the cells. Silica in ES has been showed to be associated to elevated inflammation via transcription factor NF- κ B, NLRP3 inflammasome, and reactive oxygen species (ROS), which subsequently lead to development of silicosis (Castranova, 2004; Marrocco & Ortiz, 2022). Studies have shown that silica in ES can activate the transcription factor NF- κ B, which is crucial for induction of inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6 (Liu et al., 2017; Peeters et al., 2014; Yin et al., 2022; J. Zhang et al., 2022). In addition, silica is capable of triggering the maturation of IL-1 β via NLRP3 inflammasome (Hornung et al., 2008; Kelley et al., 2019). Other studies have shown generation of ROS from cells exposed to silica where subsequently leads to increase in inflammation (Låg et al., 2018; Park & Park, 2009; Refsnes et al., 2019). KC/GRO is the rodent functional homolog of CXCL-1. It is a chemokine that recruits neutrophils to the sites of tissue injury, and it promotes cell growth. CXCL-1 can also be induced by other cytokines such as TNF- α and IL-1 β (Korbecki et al., 2022; Olivera et al., 2022). Increase in innate pro-inflammatory cytokines suggests that ES particles can cause tissue injury and lead to subsequent inflammatory response.

Besides innate inflammatory response, Th1- and Th2-associated cytokines were also assessed. IFN- γ and IL-2, Th1-associated cytokines, showed no statistically significant changes

post ES exposure. In addition, for Th2-associated cytokines, IL-4, IL-5, and IL-13, results were largely non-significant except the increase in IL-4 at the highest concentration for all ES samples and in IL-5 at 2.5 $\mu\text{g}/\text{cm}^2$ for sample D. Although it is mostly a null result, it is still informative as it suggests response from ES particle exposure is mainly innate pro-inflammatory associated, whereas Th1-associated cytokines are usually induced from intracellular pathogens such as viral or bacterial infection to cells. Th2-associated cytokines are usually linked to defending parasitic infection and allergens that are too large in size to be cleared by macrophages (Berger, 2000), yet there are studies that shown these mediators are associated to fibrosis (Akdis et al., 2020; Buchheit et al., 2024; She et al., 2021). However, this co-culture model lacks lymphocytes, so the results can only speak for the difference in Th2-associated cytokine itself instead of implying activation of Th2 adaptive immunity. Furthermore, it is expected that the increased cytokines observed are linked to innate immunity due to the time point of the assay. This assay was performed with cell culture medium supernatant collected 24 hours after ES exposure, when innate immunity was in action as it is typically activated within minute or hours after exposure to foreign substance (Marshall et al., 2018).

The only anti-inflammatory cytokine assessed in this assay was IL-10, and the level was generally lower than control in cells exposed to samples A, B, and C, with significant decrease at 1 $\mu\text{g}/\text{cm}^2$ for sample C. As an anti-inflammatory cytokine, IL-10 acts as a brake on inflammatory response. It also suppresses activation of NLRP3 inflammasome and production of pro-inflammatory cytokine like IL-1 β (Ip et al., 2017). Combining elevated levels of TNF- α , IL-1 β , and KC/GRO with the decline of IL-10 for sample C suggests a disrupted balance between pro- and anti-inflammatory signaling.

Findings from previous studies were consistent with the results. Multiple studies found that exposing ES to THP-1 cells, A549 cells, and human bronchial epithelial cells (HBEC3-KT) resulted in elevated levels of pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-8 (Grytting et al., 2021; Ramkissoon et al., 2024; Song et al., 2024). Additionally, rise in level of IL-8 has been reported in patients with ES-related silicosis, whereas the level was associated to silicosis progression and death (Blanco-Pérez et al., 2021; Campos-Caro et al., 2023).

The pro-inflammatory cytokines, TNF- α (Piguet et al., 1990), IL-1 β (Borthwick, 2016; Gasse et al., 2007), IL-6 (Le et al., 2013; Shieh et al., 2019), and KC/GRO (or CXCL-1) (Cheng et al., 2024; Ishida et al., 2017) have been shown to be associated to pulmonary fibrosis. These cytokines are linked to pulmonary fibrosis by three mechanisms according to Bolourani et al. and are consistent with other studies: 1) trigger fibroblast activation directly, 2) lead to release of other pro-fibrotic cytokines such as TGF- β in immune cells or fibroblasts, or 3) bolster the autocrine or paracrine activation of fibroblasts (Bolourani et al., 2021; He et al., 2025; Kawasaki, 2015; Mutsaers et al., 2023; She et al., 2021; Wang et al., 2023).

To investigate ES-related silicosis, fibroblast activation was assessed by staining the cells with α -SMA post-ES exposure followed by qualitatively evaluating α -SMA signal. Similar to the determination of inflammatory profile, α -SMA signal was also varied among ES samples. There was a concentration-related increase in α -SMA positive cells in sample D, while a moderate boost in sample C and Min-U-Sil 5 was observed. Overall, there was very low or no α -SMA signal in samples A and B. Sample D had the strongest fibroblast activation among all ES samples despite minimal increase of pro-inflammatory cytokines except KC/GRO, suggesting a pathway beside classic innate inflammation also contributes to the activation observed. One possible pathway was through Th2-associated mediators such as IL-4, IL-5, and IL-13, which

have been shown to be associated to fibrosis (Buchheit et al., 2024; Murray et al., 2008; Saito et al., 2003; Sempowski et al., 1996). Additionally, IL-4 and IL-13 can induce myofibroblast via c-Jun NH₂-terminal kinase (JNK) (Hashimoto et al., 2001). However, increase in IL-4 and IL-5 signaling from AT2 cells and macrophages alone is not sufficient to conclude fibroblast activation observed in sample D was associated to Type 2 immune responses as lymphocytes are absent in the co-culture model. Further investigation is required to validate this possible explanation. The moderate activation observed in sample C and Min-U-Sil 5 implies that silica is one of the many factors associated to fibroblast activation. Despite significant increase of pro-inflammatory cytokines in sample B, minimal fibroblast activation was observed. One possible explanation is inflammation alone was not enough to induce the phenotype of fibrosis.

Very limited studies have evaluated fibroblast maturation after ES exposure, but multiple silicosis studies had assessed this endpoint after silica exposure. Exposure of silica to a co-culture system of macrophages and fibroblasts, isolated from male Sprague-Dawley rats, showed an increase in α -SMA level both transcriptionally and translationally (Li et al., 2017). In addition, studies have shown elevated level of α -SMA in the lungs of mice exposed to silica via immunohistochemical analysis and immunoblotting (Lam et al., 2025; Wu et al., 2021; Xu et al., 2022).

In summary, ES exposure produced sample-dependent inflammatory responses in AT2 cell-macrophage cultures, with samples B and C inducing the most consistent increases in innate pro-inflammatory mediators. KC/GRO was increased by all four samples, suggesting that neutrophil-recruiting chemokine signaling may represent a common response to ES exposure. Qualitative α -SMA staining also varied among samples, with the strongest concentration-related pattern observed in fibroblasts co-cultured with cells exposed to sample D. Because the cytokine

and α -SMA response patterns did not closely correspond, the measured inflammatory mediators alone do not explain the apparent fibroblast response. Overall, the findings support biological differences among ES samples and identify inflammatory signaling and fibroblast-associated responses as endpoints requiring further mechanistic evaluation.

CHAPTER 5: Summary and Conclusions

5.1 Summary

The aim of this study was to investigate the effects of ES particles with varying silica content using an *in vitro* respiratory co-culture model. Cytotoxicity, epithelial barrier integrity, inflammatory mediator release, and fibroblast-associated responses were evaluated. ES exposure did not induce significant cytotoxicity across the samples, concentrations, or time points tested, although a transient increase in cytotoxicity was observed 48 hours after exposure to Min-U-Sil 5. A delayed decline in barrier integrity was observed 96 hours after exposure to samples A and D.

The inflammatory and fibroblast-associated responses varied among ES samples. Samples B and C produced the most consistent increases in innate pro-inflammatory mediators, including TNF- α , IL-1 β , and KC/GRO. Sample C also produced a decrease in IL-10 at one concentration. Sample D increased the Th2-associated cytokines IL-4 and IL-5 at selected concentrations. Qualitative α -SMA signal was the strongest in fibroblasts co-cultured with cells exposed to sample D, while sample C and Min-U-Sil 5 produced moderate increases. Samples A and B produced minimal changes in α -SMA fluorescence. Overall, the biological responses did not consistently follow silica-content ranking of samples, suggesting that silica alone did not explain the observed effects. A summary of the responses across all endpoints is presented Table 4.

Table 4. Summary of the responses to ES and Min-U-Sil 5 across the evaluated endpoints.

	Cytotoxicity	Barrier integrity	Innate pro-inflammatory cytokines	Th2 associated cytokines	Fibroblast activation
Sample A	↔	↓	↑	↔	↔
Sample B	↔	↔	↑↑	↔	↔
Sample C	↔	↔	↑↑	↔	↑
Sample D	↔	↓	↑	↑	↑↑
Min-U-Sil 5	Transient ↑	↔	N/A	N/A	↑

Arrows summarize the overall response relative to the control: ↔, no clear change; ↑ or ↓, increase or decrease; ↑↑, strongest response. α -SMA results were evaluated qualitatively.

5.2 Integration of findings and possible pathways

At the concentrations examined, ES exposure did not cause significant cytotoxicity, suggesting that cell death may not be the most sensitive endpoint for detecting early effects of ES particles at relatively low concentrations. Previous studies reporting cytotoxicity following ES or silica exposure generally used concentrations of at least 20 $\mu\text{g}/\text{cm}^2$ or 200 $\mu\text{g}/\text{mL}$ (Pavan et al., 2016; Ramkissoon et al., 2024). Similarly, epithelial barrier integrity was not significantly affected between 24 and 72 hours after exposure. A decrease was observed at 96 hours following exposure to samples A and D. This delayed response may reflect biological processes that require time to develop, including inflammatory or oxidative stress responses. However, because sample A did not produce a consistent increase in the measured pro-inflammatory mediators, the barrier response cannot be attributed to inflammation alone.

The inflammatory profiles differed among ES samples. Samples B and C produced the strongest increases in innate pro-inflammatory mediators, whereas sample D increased Th2-associated cytokines IL-4 and IL-5 at selected concentrations. These cytokines have been

associated with profibrotic signaling, including pathways involving TGF- β (Cheng et al., 2024; Gasse et al., 2007; Gharaee-Kermani et al., 1998; Hashimoto et al., 2001; K. Zhang et al., 1997). For sample C the increase pro-inflammatory mediators, together with the decrease in IL-10 at 1 $\mu\text{g}/\text{cm}^2$, suggests an altered balance between pro- and anti-inflammatory signaling. In contrast, the cytokine profile of sample D suggests a possible contribution of IL-4 and IL-5-associated signaling. However, the co-culture did not contain lymphocytes; therefore, these findings represent changes in Th2-associated cytokines rather than evidence of Th2 immune activation.

The distinct cytokine profiles may help explain, at least in part, the differences in α -SMA fluorescence. Sample C produced a moderate α -SMA response, whereas sample D produced the strongest concentration-related pattern. Profibrotic cytokines can influence fibroblast phenotype through TGF- β -dependent pathways or by acting directly on epithelial cells and fibroblasts (Kokubo et al., 2022). However, sample B produced a strong pro-inflammatory response but minimal α -SMA fluorescence, indicating that the magnitude of the measured inflammatory response alone did not predict the fibroblast-associated response. Thus, different combinations of cytokines or other unmeasured factors may have contributed to the sample-specific α -SMA patterns. Because TGF- β and downstream signaling were not measured, the study does not establish which mediators caused the α -SMA response.

5.3 Overall significance

This study used an ALI co-culture model consisting of AT2 cells, macrophages, and fibroblasts to examine cellular responses relevant to ES-related silicosis. The model represents selected features of the alveolar microenvironment and allows interactions among multiple cell types. It also permits fibroblast responses to be evaluated following exposure of epithelial cells

and macrophages in the apical compartment. However, direct particle contact with fibroblasts cannot be excluded unless particle movement into the basolateral compartment was assessed.

Exposure via ALI provides greater physiological relevance than conventional submerged exposure because particles are delivered to the apical surface of the respiratory epithelium. Importantly, the study detected inflammatory and barrier-related changes at concentrations that did not produce substantial cytotoxicity. The results also demonstrate that ES samples with different compositions can produce distinct biological response patterns that are not explained by silica content alone. These findings support the need to consider the broader physicochemical composition of ES dust when evaluating its occupational health hazards.

5.4 Study Limitations

Several limitations should be considered when interpreting the results. The sample-specific patterns across endpoints suggest that silica content in ES alone is not a good indicator of particle toxicity.

The small and variable sample sizes for the TEER and multiplex immunoassay measurements limited statistical power. Cytokines were measured at one time point, and the model did not contain lymphocytes; therefore, activation of Th1, Th2, or other adaptive immune responses could not be determined. Fibroblast-associated responses were evaluated qualitatively using α -SMA alone. Quantitative analysis and measurement of additional markers, such as collagen, fibronectin, TGF- β , and fibroblast activation protein- α , would be needed to confirm myofibroblast differentiation and extracellular matrix production.

Finally, the co-culture consisted of rat-derived cell lines and represents only selected components of the alveolar environment. It does not reproduce the full complexity of the human

lung or the repeated and prolonged exposures experienced by workers. These limitations affect the direct translation of the findings to human ES-related silicosis.

5.5 Future directions

This study provides evidence that ES particles can induce inflammatory, barrier-related, and fibroblast-associated responses at concentrations that do not cause substantial cytotoxicity. It also demonstrates that these responses differ among ES samples and are not explained by silica content alone. By using a co-culture model and exposure via ALI, the study provides a foundation for investigating how the physicochemical properties and reactivity of ES particles influence early biological responses relevant to ES-related silicosis.

Future studies should focus on a more comprehensive characterization of samples A-D, with emphasis on characteristics including mineral composition and crystalline phases, particle morphology, specific surface area, surface charge, elemental and metal content, resin-associated organic compounds, and particle behavior under biologically relevant conditions. Acellular assays of oxidative potential, surface radical generation, dissolution, and other measures of particle reactivity could help determine why samples with similar silica content produced different cellular responses.

Integrating these physicochemical and reactivity measurements with the inflammatory, barrier, and fibroblast-associated endpoints could help identify the particle properties most closely associated with biological activity. These relationships could provide the foundation for a predictive framework linking ES composition and reactivity with toxicological responses. Development of a validated predictive model would ultimately require independent datasets or

collaboration across studies, but the present work can help define the variables and biological endpoints that should be included.

Additional mechanistic studies should quantify α -SMA and evaluate profibrotic markers such as collagen, fibronectin, TGF- β , and connective tissue growth factor (CTGF) to clarify the progression from early cellular responses to fibroblast remodeling. From an occupational health perspective, this approach could improve hazard assessment by moving beyond nominal silica content as the primary indicator of risk. Identifying the particle characteristics that best predict biological reactivity could inform material design, workplace controls, exposure assessment, and future regulations intended to prevent ES-related silicosis.

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