

Evaluation of the exposure, dose-response and fate in the lung and pleura of chrysotile-containing brake dust compared to TiO₂, chrysotile, crocidolite or amosite asbestos in a 90-day quantitative inhalation toxicology study – Interim results Part 1: Experimental design, aerosol exposure, lung burdens and BAL¹

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ABSTRACT

This 90-day repeated-dose inhalation toxicology study of brake-dust (BD) (brakes manufactured with chrysotile) in rats provides a comprehensive understanding of the biokinetics and potential toxicology in the lung and pleura. Exposure was 6 h/d, 5d/wk., 13wks followed by lifetime observation (~20 % survival). Control groups included a particle control (TiO₂), chrysotile, commercial crocidolite and amosite asbestos.

Aerosol fiber distributions of the chrysotile, crocidolite and amosite were similar (fibers L > 20 μm/cm³: chrysotile-Low/High 29/72; crocidolite 24; amosite 47 fibers/cm³; WHO-fibers/cm³: chrysotile-Low/High 119/233; crocidolite 181; amosite 281 fibers/cm³). The number of particles/cm³ in the BD was similar to that in the chrysotile, crocidolite & amosite exposures (BD 470–715; chrysotile 495–614; crocidolite 415; amosite 417 particles/cm³).

In the BD groups, few fibers L > 20 μm were observed in the lungs at the end of exposure and no fibers L > 20 μm at 90d post exposure. In the chrysotile groups, means of 204,000 and 290,000 fibers(L > 20 μm)/lung were measured at 89d. By 180d, means of 1 and 3.9 fibers were counted on the filter corresponding to 14,000 and 55,000 fibers(L > 20 μm)/lung.

In the crocidolite and amosite groups mean lung concentrations were 9,055,000 and 11,645,000 fibers (L > 20 μm)/lung at 89d. At 180d the means remained similar with 8,026,000 and 11,591,000 fibers (L > 20 μm)/lung representing 10–13% of the total lung fibers.

BAL determined the total number of macrophages, lymphocytes, neutrophils, eosinophils, epithelial-cells and IL-1 beta, TNF-alpha and TGF-beta. At the moderate aerosol concentrations used in this study, neutrophil counts increased ~5 fold in the amphibole asbestos exposure groups. All other groups and parameters showed no important differences at these exposure concentrations. The exposure and lung burden results provide a sound basis for assessing the potential toxicity of the brake dust in comparison to the TiO₂ particle control and the chrysotile, crocidolite and amosite asbestos control groups. The BAL results provide an initial indication of the differential response. Part 2 presents the presentation and discussion of the histopathological and confocal microscopy findings in this study through 90 days post exposure.

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¹ BAL - bronchioalveolar lavage

1. Introduction

The objective of this study was to assess the *in vivo* pathological response and fiber distribution within the lung and pleural cavity following the inhalation of brake dust from brake drums which were manufactured with chrysotile in rats. Previous studies of brake dust containing chrysotile (Bernstein et al., 2014; Bernstein et al., 2015 & Bernstein et al., 2018) have shown that the brake dust produces no pathological response following inhalation either in a short term 5 day exposure or a 28 day exposure. These studies have shown as well that the chrysotile used in the brake dust had minimal effect while the amphibole asbestos, crocidolite, produced significant pathological response in both the lung and pleural cavity. The 90-day sub-chronic inhalation toxicity study is often a pivotal study for assessing no-effect levels as well as longer term toxicity. The standard protocol design for a 90-day sub-chronic inhalation toxicity study specifies a non-exposure recovery period of up to 4 weeks (OECD 413, 2018; EPA 712-C-98-204, 1998). As fiber related disease in humans has a long latency period, to fully assess the long-term potential of the fibers to cause disease, this study was designed with a life-time (rat) non-exposure period in which the remaining rats were sacrificed when < 20% remained in any group. With 9 exposure groups and multiple endpoints, the methods and results from this study are presented in two publications. Part 1, presented here, provides the methods and results from the experimental design, aerosol exposure, lung burdens and the bronchial alveolar lavage (BAL) results. Part 2 (Bernstein et al., 2019) presents the methods and results from the histopathological examination of the lungs and the confocal microscopy examination of the lung and the pleural cavity. In addition, the fibrosis quantification performed as part of the histopathology examination and the collagen quantification of the lung as performed by the confocal examination are also presented.

The use of braking systems for automobiles had evolved from the earliest automobiles. Initially, friction materials were used that consisted of materials like camel hair, cotton belting, elmwood and cotton based materials impregnated with different ingredients (Harper, 1998; Paustenbach et al., 2004). These initial materials, however, were limited in their ability to withstand heat and control speed. From the early 1900s chrysotile fibers were found to be an effective replacement for these earlier materials. The chrysotile fibers maintained their integrity under higher temperatures which allowed the driver to brake at increased vehicle speeds (Harper, 1998). Because of these unique characteristics, chrysotile became the material of choice for vehicle brakes.

With the use of chrysotile, researchers began to investigate the degree of exposure to the fibers experienced by mechanics servicing the brakes. Short duration activities, such as removal of brake-wear debris (e.g., brake dust) from brake assemblies (often using compressed air or a dry brush) and the machining of brake linings (often by grinding or bevelling the lining surfaces to provide a better fit with the drum) have been reported to produce occupational dust exposures (Richter et al., 2009).

Although chrysotile containing brakes are not manufactured in the United States they can be imported, and they are still manufactured and/or imported in 110 countries.² US domestic consumption of chrysotile in 2018 was estimated to be 750 tons.³ The U.S. Census Bureau indicated that companies in the United States imported asbestos-containing brake pads and linings from Brazil, China, Colombia, India, and Mexico in 2016 (Harmonized Tariff Schedule code 6813.20.00.10 & 15 & 20 & 25)². In addition, the United Nations Commodity Trade Database indicates that Indonesia, Kazakhstan, Malaysia, Russia, and Ukraine also may have exported asbestos-containing brakes in 2018 (Harmonized Tariff Schedule code 681320, comtrade.un.org/db/). The potential for exposure to chrysotile containing brake dust remains.

2. Methods

The acclimation, aerosol generation exposure, and post exposure phase of this study were performed by the Citoxlab Hungary Ltd. (H-8200 Veszprém, Szabadságpuszta, Hungary) in a barrier maintained state-of-the-art inhalation facility. This study was conducted in compliance with the Hungarian Principles of Good Laboratory Practice (GLP) Regulations 42/2014 (VIII. 19). The tissue digestion, low temperature ashing and pathological examination were performed by the Fraunhofer Institute for Toxicology and Experimental Medicine (Hannover, Germany). The histopathological examination was performed by a German board certified (Fachtierarzt(FTA)) Veterinary Pathologist. The fiber counting and sizing were performed by Gesellschaft für Schadstoffanalytik mbH (GSA) (Ratingen, Germany). The confocal microscopy was performed by Rogers Imaging (Natick, Massachusetts, USA).

2.1. Test articles

2.1.1. Brake dust

The brake dust was produced directly from chrysotile-containing friction products (automotive drum brake shoes) by the RJ Lee Group Ltd. (Monroeville, PA, USA). The brake shoes were obtained from Davies McFarland & Carroll (Pittsburgh, PA). The shoes were designed to fit the drum brakes of mid-1960's Chevrolet Impala model cars and were typical of those used at that time. The brake dust was produced by grinding the brake shoes using a commercial AMMCO arc grinder (Model 8000, S/N 24788) with a modified dust collection system. AMMCO arc grinder was used as it is one of the best recognized names in brake lathes and brake service tools known for their use with cars and trucks and is the world's leading manufacturer of brake lathes, selling more than a quarter of a million since 1952. The friction material was evaluated and found to contain approximately 30% (by area⁴) chrysotile (analyzed in accordance with EPA 600/R-93/116, Perkins and Harvey, 1993). No amphibole asbestos minerals have been observed in any of the aerosol or lung samples from these brake shoes or in the chrysotile used in this study.

The AMMCO arc grinder (Model 8000, S/N 24788) is a motorized sander that is swept across the surface of the brake shoe. It was fitted with a modified dust collection system consisting of an attached 8 × 10 in. quartz micro-fiber filter that was used in place of the collection bag. A Tisch high volume air sampler sampling pump (Tisch Environmental Inc., Ohio, USA) was used following the filter to provide uniform sampling suction over the course of the grinding operation. All brake dust preparation took place at the RJ Lee Group facility in a room equipped with an Aramsco Comanche® HEPA ventilation unit (Model 55,011) with a nominal flowrate of 1800 cfm (50 m³/min). The composition of the brake dust was determined quantitatively using inductively coupled plasma mass spectrometry (ICP-MS) following the German norm DIN EN ISO 17294-2 (INDIKATOR GmbH, Wuppertal, Germany). The results are presented in (Bernstein et al., 2014 Supplementary data, Tables S-1 and S-2).

Bernstein et al., 2014, Supplementary Data, Figures S1-6) presented SEM photomicrographs of the brake dust including the chrysotile fibers present in comparison to the pure chrysotile exposure and the amphibole asbestos exposure. The chrysotile fibers in the brake dust groups appear very similar to those in the pure chrysotile group.

⁴ The EPA method (EPA 600/R-93/116) is a semi-quantitative analysis which involves the use of calibrated visual area estimation and/or point counting by polarized light microscopy. Observation of particles or fibers while oriented between polarizing filters whose privileged vibration directions are perpendicular (crossed polars) allows for determination of isotropism/anisotropism, extinction characteristics of anisotropic particles, and calculation of birefringence (Perkins and Harvey, 1993).

² <https://comtrade.un.org/db/mr/rfCommoditiesList.aspx>

³ <https://www.usgs.gov/centers/nmic/asbestos-statistics-and-information>

2.1.2. Chrysotile

The chrysotile fiber used in this study had the mineralogical grade of 7H19.⁵ The chrysotile sample was chosen based upon an evaluation of which chrysotile grade was ordered or supplied for use in brake manufacturing in a random search of 67 formulations dating from 1964 to 1986. The sample used in this study was obtained directly from Mine Jeffery Canada (formerly the Johns-Manville Mine). The 7H19 sample received contained some large bundles of fibers. To separate these bundles into respirable fibers without significantly reducing the fiber length, the bulk was passed twice through a Cyclotec Sample Mill (FOSS Tecator, Denmark), which rolls the sample against the inner circumference and then separates the fibers through a 2 mm mesh screen. The separation process takes a few seconds for each pass.

2.1.3. Crocidolite asbestos

The crocidolite asbestos used previously in animal studies had been largely either the Union for International Cancer Control (UICC) or US National Institute of Environmental Health Sciences (NIEHS) prepared crocidolite. Both of these samples were ground extensively > 30 years ago using large scale industrial mills resulting in a size distribution not typical of the commercial product (Bernstein et al., 2013). The present study used a crocidolite asbestos sample from the Voorspoed mine in South Africa, obtained from the National Institute of Occupational Health—NIOH, South Africa. This mine is located in Limpopo Province, which at the time when mining took place was called Transvaal Province. The crocidolite asbestos sample from the Voorspoed mine has very long fiber strands, which may not be rat respirable. Therefore, the crocidolite samples were minimally broken-up by milling using a Moulinex AR100 grinder for less than one minute.

2.1.4. Amosite asbestos

The amosite asbestos was obtained from the Fraunhofer Institute (Hanover, Germany) which had obtained it from Johns Manville, Technical Center, Fiber Preparation Laboratory, Colorado, USA. The amosite sample was size selected using by aqueous suspension techniques by the Johns Manville, Technical Center to maintain fiber length and have diameters which are largely rat respirable (Hesterberg et al., 1998).

The chemical compositions of chrysotile, a serpentine asbestos, and crocidolite and amosite, both amphibole asbestos, have been described previously (Shedd, 1985; Virta, 2002).

2.1.5. Titanium dioxide (TiO₂)

The TiO₂ was chosen to have a particle size in the same range as the particles in the brake dust exposure (after micronization). The sample used was Titanium(IV) oxide ReagentPlus®, ≥99% obtained from Sigma-Aldrich, product number 14021-1KG.

2.2. Experimental animals

Young adult male Wistar rats (CrI: Wi(Han), Specific Pathogen Free from Charles River Deutschland, Sulzfeld, Germany), 8 weeks old at the start of treatment, were used.

Rats were housed during non-exposure periods by two or three animals per cage in a HEPA filtered barrier-maintained housing facility. Each group of rats were housed separately to prevent the possibility of cross contamination between groups. Staff were required to wear a full face, filtered, positive air-supplied respirator, gloves and laboratory overalls in the animal housing rooms and inhalation exposure laboratory.

All procedures conformed to the Institutional Animal Care and Use Committee (IACUC). Citoxlab Hungary Ltd.'s facility is AAALAC accredited.

2.3. Experimental design

The experimental design, study plan and timing of the exposure and non-exposure recovery period is shown in Fig. 1. The interim sacrifices are indicated on top of the time-line bar. The time-line bar shows the study day referred to herein. The timing of the inhalation and exposure period are shown below the time-line bar. The current interim publications cover the results through 180 days (3 months post-exposure) with the exception of the results for which includes the full set of data as this was readily processed.

Groups of laboratory rats were exposed by inhalation for 6 h per day 5 days per week for 13 weeks. Different subsets of animals were scheduled to be sacrificed immediately following 45 and 89 days of exposure (approximately 0.2–1 h after end of exposure) and at 3, 6, 9, 12, 18 and 24 months after the last exposure (experimental days 180, 270, 360, 450, 540). The final sacrifice is planned, when one of the groups reach 20% survival. The following groups were exposed:

- Group 1: Air Control (Filtered air alone)
- Group 2: Low dose (LD) level of brake dust (0.20 mg/m³)
- Group 3: Mid dose (MD) level of brake dust (0.34 mg/m³)
- Group 4: High dose (HD) level of brake dust (0.67 mg/m³)
- Group 5: Titanium dioxide particle control (0.70 mg/m³)
- Group 6: Low dose level of Chrysotile 7H19 (0.27 mg/m³)
- Group 7: High dose level of Chrysotile 7H19 (0.64 mg/m³)
- Group 8: High dose level of Crocidolite asbestos (Voorspoed mine) (1.28 mg/m³)
- Group 9: High dose level of Amosite asbestos (2.32 mg/m³)

The following analyses were conducted for each group:

- Aerosol exposure characterization (presented in Part 1)
- Fiber / Particle lung burden evaluation (presented in Part 1)
- Bronchoalveolar lavage examination (presented in Part 1)
- Histopathology examination (presented in Part 2)
- Confocal microscopy of the lung including collagen quantification (presented in Part 2)
- Confocal low temperature microscopy of the lung and chestwall including collagen quantification (presented in Part 2)

The low dose exposure concentrations for each test article for this study were selected based upon the exposure concentrations that were required to achieve the similar cumulative exposure as in the earlier 28-day range finding study (Bernstein et al., 2018) and on aerosol generation pre-tests for the present study. The higher exposure concentrations were set as multiples of the lower concentrations. As the SEM fiber measurements were not quickly available due to the time required for processing and analysis, the exposures were adjusted higher than calculated.

For groups 2 through 4, the exposure concentrations were set gravimetrically as there was a relatively low concentration of chrysotile fibers (fiber concentrations were characterized subsequently). For groups 6 through 9, the exposure concentrations were based upon the number of fibers longer than 20 μm/cm³. A negative control group 1 was exposed in a similar set-up to filtered air and a particle control group 5 was exposed to titanium dioxide.

The relationship of fiber number/cm³ to gravimetric concentration (mg/m³) was determined during aerosol generation trials prior to the study start. During the study, day to day concentrations were controlled based upon gravimetric concentrations.

2.3.1. Exposure system

Animals were acclimatized to the test apparatus (restraint procedures) in two steps in order to lessen the stress during exposure. During the first week of acclimation, animals were progressively placed in the animal restraint tubes for 1, 2, 3, 4 and 6 h. During the second week of

⁵ Canadian chrysotile classification, Cossette and Delvaux (1979)

Study plan & timing



Fig. 1. Study plan and timing for the sub-chronic inhalation toxicology study.

acclimation, animals were placed into the inhalation tubes, which were then attached to the nose only exposure and put onto the tower for one, three and six hours, respectively over 3 days.

The animals were exposed by the inhalation route using a nose only, flow past, dynamic flow exposure unit, consisting of two, concentric stainless steel cylinders, the inner plenum and the outer chamber with 110 circularly arranged exposure ports based on the concept described by Cannon et al. (1983) (TSE Systems GmbH, Bad Homburg, Germany). The TSE system has been modified to better facilitate airflow and delivery of the test material to the animals. The exposure units were housed in individual closed rooms under negative pressure in order to avoid cross-contamination and operated as described previously (Bernstein et al., 2018).

2.3.2. Exposure atmosphere generation

The Model CR3020 (CR Machinery SA, Coppet, Switzerland) generators were used for aerosolization of the fibers. The set-up, operation and flowrates used for this system are same as described for the 28-day study in Bernstein et al. (2018).

The set-up for TiO₂ was the same as used for the brake dust and the set-up for amosite was the same as used for crocidolite in the 28-day study (Bernstein et al., 2018).

2.3.3. Exposure system monitoring

The aerosol concentration was monitored through filter sampling taken directly from one of the aerosol supply ports in the flow-past exposure system at a sampling flow rate similar to the supply flow rate of air to each animal (1.0 l/min). These filters were used for gravimetric analysis and for bivariate length and diameter analysis by SEM. The temperature and the relative humidity of the exposure atmosphere were determined continuously by the animal exposure facility computer system.

Gravimetric determinations of aerosol concentration were performed at least once daily from filter samples collected for approximately 6 h per day for each group. In addition, a minimum of 2 consecutive filters were taken for approximately 2 h each for Groups 2 to 9. Samples were collected on Millipore HVLP 4700 (HVLPO4700 | Durapore Membrane Filter, PVDF, hydrophilic, 0.45 μm, 47 mm, white, plain) filters, loaded in a 47 mm in-line stainless steel filter sampling device.

Aerosol samples for bivariate analysis of fiber size distribution and counting were collected onto NUCLEOPORE® filters (PC membrane, diameter 47 mm, pore size 0.2 μm - SN 111.106, Whatman Ltd.) for approximately 2 h successively during each exposure period in parallel with the gravimetric sampling. For group 1 (air control), one sample per treatment day was collected over approximately 5 h per day.

Counting rules for the evaluation of aerosol and lung samples by scanning electron microscopy:

The counting rules and sample preparation for scanning electron microscopy are described in detail in Bernstein and Kunzendorf (2018).

The stopping rules for counting each sample were defined as follows.

Fibrous objects: The minimum numbers of fibers examined were:

- fibers with a length < 5 μm = 100 fibers (200 fiber ends)
- fibers with a length between 5 and 20 μm = 200 fibers (400 fiber ends)
- fibers with a length > 20 μm = 100 fibers (200 fiber ends)

Fields of view were examined for each length category until the defined minimum number of fibers for each length category was recorded or a maximum of 1 mm² of the filter surface was examined in case the fiber minimum number for the length category was not reached. For samples of the control group a filter area of 0.5 mm² was evaluated. These counting rules were based on the number of fibers per sample necessary in order to have statistical reproducibility of the means (EUR, 18748 EN, 1999). For non-fibrous objects, fields of view were examined until a total of 100 particles were recorded or when the afore mentioned stopping criteria for fibers were reached.

Particle Size Analysis: The aerodynamic particle size of the aerosols was planned using a 7-stage cascade impactor of Mercer style (TSE Systems GmbH, Bad Homburg, Germany). The particle was determined for the brake dust high dose. The particle size of the TiO₂ was below the lower limit of the impact. For the pure fiber exposed group, due to the low gravimetric concentrations, and fiber bounce on the stages, stable measurements were not possible.

Clinical observations: Animals were observed for mortality/morbidity and for other clinical symptoms at least once daily during the training period, twice daily before and after exposure during the treatment period, and once a week during the post exposure observation. Observations included changes in the skin and fur, eyes and mucous membranes as well as alterations in respiratory, circulatory, autonomic and central nervous system, somatomotor activity and behaviour pattern. Particular attention was directed to observation of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. Individual body weights were recorded on the day before the exposure and weekly during the 13-week exposure and the lifelong recovery period. Food consumption was recorded weekly.

2.4. Gross pathology and organ weight

Animals were anesthetized with an i.p. overdose of pentobarbital sodium (0.1 ml/100 g from the 40% Euthanimal™) and humanely killed by cutting the vena cava caudalis after opening the abdominal cavity. The diaphragm was cut carefully allowing the lungs to collapse. Heart, esophagus, upper half of trachea, thymus and lung associated lymphnodes were sampled. The physical condition of the animals prior to euthanasia and the examination of the internal organs were recorded. Moribund animals or those found dead were necropsied as soon as possible and the findings recorded.

Table 1
 Mean aerosol concentration and size distribution of the exposure atmosphere in the air control group 1, brake dust groups 2, 3, 4, titanium dioxide group 5 chrysotile groups 6, 7, crocidolite asbestos group 8 and amosite asbestos group 9.

Exposure group	Gravimetric concentration mg/m ³ (SD)	Total number of Fibers counted on the filter ^a	Number of total fibers/cm ³	WHO Fibers/cm ³	Percent WHO fibers	Number of fibers L ≥ 20 μm/cm ³	Percent of all fibers L ≥ 20 μm/cm ³	Mean Number Particles/cm ³	Diameter Range (μm)	Length Range (μm)	GMD (μm) (Std. Dev.)	GML (μm) (Std. Dev.)	Mean Diameter (μm) Std. Dev.	Length weighted arithm. Diameter (μm)	Length weighted geom. Diameter (μm)	Aspect ratio
(Group 1) Air control	0 (0)	605	6.58	0.15	2.3	0	0	110	0.03–1.8	0.3–42.4	0.27 (1.89)	1.58 (1.83)	0.33 (0.24)	0.40	0.41	7.78
(Group 2) Brake dust – low dose	0.20 (0.20)	1297	36.44	2.42	6.6	0.17	0.5	470	0.3–2.9	0.3–83	0.15 (1.88)	1.72 (1.96)	0.19 (0.16)	0.26	0.20	15.56
(Group 3) Brake dust – mid dose	0.34 (0.18)	1784	48.83	4.85	9.9	0.43	0.9	540	0.01–2.1	0.3–90	0.16 (1.73)	2.20 (2.05)	0.19 (0.14)	0.25	0.19	18.10
(Group 4) ^b brake dust – high dose	0.67 (0.24)	2120	78.44	6.63	8.5	0.52	0.7	715	0.02–2.4	0.2–120	0.15 (1.91)	1.86 (2.15)	0.19 (0.15)	0.24	0.20	17.58
(Group 5) ^c Titanium dioxide	0.70 (0.27)	1142	24	0.33	1.4	0	0	2752	0.03–2.1	0.3–18.7	0.23 (1.77)	1.31 (1.97)	0.27 (0.17)	0.03	0.43	7.04
(Group 6) Chrysotile – low dose	0.27 (0.16)	4952	665	119	17.8	28.38	4.4	614	0.01–0.27	0.2–586	0.11 (1.86)	2.52 (2.65)	0.14 (0.11)	0.15	0.12	50.08
(Group 7) Chrysotile – high dose	0.64 (0.22)	5219	753	233	30.9	72.33	9.6	495	0.02–2.2	0.3–333	0.11 (1.85)	3.89 (3.31)	0.14 (0.12)	0.16	0.11	82.53
(Group 8) Crocidolite – high dose	1.28 (0.36)	5212	516	181	35.1	23.5	4.6	415	0.02–2.1	0.3–322	0.24 (1.80)	3.72 (2.62)	0.28 (0.17)	0.35	0.28	24.44
(Group 9) Amosite – high dose	2.32 (0.88)	5217	768	281	36.6	47.66	6.2	417	0.01–2.9	0.4–311	0.28 (1.80)	3.85 (2.80)	0.33 (0.20)	0.43	0.33	22.78

WHO fibers: WHO fibers are defined by the World Health Organization to have L > 5 μm, D < 3 μm and aspect ratio (L/D) of 3:1 (WHO, 1985; NIOSH, 1994).

SD: Standard deviation; GMD: Geometric mean diameter; GML: Geometric mean length; MMAD: Mass median aerodynamic diameter.

^a The total number of fibers counted on the filter is based upon the rules specified in section “Fiber/Particle Analysis and Lung Digestion” above.

^b For the brake dust group 4, the MMAD = 2.6 μm (Geometric standard deviation = 2.54) as determined by the impactor measurement.

^c The geometric mean diameter of the TiO₂ particles as determined by SEM was 0.39 μm (below the lower cut-off of the impactor).

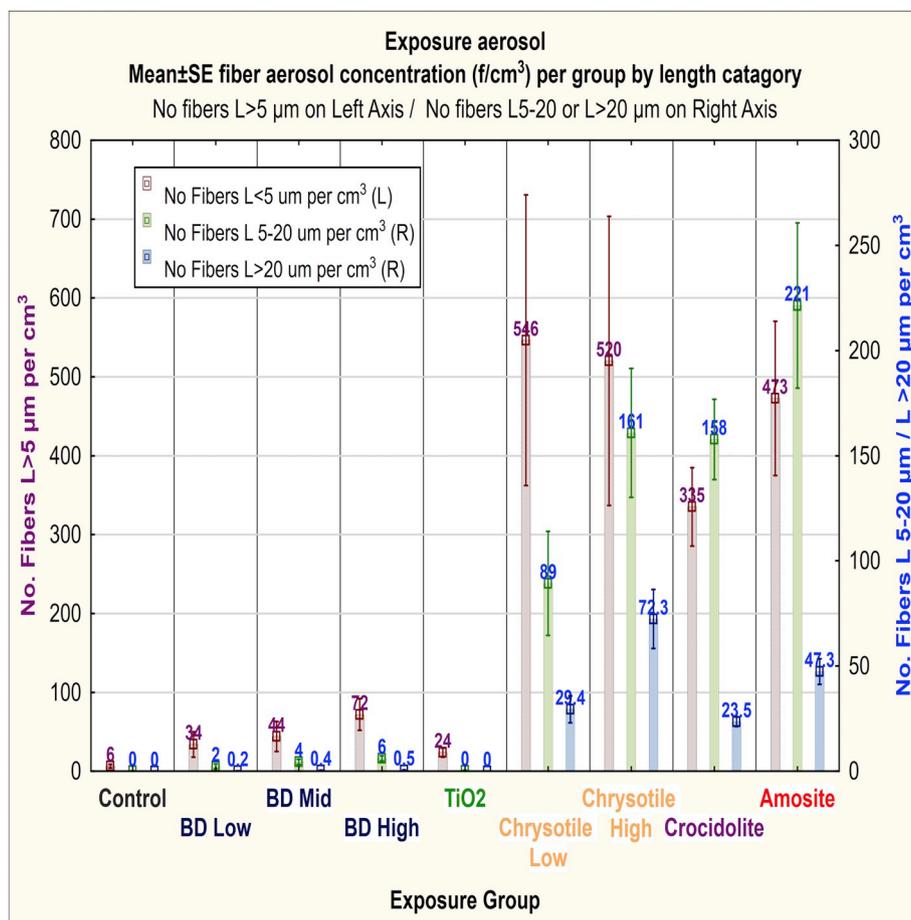


Fig. 2. Exposure aerosol – mean ± SE of the number of fibers in each group by the length category: < 5 μm, 5–20 μm & > 20 μm. The numbers shown above the bars are the mean values.

2.5. Bronchoalveolar lavage

Bronchoalveolar lavage was performed from three rats/group at 45 and 89 days during the exposure period and at 3 and 9 months post-exposure period. The rats were euthanized with intraperitoneal injection of pentobarbital. The trachea was then exposed and cannulated with a plastic cannula and the cannula secured with surgical thread. The abdominal cavity was opened and the descending aorta cut. The diaphragm was then cut to deflate the lung. Five lavages were performed on each animal. With the first lavage, a syringe with 5 ml isotonic saline was attached to the cannula; the saline slowly inserted via the trachea into the lung, while massaging the lung with a finger placed into the chest cavity through the cut diaphragm. Massaging the lung during lavage is included to improve the uniform distribution of lavage medium throughout the lung and thus improve cell yield (OECD 2015). The fluid was withdrawn and reinstalled, while the lung was massaged, and withdrawn a second time. The volume of lavage saline was chosen based on the optimal recovery of macrophages from the rat lung as determined by Brain and Frank, 1968.

The first lavage fluid was centrifuged (1125 g for 10 min at 4 °C) and the acellular BAL fluid was separated and saved for analysis (see below); the cells were saved from this first lavage.

For lavages 2–5, 7 ml of saline was used following the procedure above. The lavage fluid was centrifuged as above; the supernatant was discarded and the cells were saved. The cells from the first lavage were combined with those from lavages 2–5 by centrifuging and resuspending in known volume of saline.

Cell counts and differentials were determined using a Countess™ automated cell counter (Invitrogen Corp.). Cell differentials were

determined following staining of the slide with modified Wright-Giemsa stain. Lactate dehydrogenase activity was determined on fresh BAL fluid using VITROS LDH slides (Ortho Clinical Diagnostics #8384489). The following cytokines were determined by ELISA kit: TGF-β1 (BioLegend cat no. 737707), TNF-α (Invitrogen cat no. KRC3011), IL-1β (Thermo Scientific cat no. ERL1B).

Tissue preparation and methods for histopathology and confocal microscopy:

The tissue preparation, methods and results for histopathology and confocal microscopy are presented in Part 2 (Bernstein et al., 2019).

2.6. Statistical analyses

One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 8.1.2 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

Graphs and summary statistics were prepared using STATISTICA (data analysis software system), TIBCO Software Inc. (2018) <http://tibco.com>.

3. Results

3.1. Inhalation exposure

The aerosol concentrations and the size distributions of the fibers of all groups are shown in Table 1. As mentioned above, the aerosol concentrations were set based upon a similar cumulative dose as in the previous 28-day study (Bernstein et al., 2018).

The number of fibers in each group per length category is shown in

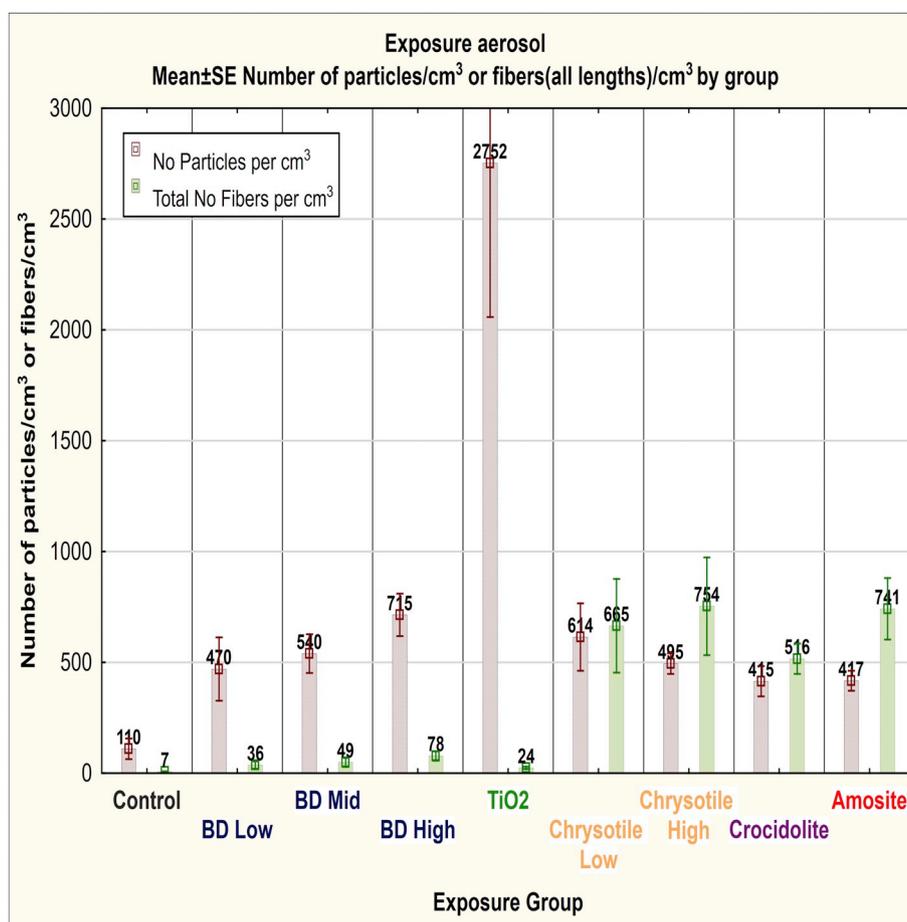


Fig. 3. Exposure aerosol – mean \pm SE of the number of the total number of fibers (all lengths with 3:1 aspect ratio) and particles in each group. The numbers shown above the bars are the mean values.

Fig. 2. The fiber preparation techniques and aerosol generation methods described above resulted in comparative exposures of the chrysotile, crocidolite and amosite in each size category. As mentioned, the few fibers present in the brake dust is reflected in these results as well. The mean, minimum, maximum and standard deviation (Std.Dev.) for each size fiber fraction and particle number are shown in Table S-1 (supplemental data). Also shown is the number of fibers $L > 20 \mu\text{m}$ evaluated on the filters according to the counting rules described above.

The mean number of particles/cm³ in the brake dust groups ranged from 470 in the low dose to 715 in the high dose (Fig. 3). This was in the same range as the number of particles/cm³ in the chrysotile exposure groups which range from 495 to 614. The TiO₂ exposure group had a mean of 2752 particles/cm³. The crocidolite and amosite exposure groups had similar number of particles/cm³ 415 and 417, respectively.

The brake dust groups had fewer than 1 fiber $L > 20 \mu\text{m}$ /cm³. The chrysotile exposure groups had means of 29 and 72 fibers ($L > 20 \mu\text{m}$)/cm³. This was comparable to the amphibole groups which had means of 24 and 47 fibers ($L > 20 \mu\text{m}$)/cm³, respectively for crocidolite and amosite. In the original study design similar concentrations of fibers $L > 20 \mu\text{m}$ were planned for crocidolite and amosite, however, due to logistics at GSA in counting & sizing the fibers at the study start up, this differential occurred.

The detailed summary statistics for each exposure group are presented in Table S-1 (Supplemental data).

The bivariate length and diameter distributions of all fibers evaluated during the exposure period are shown for each group in Figs. S-1 to S-9 (supplemental data). The fiber aerosol diameter distribution for all fibers and for fibers $L > 20 \mu\text{m}$ are presented in Table S-2

(supplemental data). In the brake dust exposed groups, between 91 and 99% of the aerosol fibers were rat respirable ($< 1 \mu\text{m}$ fiber diameter⁶). For fibers $\geq 20 \mu\text{m}$ in length in the low dose brake dust group 64% were rat respirable, while in the mid and high dose groups 91 and 86% were rat respirable. As mentioned above, there were few fibers present in the low dose brake dust and this number was based on the evaluation of 7 fibers.

As described previously (Bernstein et al., 2018) the chrysotile aerosol sample had numerous thin longer fibers with an occasional fiber bundle. In the current study $> 99\%$ of the chrysotile fibers (all lengths) present were rat respirable ($< 1 \mu\text{m}$ diameter). For fibers $> 20 \mu\text{m}$ in length 99% were also rat respirable. In the crocidolite asbestos aerosol sample, the number of long thin fibers was even more apparent as shown in Fig. S-2 (supplementary data). $> 98\%$ of the crocidolite fibers (all lengths) present were rat respirable ($< 1 \mu\text{m}$ fiber diameter). For fibers $> 20 \mu\text{m}$ in length 96% were also rat respirable. Similarly, for the amosite exposure group 95% of all fibers were rat respirable and 95% of the fibers $L > 20 \mu\text{m}$ were also rat respirable.

The chrysotile low and high dose groups had long fibers ranging in length up to 586 μm and 333 μm , respectively. This was similar to the amphibole asbestos groups, crocidolite and amosite, which had fibers ranging in length up to 322 μm and 311 μm , respectively.

3.2. Lung Fiber burdens

The number of fibers in the lung during exposure at 45 days, at the

⁶ Rat respirable fiber, see EPA-748-R-001, 1995, Bernstein et al., 1994, Morgan, 1995

Table 2

LUNG – Number of particles and fibers by length category < 5 μm , 5–20 μm , > 20 μm & total (all lengths with 3:1 aspect ratio) per lung (Mean \pm SD). The column on the right shows the number of fibers L > 20 μm evaluated on the filter according to the SEM counting rules. (Groups: 1- Air control; 2-Low dose BD; 3-mid dose BD; 4-high dose BD; 5-TiO₂; 6-low dose chrysotile; 7-high dose chrysotile; 8-crocidolite; 9-amosite).

Exposure group	Time days	No particles		No fibers L < 5 μm		No fibers L 5–20 μm		No fibers L > 20 μm		Total no fibers		Number of fibers L > 20 μm evaluated	
		per lung		per lung		per lung		per lung		per lung			
		Means	Std Dev	Means	Std Dev	Means	Std Dev	Means	Std Dev	Means	Std Dev	Means	Std Dev
1	45	13,667,000	6,192,200	21,150	42,290	0	0	0	0	21,100	42,300	0	0
1	89	100,050,000	19,561,000	437,000	434,800	10,570	13,500	0	0	448,000	431,000	0	0
1	180	13,622,000	4,996,000	0	0	0	0	0	0	0	0	0	0
2	45	25,610,000	15,498,000	1,668,000	1,713,000	68,730	46,880	0	0	1,736,000	1,755,000	0	0
2	89	81,391,000	12,958,000	2,299,000	1,666,000	276,700	127,100	0	0	2,576,000	1,790,000	0	0
2	180	41,923,000	16,020,000	1,095,000	636,300	77,540	58,980	0	0	1,172,000	690,700	0	0
3	45	107,240,000	88,853,000	8,838,000	8,727,000	740,200	456,700	7050	8140	9,585,000	9,136,000	0.5	0.6
3	89	66,989,000	24,258,000	13,150,000	8,656,000	1,385,000	782,500	3520	7050	14,540,000	9,382,000	0.3	0.5
3	180	47,573,000	12,765,000	2,819,000	706,100	338,300	157,900	0	0	3,157,000	849,900	0	0
4	45	85,834,000	62,028,000	8,305,000	7,428,000	516,400	341,200	8820	17,600	8,831,000	7,757,000	0.6	1.3
4	89	86,390,000	53,366,000	24,066,000	17,214,000	986,800	344,900	3520	4070	25,060,000	17,540,000	0.3	0.3
4	180	33,916,000	15,338,000	1,621,000	1,354,000	66,960	35,710	0	0	1,688,000	1,386,000	0	0
5	45	128,590,000	28,770,000	2,082,000	318,200	215,100	133,500	3530	7050	2,300,000	243,300	0.3	0.5
5	89	205,560,000	63,747,000	521,600	379,900	15,860	3520	0	0	537,500	382,500	0	0
5	180	270,870,000	67,074,000	0	0	0	0	0	0	0	0	0	0
6	45	21,540,000	18,012,000	15,369,000	6,608,300	2,037,400	1,136,600	100,900	95,510	17,507,000	7,458,500	6.1	4.8
6	89	161,570,000	26,748,000	44,427,000	19,172,000	8,632,300	2,897,200	204,400	55,500	53,264,000	21,662,000	14.5	3.9
6	180	29,073,000	14,890,000	5,279,200	1,204,800	939,300	200,600	14,100	17,270	6,232,500	1,341,600	1	1.2
7	45	80,160,000	10,708,000	6,582,600	2,770,800	1,542,600	913,670	42,310	38,620	8,167,500	3,719,100	3	2.7
7	89	136,110,000	55,960,000	55,186,000	19,668,000	11,396,000	3,777,100	290,800	102,200	66,873,000	23,298,000	20.6	7.3
7	180	45,708,000	3,644,600	7,959,600	982,620	2,555,900	293,920	54,630	12,040	10,570,000	1,245,600	3.9	0.9
8	45	71,573,000	10,875,000	31,181,000	12,383,000	19,467,000	4,052,900	4,911,800	1,116,600	55,560,000	16,789,000	100.3	0.3
8	89	81,470,000	40,753,000	30,333,000	10,931,000	28,805,000	7,759,400	9,054,600	2,346,400	68,192,000	18,737,000	100	0
8	180	103,910,000	15,228,000	32,798,000	14,654,000	24,982,000	6,178,700	8,026,600	1,478,000	65,807,000	20,036,000	100	0
9	45	71,609,000	39,837,000	47,424,000	18,658,000	29,912,000	4,837,500	7,063,900	864,380	84,399,000	23,651,000	100	0
9	89	121,630,000	20,188,000	57,730,000	16,012,000	40,794,000	6,406,300	11,645,000	1,382,900	110,170,000	22,299,000	100	0
9	180	123,480,000	48,300,000	39,715,000	19,757,000	44,003,000	5,895,700	11,591,000	1,121,700	95,310,000	24,729,000	100.6	0.5

end of exposure at 89 days and 3 months post exposure at 180 days are shown for each group in Table 2 and Fig. 4 a-i. The fraction of fibers in the size categories length < 5 μm , 5–20 μm and > 20 μm and the percentage of fibers in each category relative to the amount at 89 days at the end of exposure is summarized in Table 3. The full summary statistics for each group and time point are presented in Tables S-3 to S-5 (supplementary data). The bivariate size distributions for each group at each time point are shown in Figs. S-10 to S-35 (supplementary data).

The few chrysotile fibers L > 20 μm present in the brake dust aerosol (< 1 fiber L > 20 $\mu\text{m}/\text{cm}^3$) are reflected in the low lung concentrations. In lungs from the low dose brake dust, no long fibers were observed. In the mid and high dose brake dust groups, less than one fiber was observed on the filter (according to the SEM Counting rules) accounting for < 0.001 of the total fibers. By 3 months post exposure (180 days) no long fibers were observed and between 72 and 93% of the fibers remaining at 89 days were cleared by 3 months post exposure.

The number of the fibers in the low and high dose chrysotile aerosol was considerably greater ranging from 28 to 72 fibers(L > 20 $\mu\text{m})/\text{cm}^3$. This resulted in 200,000 fibers longer than 20 μm in the low dose and 290,000 fibers longer than 20 μm high dose chrysotile groups. By three months post exposure 7% remained in the low dose and 19% in the high dose representing 0.002 and 0.005 of the total fibers present. The maximum fiber length of these long fibers was reduced from 86 and 73 μm respectively at 89 days to 30 and 38 μm respectively at three months post exposure.

This is in contrast to the amphibole fiber exposure groups 8 and 9, crocidolite and amosite respectively. The mean crocidolite exposure aerosol for fibers longer than 20 μm was 24 fibers/ cm^3 and the mean amosite exposure aerosol for fibers longer than 20 μm was 48 fibers/ cm^3 and were similar to those for chrysotile. At the end of exposure at 89 days, there were more than mean of 9,000,000 fibers (L > 20 μm)

per lung in the crocidolite exposed animals and 11,600,000 fibers (L > 20 μm) per lung in the amosite exposed animals, representing 13 and 10% of the total fibers. By three months post exposure there was no statistically significant reduction in the amount remaining in the lungs in these groups.

3.3. Bronchioalveolar lavage

The bronchial alveolar lavage was performed on a subset of animals at 45, 89 (end of exposure) 180 (3 months post exposure) and 365 days (9 months post exposure). The total number of macrophages, lymphocytes, neutrophils, eosinophils and epithelial cells and their percentages were evaluated. In addition, concentrations of cytokines IL-1 beta, TNF alpha and TGF beta were measured.

The means (SE) of each measurement are shown in Figs. 5a-d and Fig. 6a-d. The analysis of variance, Dunnett's multiple comparisons test and mean values are presented in Tables S6-S10 (Supplementary data).

The mean difference that were statistically significant as compared to controls were:

Macrophages: Crocidolite on days 45, 89 and Amosite on days 45,89 and 180 showed a statistically significant decrease in the number of macrophages lavaged. This ranged from a mean of 9 and 10% on day 45, and 9 and 8% on day 89 for crocidolite and amosite respectively and 7% for amosite on day 180 (Fig. 5a).

Neutrophils: Crocidolite on days 45, 89 and Amosite on days 45,89 and 180 showed an statistically significant increase in the number of neutrophils lavaged. This ranged from a mean of 545% and 480% on day 45 and 246% and 223% on day 89, for crocidolite and amosite respectively and 307% for amosite on day 180 (Fig. 5b).

All other parameters were not statistically significant at any time point.

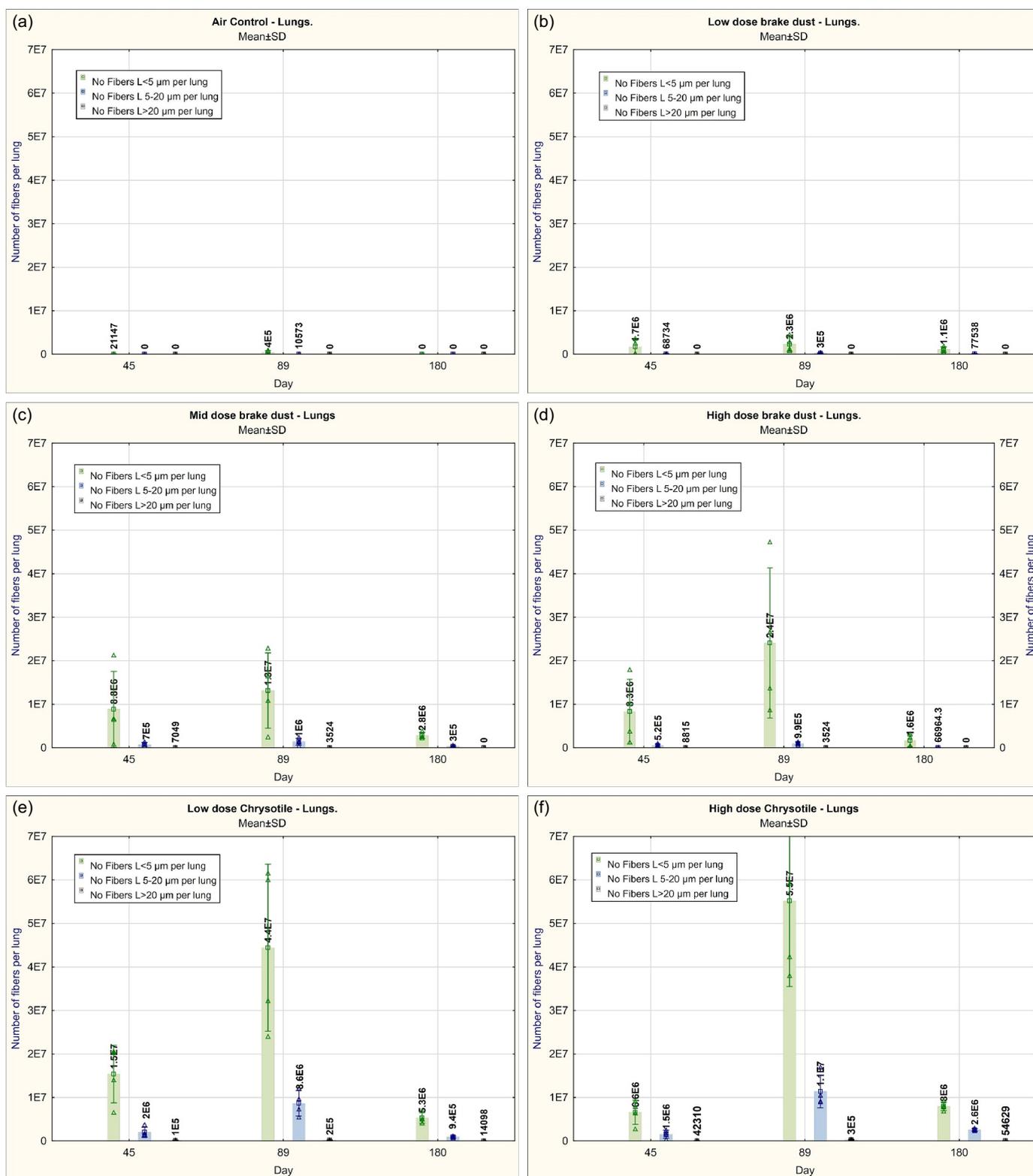


Fig. 4. a–i: Fiber size distributions in the lung at 45, 89 and 180 days. Shown are the mean ± SD number of fibers in the length categories < 5 μm, 5–20 μm and > 20 μm. The numbers shown above the bars are the mean values. a: Air control, number of fibers per lung. b: Low dose brake dust, number of fibers per lung. c: Mid dose brake dust, number of fibers per lung. d: High dose brake dust, number of fibers per lung. e: TiO₂, number of fibers per lung. f: Chrysotile low dose, number of fibers per lung. g: Chrysotile high dose, number of fibers per lung. h: Crocidolite, number of fibers per lung. i: Amosite, number of fibers per lung.

4. Discussion

This is the first study to evaluate the potential toxicity of brake dust from brake drums manufactured with chrysotile in a multi-dose sub-

chronic 90 day inhalation toxicity study. To fully evaluate the chronic effect of such exposure, the non-exposure follow-up period in this study was extended to the animal's life-time. In addition, a particle control of TiO₂ was included as well as control groups of chrysotile (2 doses),

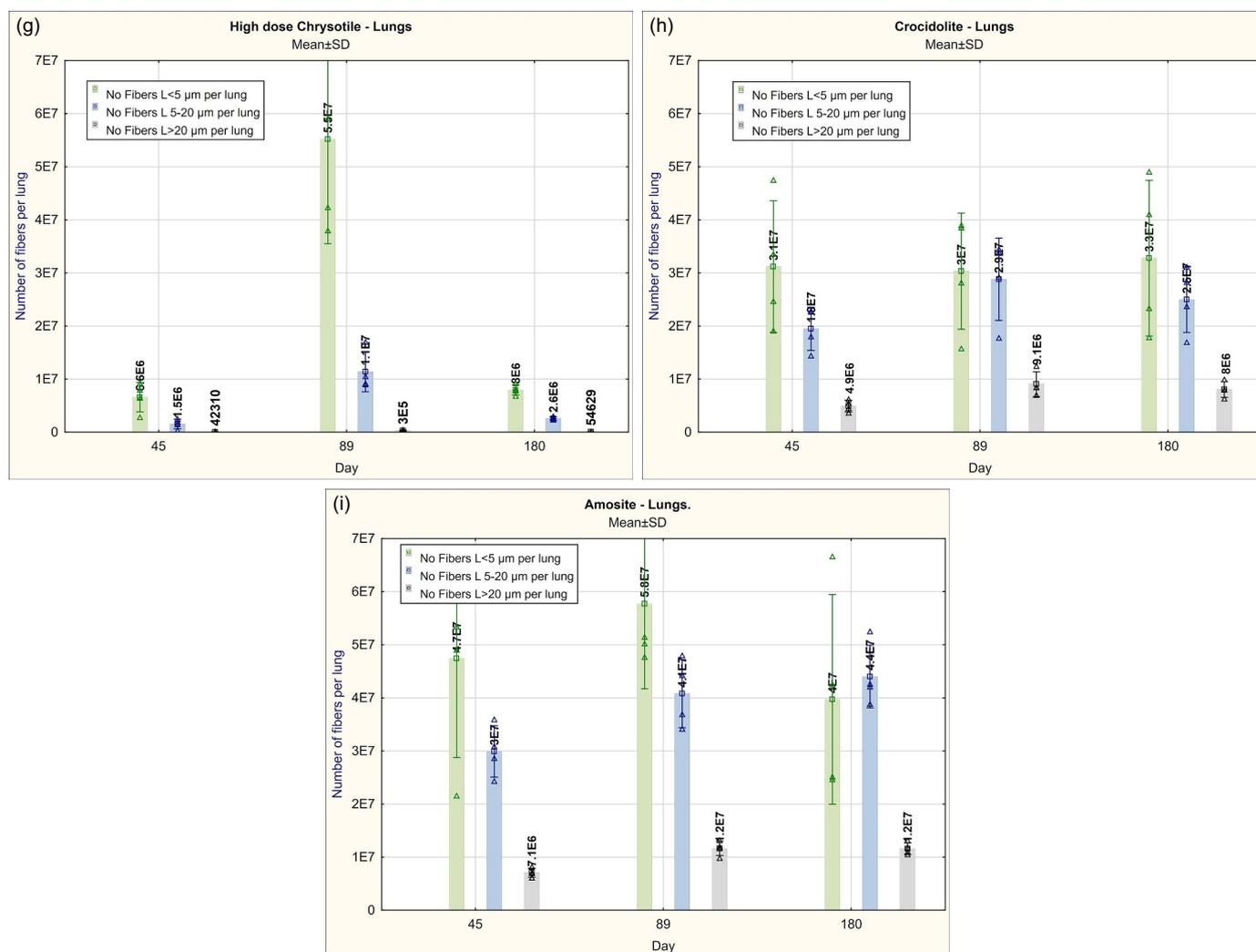


Fig. 4. (continued)

crocidolite and amosite asbestos. The chrysotile, crocidolite and amosite asbestos control groups were also unique in that the exposure concentrations were considerably lower than most historical asbestos inhalation studies. The WHO aerosol fiber concentrations ranged from 119 to 233 fibers/cm³, which is within a few orders of magnitude of the current ACGIH TLV Of 0.1 fibers/cm³.

Many previous studies of asbestos were based upon gravimetric exposure concentrations of 10 mg/m³ which correspond to > 100,000 fibers/cm³ (Bernstein et al., 2013) where it would be reasonable to expect that the number of particles and short fibers present in the exposure would be sufficient to overload the lung through impairment of macrophage function. High concentrations of insoluble nuisance dusts can result in lung overload which compromises the clearance mechanisms of the lung though reduced macrophage function and clearance, and even result in inflammation and a tumorigenic response in the rat (Bolton et al., 1983; Muhle et al., 1988; Morrow, 1988; Morrow et al., 1996; Oberdörster, 1995). The exposures in this study were chosen in order to avoid the phenomenon of “lung overload,” and were based on the responses observed in the 5 day and 28 day study that were performed previously.

Brake dust emissions have been evaluated by Blake et al. (2003) from four nearly identical automobiles from 1960s that were fitted with new replacement chrysotile-containing brake shoes and then driven over a predetermined public road course for about 2250 km. Each car was brought separately into a repair facility; the brakes removed and

replaced with new asbestos-containing shoes that were filed, sanded and ground as required. The airborne chrysotile fiber exposures as reported for each test remained below currently applicable limit of 0.1 fibers/cm³ (historical mean for workers servicing automobiles and light trucks was an eight-hour time-weighted average of 0.04 fibers/cm³). In this study, fiber concentrations were 2, 5 and 7 fibers(WHO)/cm³ in the brake dust groups 2, 3 and 4. In comparison, the mean chrysotile aerosol exposure concentration was 119 and 233 fibers(WHO)/cm³ in the chrysotile groups 5 and 6. Therefore, the chrysotile fiber exposure concentration in the chrysotile exposure groups of this study was 2975 to 5825 times the mean historical brake dust exposure TWA and 50 to 175 times the levels used in the brake dust exposed groups.

The brake dust that was evaluated was obtained from automobile brakes that were manufactured using chrysotile as one of the components. The original friction material contained approximately 30% chrysotile (analyzed in accordance with EPA 600/R-93/116 see Perkins and Harvey (1993), Bernstein et al., 2018); Blau (2001) has reported that brakes typically contained between 30 and 70% asbestos. The brake dust aerosols contained a mean of 1297 to 2120 total fibers/cm³, however, there were only between 2 and 7 WHO fibers/cm³ (means) depending on exposure group. Rhee (1974) determined that the wear of asbestos friction materials is controlled by a pyrolysis mechanism at elevated temperatures (above 450 °F drum temperature) and by adhesive and abrasive mechanisms at low temperatures. The wear mechanisms of the brake drums appear to favor the production of very

Table 3

LUNG - Fraction of each size category in the lung: Particles, fibers < 5 µm, 5–20 µm, > 20 µm & total (all lengths with 3:1 aspect ratio) per lung (Mean ± SD). The column on the right shows the fraction of fibers L > 20 µm evaluated on the filter according to the SEM counting rules. (Groups: 1- Air control; 2-Low dose BD; 3-mid dose BD; 4-high dose BD; 5-TiO₂; 6-low dose chrysotile; 7-high dose chrysotile; 8-crocidolite; 9-amosite).

Exposure group	Time days	No particles per lung (Means)	No particles - % of 89 days	No fibers L < 5 µm per lung (Means)	No fibers L < 5 µm - % of 89 days	No fibers L 5–20 µm per lung (Means)	No fibers L 5–20 µm - % of 89 days	No fibers L > 20 µm per lung (Means)	No fibers L > 20 µm - % of 89 days	Total No fibers per lung (Means)	Total No fibers - % of 89 days	number of fibers L > 20 µm evaluated (Means)
1	45	13,667,000	14	21,100	5	0	0	0	0	21,100	0	0.0
Fraction of total no fibers				1.00		0.00		0.00				
1	89	100,052,000	100	437,000	100	10,570	100	0	0	448,000	0	0.0
Fraction of total no fibers				0.98		0.02		0.00				
1	180	13,622,000	14	0	0	0	0	0	0	0	0	0.0
Fraction of total no fibers				0.00		0.00		0.00				
2	45	25,610,000	31	1,667,600	73	68,730	25	0	0	1,736,000	0	0.0
Fraction of total no fibers				0.96		0.04		0.00				
2	89	81,391,000	100	2,299,400	100	276,700	100	0	0	2,576,000	0	0.0
Fraction of total no fibers				0.89		0.11		0.00				
2	180	41,923,000	52	1,094,800	48	77,540	28	0	0	1,172,000	0	0.0
Fraction of total no fibers				0.93		0.07		0.00				
3	45	107,241,000	160	8,837,900	67	740,200	53	7000	200	9,585,000	66	0.5
Fraction of total no fibers				0.92		0.08		0.001				
3	89	66,989,000	100	13,148,000	100	1,385,100	100	3500	100	14,537,000	100	0.3
Fraction of total no fibers				0.90		0.10		0.000				
3	180	47,573,000	71	2,818,600	21	338,300	24	0	0	3,157,000	22	0.0
Fraction of total no fibers				0.89		0.11		0.000				
4	45	85,834,000	99	8,305,500	35	516,000	52	8800	251	8,831,000	35	0.6
Fraction of total no fibers				0.94		0.06		0.001				
4	89	86,390,000	100	24,066,000	100	987,000	100	3500	100	25,056,000	100	0.3
Fraction of total no fibers				0.96		0.04		0.000				
4	180	33,916,000	39	1,621,500	7	67,000	7	0	0	1,688,000	7	0.0
Fraction of total no fibers				0.96		0.04		0.000				
5	45	128,594,000	63	2,081,900	399	215,000	1344	3500	0	2,300,000	0	0.3
Fraction of total no fibers				0.91		0.09		0.002				
5	89	205,564,000	100	521,600	100	16,000	100	0	0	537,000	0	0.0
Fraction of total no fibers				0.97		0.03		0.00				
5	180	270,871,000	132	0	0	0	0	0	0	0	0	0.0
Fraction of total no fibers				0.00		0.00		0.00				
6	45	21,540,000	13	15,369,000	35	2,037,000	24	100,900	49	17,507,000	33	6.1
Fraction of total no fibers				0.88		0.12		0.006				
6	89	161,573,000	100	44,427,000	100	8,632,000	100	204,400	100	53,264,000	100	14.5
Fraction of total no fibers				0.83		0.16		0.004				
6	180	29,073,000	18	5,279,200	12	939,300	11	14,100	7	6,232,500	12	1.0
Fraction of total no fibers				0.85		0.15		0.002				
7	45	80,160,000	59	6,582,600	12	1,543,000	14	42,300	15	8,167,500	12	3.0
Fraction of total no fibers				0.81		0.19		0.005				
7	89	136,107,000	100	55,186,000	100	11,396,000	100	290,800	100	66,873,000	100	20.6
Fraction of total no fibers				0.83		0.17		0.004				
7	180	45,708,000	34	7,959,600	14	2,556,000	22	54,600	19	10,570,000	16	3.9
Fraction of total no fibers				0.75		0.24		0.005				
8	45	71,573,000	88	31,181,000	103	19,467,000	68	4,912,000	54	55,560,000	81	100.3
Fraction of total no fibers				0.56		0.35		0.088				
8	89	81,470,000	100	30,333,000	100	28,805,000	100	9,055,000	100	68,192,000	100	100.0
Fraction of total no fibers				0.44		0.42		0.133				
8	180	103,913,000	128	32,798,000	108	24,982,000	87	8,027,000	89	65,807,000	97	100.0
Fraction of total no fibers				0.50		0.38		0.122				
9	45	71,609,000	59	47,424,000	82	29,912,000	73	7,064,000	61	84,399,000	77	100.0
Fraction of total no fibers				0.56		0.35		0.084				
9	89	121,630,000	100	57,730,000	100	40,794,000	100	11,645,000	100	110,170,000	100	100.0
Fraction of total no fibers				0.52		0.37		0.106				
9	180	123,477,000	102	39,715,000	69	44,003,000	108	11,591,000	100	95,310,000	87	100.6
Fraction of total no fibers				0.42		0.46		0.122				

The mean number of fibers L > 20 µm per lung is shown in bold.

short (< 5 µm) chrysotile fibers. Fibers longer than 20 µm were even fewer with means ranging between 0.2 and 0.5 fibers(L > 20 µm)/cm³. Based on the gravimetric and WHO fiber concentration of the chrysotile exposure group, the WHO fibers in the brake dust aerosol were < 2% of the total brake dust mass.

In rats after 89 days inhalation to brake dust, the fewer long fibers (> 20 µm) in the aerosol accounted for no more than for 0.001 of the total number of fibers deposited in the lungs. By 90 days post exposure no long fibers were observed in the lungs of any of the brake dust groups.

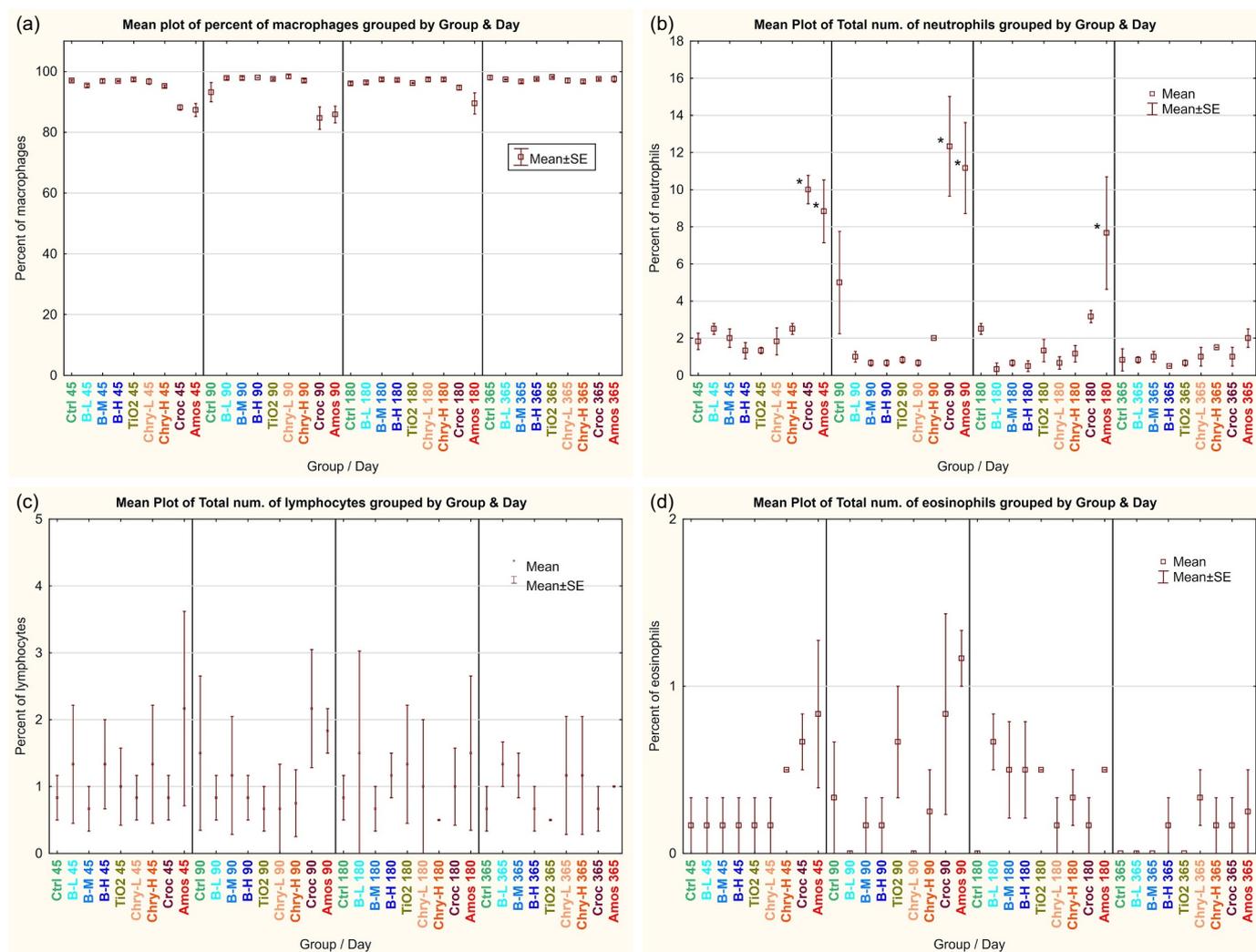


Fig. 5. a–d: Bronchial alveolar lavage (BAL): Percent (Mean ± SE) macrophages, neutrophils, lymphocytes & eosinophils per group and time point. a: Mean percent macrophages in BAL. b: Mean percent neutrophils in BAL. c: Mean percent lymphocytes in BAL. d: Mean percent eosinophils in BAL.

The biosolubility/clearance of the brake dust plus chrysotile as compared to chrysotile and crocidolite asbestos alone has been reported by Bernstein, 2014, Bernstein et al., 2015). The biosolubility of chrysotile results in accelerated clearance. Chrysotile is a thin rolled sheet of magnesium on the outside and silica on the inside, which is acid soluble (Kobell, 1834; Whittaker, 1957, 1963; Tanji et al., 1984; Titulaer et al., 1993).

Amphibole asbestos fibers, such as crocidolite, are encased in silica and are insoluble at any pH that can occur in physiological conditions (Skinner et al., 1988; Whittaker, 1960). Those fibers, once deposited in the lung which are too long to be cleared by the macrophage, persist in the lung and quickly initiate a sustained inflammatory response, leading to persistent tissue injury and a subsequent fibrogenic response (Bernstein et al., 2018).

In the brake dust exposed groups, few fibers longer than 20 μm were observed in the lungs at the end of exposure (89 days). Means of between 0 (group 2) and 0.3 (group 4) fibers were counted on the filter, corresponding to 0 and 3524 fibers(L > 20 μm)/lung. By 180 days (90 days following cessation of exposure), no fibers longer than 20 μm were observed.

In the chrysotile exposed groups, even though there were means 28 and 72 fibers(L > 20 μm)/cm³ in the aerosol in groups 6 and 7, respectively, in the lungs at the end of exposure (89 days) means of between 15 (group 6) and 20 (group 7) fibers were counted on the filter, corresponding to means of 204,000 and 290,000 fibers(L > 20 μm)/lung. By 180 days (90 days following cessation of exposure), means 1 and 3.9 fibers were counted on the filter corresponding to means of 14,000 and 55,000 fibers(L > 20 μm)/lung.

With both amphibole asbestos groups, crocidolite (group 8) and amosite (group 9) there were means of between 28 and 72 fibers (L > 20 μm)/cm³ in the aerosol, respectively. In the lungs, at 89 days (end of exposure) the full complement of 100 fibers were counted on the filters in each group, corresponding to means of 9,055,000 and 11,645,000 fibers(L > 20 μm)/lung in groups 8 and 9. By 180 days (90 days following cessation of exposure), 100 fibers were still counted on the filter corresponding to means of 8,026,000 and 11,591,000 fibers(L > 20 μm)/lung. This represented 10 to 13% of the total number of fibers in the lungs at both 89 and 180 days.

While previous studies have reported the clearance half-time of chrysotile as compared to amphibole asbestos following a 5-day

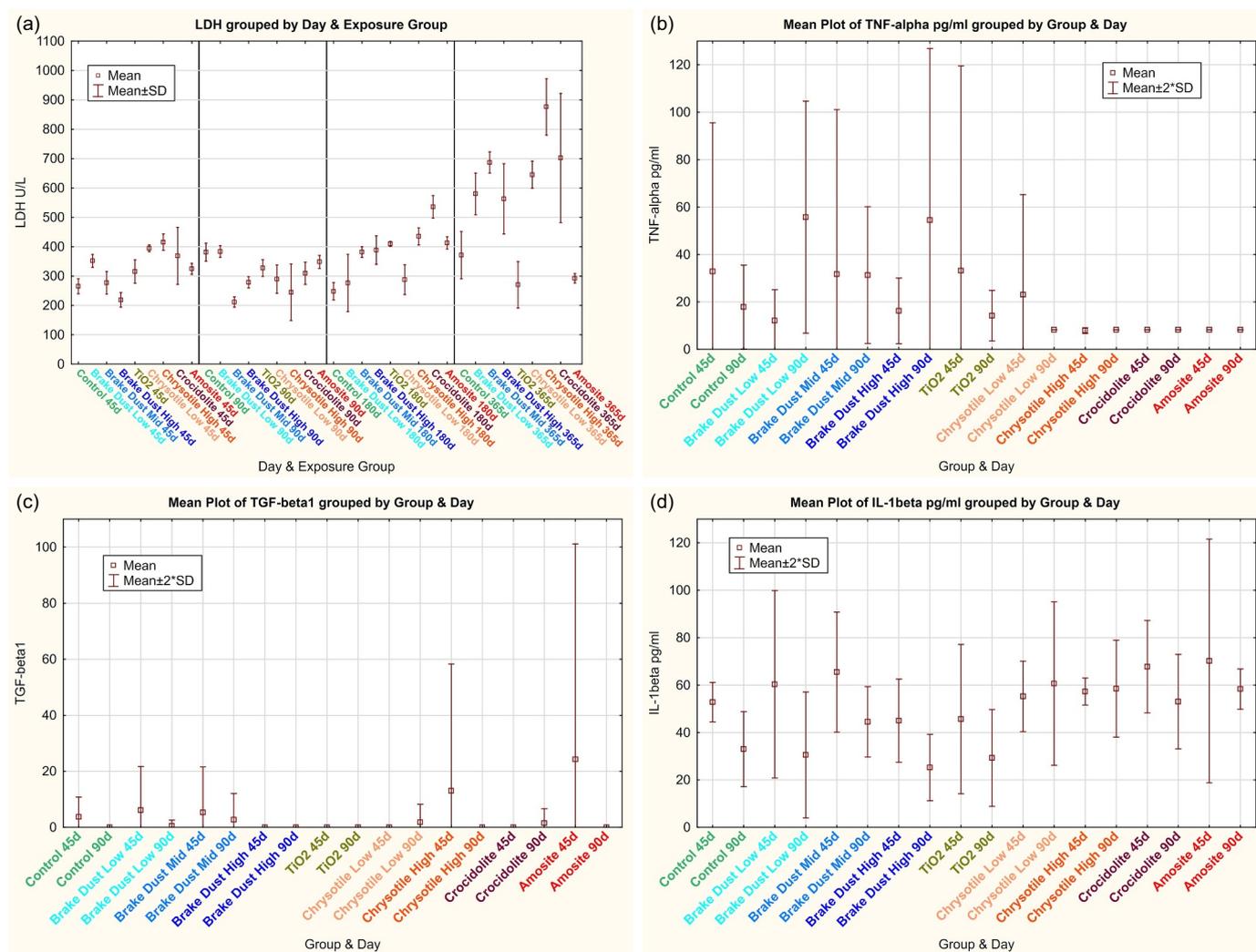


Fig. 6. a–d: Bronchial alveolar lavage (BAL): LDH, TNF-alpha, TGF-beta1 & IL-1 beta per group and time point (Mean ± SD). The units for each are shown on each plot. a: Mean LDH in BAL. b: Mean TNF-alpha in BAL. c: Mean TGF-beta1 in BAL. d: Mean IL-1 beta in BAL.

exposure (Musselman et al., 1994; Hesterberg et al., 1996; Hesterberg et al., 1998; Bernstein et al., 2005, 2011). These studies have shown that following similar fiber aerosol exposures, that chrysotile clears rapidly (0.7 to 11 days) while amphibole asbestos such as amosite and crocidolite remain for the rat's lifetime.

The results from this study allow the assessment of the clearance half times of the different exposures at the end of the 13 week exposure period. The mean lung concentration, percent remaining at 180 days (of that at 89 days) and the $T_{1/2}$ clearance half-time (days) are shown in Table 4.

Elder et al. (2005) evaluated the retention and subsequent clearance of carbon black following sub chronic (90 day) inhalation exposure at a range of exposure concentrations ranging from a no-observable-adverse-effects-level (NOAEL) to lung particle overload (1.1, 7.6, 50.3 mg/m³) in multiple species (rats, mice, hamsters). The authors reported particle retention clearance half-times following 13 weeks of exposure to carbon black in rats as follows (Table 5):

Elder et al. (2005) reported the normal retention half times of ~70 days for rats which is seen at the low dose of 1.1 mg/m³. At higher exposure concentrations, of 7.6 mg/m³ prolonged retention (115 d half-

time) due to particle load is observed while at 50 mg/m³ no significant clearance was observed.

4.1. Bronchioalveolar lavage BAL

The results of the bronchioalveolar lavage analyses reveal a markedly different profile at the moderate exposure concentrations used in this study as compared to historical studies that used considerably higher exposure concentrations.

At the inhalation exposure concentrations in this study, the only statistically significant mean differences as compared to the air control group are with macrophages and neutrophils in the amphibole groups (crocidolite and amosite). There was a statistically significant decrease in the number of macrophages in the lavage fluid at both 45 days and at 89 days (end of exposure) in the crocidolite and amosite groups (~9%) and in the amosite group at 180 days (~7%). This may have been due to the macrophages phagocytizing the insoluble longer fibers, which then effectively anchored them in place in the lung.

This was accompanied by a statistically significant increase in the number of neutrophils in the same groups and time points. At 45 days,

Table 4

The mean lung concentration, percent remaining at 180 days (of that at 89 days) and the $T_{1/2}$ clearance half-time (days) (calculated from the end of exposure (89 days) to 90 days post exposure (180 days)).

Exposure group	Time (days)	No fibers L < 5 μm			No fibers L 5–20 μm			No fibers L > 20 μm		
		per lung			per lung			per lung		
		(Means)	% remaining at 180 days	$T_{1/2}$ (days)	(Means)	% remaining at 180 days	$T_{1/2}$ (days)	(Means)	% remaining at 180 days	$T_{1/2}$ (days)
Air control	45				0			0		
	89	437,000			10,570			0		
	180	0			0			0		
Brake dust low	45	1,667,600			68,730			0		
	89	2,299,400		84	276,700		49	0		Complete Clearance ^a
	180	1,094,800	48		77,540	28		0	0	
Brake dust mid	45	8,837,900			740,200			7000		
	89	13,148,000		41	1,385,100		44	3500		
	180	2,818,600	21		338,300	24		0	0	
Brake dust high	45	8,305,500			516,000			8800		
	89	24,066,000		23	987,000		23	3500		Complete Clearance ^a
	180	1,621,500	7		67,000	7		0	0	
TiO ₂	45	2,081,900			215,000			3500		
	89	521,600		Complete Clearance ^a	16,000		Complete Clearance ^a	0		Complete Clearance ^a
	180	0	0		0	0		0	0	
Chrysotile low	45	15,369,000			2,037,000			100,900		
	89	44,427,000		29	8,632,000		28	204,400		23
	180	5,279,200	12		939,300	11		14,100	7	
Chrysotile high	45	6,582,600			1,543,000			42,300		
	89	55,186,000		32	11,396,000		42	290,800		37
	180	7,959,600	14		2,556,000	22		54,600	19	
Crocidolite	45	31,181,000			19,467,000			4,912,000		
	89	30,333,000		No clearance	28,805,000		438	9,055,000		518
	180	32,798,000	108		24,982,000	87		8,027,000	89	
Amosite	45	47,424,000			29,912,000			7,064,000		
	89	57,730,000		167	40,794,000		No clearance	11,645,000		13,446
	180	39,715,000	69		44,003,000	108		11,591,000	100	

^a Complete Clearance: There were no fibers observed at 180 days.

there was ~5 fold increase in the numbers of neutrophils, at 89 days, a 2 fold increase in both crocidolite and amosite, while at 180 days only amosite exhibited a 3 fold increase as compared to the air controls. All other groups showed no statistically significant differences with the controls.

No statistically significant differences of any group in comparison to the air control were observed at any time point in eosinophils, lymphocytes, IL-1 beta, TNF-alpha, TGF-beta1 or LDH in the BAL fluid.

The large majority of previous studies with asbestos exposure, which reported associations of exposure to endpoints measured in the BAL fluid were performed using non-inhalation techniques such as intratracheal installation or other bolus applications (e.g. Cyphert et al., 2016a, 2016b). Those studies performed by inhalation as reviewed by Mossman et al. (2011) were shorter term studies performed at very high exposure concentrations. As an example, Liu and Brody (2001) reported increased TGF-beta1 following a single 5-h inhalation exposure of NIEHS chrysotile at 10 mg/m³ respirable mass. The fiber number

exposure concentration at 10 mg/m³ respirable mass has been estimated to be > 100,000 fibers(WHO)/cm³ (Bernstein et al., 2015), which is considerably greater than the approximately 100 to 300 fibers (WHO)/cm³ in the chrysotile, crocidolite and amosite exposure groups in this study.

These exposure and lung burden results provide a sound basis for assessing the potential toxicity of the brake dust in comparison to the TiO₂ particle control and the chrysotile, crocidolite and amosite asbestos control groups. The multi-dose approach for evaluating the brake dust and chrysotile allow the assessment of possible dose response relationships. The single doses for TiO₂ and crocidolite and amosite asbestos were chosen to be comparable to the high dose for the brake dust and chrysotile respectively. The BAL results provide an initial indication of the differential response. The full interpretation of the potential toxicological effects of these exposures is presented in Part 2 which presents and discuss the histopathological and confocal microscopy findings in this study through 90 days post exposure.

Table 5

Carbon black - Mean (\pm SD) aerosol exposure (mg/m³), clearance half time (days) and exposure level design. Normal retention half times of ~70 days for rats (From Elder et al., 2005).

Carbon black	Mean (\pm SD) aerosol exposure, mg/m ³	Clearance half time	Design
Exposure group		Days	
Low	1.1 \pm 0.2	64	No-observable-adverse-effects-level (NOAEL)
Mid	7.6 \pm 1.9	115	Prolonged retention due to particle load
High	50.3 \pm 5.6	No significant clearance	Full lung particle overload

5. Conclusions

This 90-day repeated dose inhalation study is unique in that it used moderate exposures at concentrations within orders of magnitude to that which humans have been exposed. In the brake dust exposed groups, less than 2% of the fibers in the original brakes are found in the aerosols, with very few fibers present longer than 20 µm. The comparative chrysotile, crocidolite and amosite asbestos fiber exposure groups had comparative fiber concentrations for both the WHO and the fibers longer than 20 µm. The TiO₂ particle control group, provided a comparable inert particle exposure to that used for the brake dust.

The lung burden results showed that in the brake dust exposed groups, few fibers longer than 20 µm were observed in the lungs at the end of exposure (89 days) and at 180 days (90 days following cessation of exposure), no fibers longer than 20 µm were observed.

In the chrysotile exposed groups, means of 204,000 and 290,000 fibers(L > 20 µm)/lung were measured at 89 days. By 180 days (90 days following cessation of exposure), means of 1 and 3.9 fibers were counted on the filters corresponding to means of 14,000 and 55,000 fibers(L > 20 µm)/lung.

With both amphibole asbestos groups, crocidolite (group 8) and amosite (group 9) at 89 days (end of exposure) means of 9,055,000 and 11,645,000 fibers(L > 20 µm)/lung were determined. By 180 days (90 days following cessation of exposure), means of 8,026,000 and 11,591,000 fibers(L > 20 µm)/lung were found. This represented 10 to 13% of the total number of fibers in the lungs at both 89 and 180 days.

The bronchioalveolar lavage measurements, which determined the total number of macrophages, lymphocytes, neutrophils, eosinophils and epithelial cells as well as IL-1 beta, TNF alpha and TGF beta, were also unique. At the moderate aerosol concentrations used in this study, BAL macrophages decreased and neutrophils showed statistically significant increases in the amphibole asbestos groups, crocidolite and amosite. All other groups and BAL parameters were not statistically different at these exposure concentrations.

The exposure and lung burden results provide a sound basis for assessing the potential toxicity of the brake dust in comparison to the TiO₂ particle control and the chrysotile, crocidolite and amosite asbestos control groups. The BAL results provide an initial indication of the differential response. Part 2 presents the presentation and discussion of the histopathological and confocal microscopy findings in this study through 90 days post exposure.

Authors' contribution

D.M. Bernstein

Conceived and designed the analysis: Prepared the study protocol in coordination with the other authors

Collected the data: Coordinated the collection of all the data for each analysis

Contributed data or analysis tool: Performed the summary and statistical analysis

Wrote the paper: With the contributions from the authors

Other contribution: Project study director

B. Toth

Collected the data

Contributed data or analysis tool

Performed the analysis

Wrote the paper

Other contribution

R.A. Rogers

Conceived and designed analysis

Collected the data

Contributed data or analysis tool

Performed the analysis

Wrote the paper

D. Kling

Collected the data

Contributed data or analysis tool

Performed the analysis

P. Kunzendorf

Conceived and designed analysis

Collected the data

Contributed data or analysis tool

Performed the analysis

Wrote the paper

J.I. Phillips

Conceived and designed analysis

Other contribution

H. Ernst

Conceived and designed analysis

Collected the data

Contributed data or analysis tool

Performed the analysis

Wrote the paper

Declaration of Competing Interest

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Authors contributions

See authors contributions form

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.taap.2019.114856>.

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