Ann. occup. Hyg., Vol. 48, No. 2, pp. 91–104, 2004
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Published by Oxford University Press
DOI: 10.1093/annhyg/meg093

Subchronic Inhalation Study of Stone Wool Fibres in Rats

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Received 17 March 2003; in final form 28 July 2003

Pathology results after subchronic inhalation in rats of three separate fibres representing the new biosoluble high-aluminium low-silica HT type stone wool are given, and the results were compared with the results from a similar study done with the traditional stone wool MMVF21. Male Wistar rats were exposed at one exposure level by nose-only inhalation to well-characterized fibre test atmospheres. The fibres had been size selected to be largely rat respirable. The target dose was an exposure to 150 long fibres/ml (length > 20 μm) in each group, and this dose was achieved for all the fibres. The negative control groups were exposed to filtered air. The exposure duration was 6 h/day, 5 days/week for 3 months, with a subsequent non-exposure period lasting 3 months. The rats were killed 1 week after the last exposure and additional post-exposure kills were performed at 1.5 and 3 months to monitor the progression of pulmonary change and fibre numbers in the lung. The assessments included bronchoalveolar lavage fluid (BALF) for evaluation of inflammatory response (e.g. protein content, enzymes, increase in polymorphnuclear leucocytes) and measurement of cell proliferation, assessment of early fibrosis through histological examination and comparison of body weight and lung lobe weights. After exposure of rats to the new biosoluble fibres no biologically significant effects were observed except that a statistically significant increase in lung weight was observed up to 1.5 months post-exposure in all three treatment groups. At 3 months post-exposure, the small increase was no longer significant. The increase in lung weight was still present in the MMVF21 group at the 3 months post-exposure kill. After 3 months exposure, lung retention of long fibres (length > 20 μm) varied from 0.4 to 5.2 × 10^6 per lung for the biosoluble fibres. At 3 months post-exposure, the long fibre concentration in the lungs had decreased to 1–7% of this figure. The fibre with the relatively highest biopersistence (RIF41001) showed the highest fibre retention. The retention of the more biopersistent traditional stone wool MMVF21 was 5.7 × 10^6 per rat lung after 3 months exposure and had decreased to 64% of this figure at 3 months post-exposure. There was no clear difference in the bronchoalveolar lavage fluid cell concentration and percentage of cells between MMVF21 and the HT groups. Fibre inhalation caused a significant increase after 3 months in all the biochemical parameters measured in the BALF. Cell proliferation was enhanced at the end of exposure for MMVF21 for all three labelling indices, but only for the bronchiolar epithelium in the RIF41001 group and for alveolar parenchymal cells in the RIF43006-1 group. At the termination of the 3 month exposure period, as well as after 1.5 and 3 month recovery periods, minimal morphological changes were diagnosed in the biosoluble fibre groups. These changes included alveolar macrophage aggregation and/or microgranulomas at the bronchiolar–alveolar junction in the few rats affected. No fibrogenic potential was noted for any of the three fibres. No clear-cut difference between the different biosoluble fibre types was noted. In the MMVF21 group, minimal interstitial fibrosis was observed that gradually decreased after the 1.5 and 3 month non-exposure periods. In this study, the pathological changes found in the lungs of exposed rats were in accordance with the pathology previously reported from full lifespan inhalation studies. This may indicate that for fibres belonging to the man-made vitreous fibres group a well conducted biopersistence study

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is sufficient to predict possible pathogenic effects for new fibre types. The biological parameters examined in a 90 day study may indicate little additional information to contribute to the prediction of the outcome of carcinogenicity studies.

Keywords: man-made vitreous fibres; biosoluble stone wool; high-aluminium low-silica (HT) wool; subchronic inhalation; pathogenicity; rat

INTRODUCTION

Most of the man-made vitreous fibres (MMVFs) [also known as synthetic vitreous fibres and man-made mineral fibres (MMMF)] produced are used as thermal or acoustic insulation. Rock (stone) wool is used predominantly in Europe. In recent years, high-aluminium, low-silica wools (HT wools) with reduced biopersistence are increasingly replacing traditional stone wools in this application (IARC, 2002).

The adverse effects of MMVFs of concern are mechanical skin irritation caused by friction of coarse fibres and a possible hazard of developing respiratory diseases, including lung cancer, after long-term exposure to respirable fibres. The most important endpoints that have been associated with exposure to MMVFs include chronic persistent inflammation, fibrosis and cell proliferation in the lungs and mesothelial lining. There is a consistent relationship between persistent inflammation, fibrosis and tumour development in animal models (McConnell *et al.*, 1999). Pathogenic fibres such as asbestos cause severe pulmonary fibrosis (asbestosis) as well as pulmonary carcinomas. In many experimental inhalation studies with such fibres, significant tumour production has been found to occur only when there has also been severe widespread fibrosis (Davis and Cowie, 1990). In animal studies, different MMVFs have shown a range of severity of inflammation and fibrosis with the severe effects being found for biopersistent fibres (Hesterberg *et al.*, 1998). The biopersistence of fibres deposited in the respiratory tract results from a combination of physiological clearance processes (mechanical translocation/removal) and physico-chemical processes (chemical dissolution and leaching, mechanical breaking) (IARC, 2002).

In Europe, DG XI Directive 97/69/EC (European Commission, 1997) provides guidance for MMVF classification, labelling and testing. The MMVF classification and labelling regulations follow theories generally accepted within the scientific community that relates fibre toxicity to biodurability (biopersistence or solubility) within the lung. Since long fibres are thought to be of greatest biological activity and more pathogenic than the shorter ones (Davis, 1991), the focus of the animal test has been on these fibres. In biopersistence assays, the long fibre (length > 20 μm) retention kinetics in the lung is evaluated and a number of studies in rats have suggested a correlation between the biopersistence of long fibres and their pathogenicity. The number of long fibres in the aerosol primarily defines the dosages in subchronic and chronic inhalation assays.

The International Agency for Research in Cancer (IARC), a division of the World Health Organization (WHO), in 1988 classified glass wool, rock wool and slag wool as possibly carcinogenic to humans (Group 2B) (IARC, 1988). However, in 2001 the classification was changed, and the insulation glass wool, rock (stone) wool and slag wool were classified as Group 3 (not classifiable) (IARC, 2002).

The IARC Working Group evaluated the human epidemiological and experimental data, and concluded that the results from the most recent cohort and nested case–control studies of US workers exposed to glass wool and continuous glass filaments, and of European workers exposed to rock (stone) and slag wool, did not provide consistent evidence of an association between fibre exposures and risk for lung cancer or mesothelioma. There is therefore inadequate evidence in humans for the carcinogenicity of rock (stone) wool (IARC, 2002).

The IARC Working Group considered that there is limited evidence in experimental animals for the carcinogenicity of traditional stone wool, but that there is inadequate evidence in experimental animals for the carcinogenicity of certain newly developed, less biopersistent fibres, including HT wool (IARC, 2002).

The IARC Working Group overall conclusion was that rock (stone) wools are not classifiable as to their carcinogenicity to humans (Group 3). The IARC Working Group elected not to make an overall evaluation of the newly developed fibres designed to be less biopersistent, such as HT wools, although those that have been tested appear to have low carcinogenic potential in experimental animals. This decision was made in part because no human data are available and the Working Group had difficulty in categorizing these fibres into meaningful groups based on chemical composition (IARC, 2002).

Within the European Union (EU), MMVFs are still classified as Carcinogenic category 3 (possibly carcinogenic) and additionally as an Irritant (irritating to skin) (European Commission, 1997). Commission Directive 97/69/EC, Note Q allows for derogation (exemption) from classification based on passing any one of the following tests: short-term intratracheal or inhalation biopersistence tests or long-term inhalation or intraperitoneal carcinogenicity tests.

A range of Rockwool International fibres have been tested at a qualified, independent laboratory pursuant to EU protocols for testing (Guldberg *et al.*, 2002).

The animal test results for the HT wool types were below regulatory thresholds in biopersistence tests and the HT type fibre is therefore not classified as carcinogenic within the EU. The HT fibres have also previously been tested in a long-term inhalation study in rats and produced no significant increase in the incidence of lung tumours and no mesotheliomas (Kamstrup *et al.*, 2001). In animals exposed to MMVF21, a similar result was found, but this fibre resulted in fibrosis of the lungs (McConnell *et al.*, 1994). In addition, HT fibres have been tested in a study in rats administered by intraperitoneal injection at a high dose (~10^9 fibres) and no abdominal tumours were observed (Kamstrup *et al.*, 2002). In several studies of intraperitoneal injection with similar doses of different traditional stone wool types a significant increase in mesothelioma incidence was seen (Roller *et al.*, 1996).

For MMVFs it is well established that there is a correlation between the biopersistence of long fibres and the biological activity (IARC, 2002; Maxim *et al.*, 2002). The EU protocol for 90 day inhalation studies was developed to fill a gap between the current short-term biopersistence tests and carcinogenicity studies used for regulatory purposes.

Because of the high costs of long-term inhalation and injection studies in experimental animals, it has been suggested that adequate biological and biopersistence data to predict the pathogenicity of fibres may be obtained by a 90 day inhalation study using the protocol proposed by the European Commission (European Joint Research Centre, 1999). The aim of the Rockwool International sponsored study was to investigate the biological effects and toxicity of three different HT type fibres (RIF41001, RIF42020-6 and RIF43006-1), all representing the high-aluminium low-silica wool type in a subchronic (3 month) inhalation study and to compare the results with those from a previously conducted 90 day study with the traditional stone wool MMVF21 (Bellmann *et al.*, 2003) and long-term inhalation studies with these fibre types (McConnell *et al.*, 1994; Kamstrup *et al.*, 2001)

MATERIALS AND METHODS

Overall design of studies

The animal experimental work and fibre counting in the study were conducted at the Fraunhofer Institute of Toxicology and Aerosol Research (Fh-ITA), Hannover, Germany. Johns Manville Technical Center (JMTC), Littleton, CO, did the fibre size selection. Experimental Pathology Services (EPS), Muttenz, Switzerland, performed the patho-morphological examinations.

The studies were conducted in compliance with the principles of good laboratory practice and carried out according to the EU protocol for conducting subchronic inhalation studies with MMVFs (European Joint Research Centre, 1999).

Laboratory rats were exposed by nose-only inhalation to well-characterized fibre test atmospheres that had been selected to be largely rat respirable (diameter < 1 µm). The rats were randomly assigned to the individual exposure groups.

Groups of rats were exposed to four different man-made vitreous fibres each at one nominal exposure level of 150 fibres/ml (length > 20 µm). The negative control groups were exposed to filtered air. The exposure duration was 6 h/day, 5 days/week for 3 months, with a subsequent non-exposure observation period lasting 3 months. One week after the end of the 3 months exposure the rats were killed and additional post-exposure kills were performed after 1.5 and 3 months. The findings of the studies were considered in terms of possible adverse biological effects, the necropsy and histopathological findings and were related to fibre exposure and fibre lung burden data. Fibre retention was analysed in terms of the number and bivariate size distribution of fibres in the lungs at different time intervals after exposure. The evaluations included analysis of bronchoalveolar lavage fluid (BALF) for evaluation of cell concentrations and biochemical parameters, a proliferation measurement test [5-bromo-2′-deoxyuridine (BrdU) S phase response assay], assessment of early fibrosis through conventional histopathology of lungs and using a morphometric quantitative method and a comparison of body weight and lung lobe weights.

Test substances

The present study included three different stone wool fibre types of the high-aluminium low-silica type wool (HT wool, CAS registry no. 287922-11-6), RIF41001, RIF42020-6 and RIF43006-1, all newly developed commercial insulation wool products. A previously conducted subchronic inhalation study included the traditional stone wool MMVF21 (Bellmann, 2003). The negative control groups in both studies were filtered air. The HT type fibres are characterized by a high dissolution rate at pH 4.5 and a relatively low dissolution rate at pH 7.5 (Knudsen *et al.*, 1996).

The bulk products were produced without the addition of binder or oil. Bulk fibres were size separated, using a water-based process, to be largely rat respirable and aimed at having a geometric mean diameter of ~0.8 µm and a length of ~15 µm. The possible effects of the size selection process on the chemical and surface characteristics of these fibres have been found to be insignificant based on a comparison of

the morphology of fibre surfaces using scanning electron microscopy (SEM) of the bulk fibres and the size separated fibres, with respect to the chemical composition, and the *in vitro* dissolution rates at pHs 4.5 and 7.5.

The chemical compositions are given in Table 1. Due to analytical variation and minor differences in the different batches used, variations may occur in cited values for these fibres. As shown in Table 1, the new biosoluble fibres representing the HT wools are characterized by a relatively high content of aluminium and a relatively low content of silica compared with the traditional stone wool.

Stone wool fibres ranged in density from 2.7 to 2.9 g/cm^3 (IARC, 2002).

The test fibres were characterized by SEM. A small fraction of the test substance (~0.1 mg) was suspended in 20 ml of double-distilled water. To minimize dissolution, the fibre residence time in the suspension was below 10 min. Then the suspension was sonicated for ~30 s and filtered onto a Nuclepore filter.

The preparation of the SEM sample and the counting and sizing used for stock fibres and fibres in aerosol samples and lung samples was as described below.

A part of the filter containing the fibre sample was mounted on an aluminium stub and sputtered with ~30 nm of gold. The general guidelines for counting and sizing provided by the WHO/EURO Technical Committee for Monitoring and Evaluating MMMF (1985) was followed, with the following additional procedures for mineral fibres.

Sizing of lengths and diameters was performed using SEM at a magnification of at least 2000. All objects which were seen at this magnification were counted. Fibres crossing the boundary of the field of view were counted as follows. Fibres with only one end in the field were weighted as half of a fibre and fibres with neither of their ends in the field were not measured. Diameters of fibres that were seen at 2000 magnification were measured at full screen magnification (usually up to a magnification of 12 000). No lower or upper limit was imposed on either length or diameter. The length and diameter were recorded individually for each fibre measured so that the bivariate distribution could be determined. When sizing, an object was accepted as a fibre if the ratio of length to diameter was at least 3:1. All other objects were considered particles. There was no truncation in the measurements, as for all fibres measured the full length was determined and all fibre diameters were above the limit of detection.

Enough fields of view were counted for evaluation so that at least a total of 0.15 mm^2 of the filter surface (for 25 mm diameter) was examined. Once this condition was fulfilled, fibres and particles were treated as follows:

Fibres. A size selected analysis using a minimum of 100 fibres per category for the two length categories <5 μm and >20 μm and a minimum of 200 fibres for the length category 5–20 μm was used. The distance between two fields of view for analysis was at least 10 fields. Sizing was stopped when 1 mm^2 of the filter surface was examined, even if the minimum number of fibres was not reached for a category. The total number of fibres per filter was determined by normalizing the surface area counted to the total surface area of the filter.

Particles. The recording of particles was stopped when a total of 30 particles had been counted. Mean length and diameter of the stock fibres are presented in Table 2. As can be seen, the HT fibre types were thinner and longer than the MMVF21 fibres.

Test system

Rats were selected for appropriate comparison of results from these studies with those from previous inhalation studies performed with other MMVFs. Male Wistar [strain Crl:(WI)WU], SPF (specific pathogen free) rats (Charles River, Sulzfeld, Germany) were used.

Wistar rats were recommended by an EPA workshop (Vu *et al.*, 1996) for use in subchronic and chronic inhalation toxicity studies of fibres. According to EU guidelines, the use of male Wistar rats is preferred.

The age of the animals at the start of exposure was ~9–10 weeks and the weight ~200–300 g. The animals were randomized to treatment groups stratified on

Table 1. Chemical composition of fibres by weight percentage

	RIF41001	RIF42020-6	RIF43006-1	MMVF21
SiO_2	42.7	36.8	40.6	45.9
Al_2O_3	18.9	20.7	21.9	13.8
TiO_2	1.6	1.4	1.6	3.0
FeO	6.3	5.4	5.6	6.2
CaO	18.3	18.0	15.6	17.0
MgO	7.8	10.2	10.7	9.5
Na_2O	1.7	4.4	1.4	2.5
K_2O	0.9	0.5	0.6	1.3
Other oxides	0.2	0.9	0.5	0.4
Sum	98.4	98.3	98.5	99.4

Table 2. Stock fibre dimensions [geometric mean (μm) ± SD]

Study	All fibres ($L/D > 3$)		$L > 20$ μm	
	Diameter	Length	Diameter	Length
RIF41001	0.62 (1.8)	17.3 (2.8)	0.75 (1.9)	44.2 (1.7)
RIF42020-6	0.56 (2.0)	15.7 (2.6)	0.72 (1.9)	36.5 (1.5)
RIF43006-1	0.54 (2.0)	17.2 (2.5)	0.63 (2.0)	38.1 (1.6)
MMVF21	0.92 (1.7)	10.9 (2.4)	1.13 (1.8)	35.2 (1.6)

body weight. Thirty-two rats (including two reserve animals) were initially allocated per exposure group. Ten rats were allocated for each post-exposure kill group. The five animals that were killed at each time point for histopathology were also used for the lung burden determinations.

Unique numbers identified the rats. When not being exposed, the rats were housed in groups of two in polycarbonate cages. The studies were conducted under optimum hygienic conditions behind a barrier system. The rooms were air conditioned and had a monitored environment with a temperature of 22 ± 2°C and a relative humidity of 40–70%. A period of 12 h of artificial light and a 12 h dark period were used. Pelleted standard rat maintenance diet and tap water were supplied *ad libitum* during the non-exposure periods.

For a period of 2 weeks the rats were allowed to become acclimatized and prior to exposure animals were trained for 3 weeks to become accustomed to the nose-only tubes.

Exposure

Inhalation was used as the method of administration as it closely mimics that in humans and represents the natural route of uptake. The nose-only, flow-past technique was used. In this system, the fibre aerosol is supplied to each animal individually, and exhaled air is immediately exhausted.

The draft EU guideline (European Joint Research Centre, 1999) *Sub-chronic inhalation toxicity of synthetic mineral fibres in rats*, uses the number of fibres with a length >20 μm as the basis of the choice of exposure condition and specifies three dosage groups with 15, 50 and 150 fibres >20 $\mu m/cm^3$ in the different dosage groups. The study with MMVF21 was conducted using these dosage groups, but only the data from the high dosage group was used for comparison with the HT type fibres. The study with the HT type fibres was done using the high dosage only. The duration of exposure was 6 h/day, during the hours of light, 5 days/week for 3 months.

For each nose-only exposure unit the fibre aerosol was generated by a high pressure pneumatic disperser. The disperser was fed with test substances under computerized control, i.e. with feedback to the actual aerosol concentrations measured by an aerosol photometer. The photometer gives a scatter light signal, which is nearly proportional to the particle concentration, if the particle size distribution is constant. The ratio between photometer signal and concentration was determined throughout the study by comparison with gravimetric and fibre concentration. The aerosols of the test substances were neutralized by a ^{63}Ni source to reduce the charge on the fibres.

The airflow to each animal was ~1 l/min, which is calculated to be laminar. Therefore, according to the EU guidelines, it was not necessary to measure the oxygen concentration. The airflow, the temperature and the humidity were monitored continuously and were stored as 20 min mean values.

The animals were restrained in Battelle type polycarbonate tubes; with this system animals in the supply tubes keep their noses close to the airflow at the opening of the tube. The exposure of animals was performed in identical exposure chambers of cylindrical shape, each housing up to 48 animals (three levels of 16 animals each).

Observations, examinations and measurements

The rats were examined daily for clinical signs, morbidity and mortality. They were individually examined outside the cage once a week. Body weight was recorded once each week during the first 3 months, then every second week.

Necropsy was performed on all animals. At the scheduled kills the lungs were removed *in toto*, weighed without the trachea and carefully examined. The right lobes were removed, weighed and deep frozen for lung fibre burden analysis (see below). The left lung lobe was perfused with fixative via the trachea at a pressure of 20 cm water for 2 h.

Replicate sections for histopathology were stained with Trichrome (Masson-Trichome method) or haematoxylin and eosin. The lungs were examined and classified histopathologically according to the EPS grading system, as described below. Histological changes were described according to distribution, severity and morphological character of the following parameters: alveolar bronchiolization, micrograngulomas and collagen deposition at the bronchiolar–alveolar junction, pleural collagen deposition and macrophages in the alveolar lumina. The severity was scored as one of the following grades: no lesions, minimal, slight, moderate, marked or massive (grades 0–5, respectively). A Wagner score for inflammatory change and fibrosis was given for all rats at scheduled kills (McConnell *et al.*, 1984). In this system a grade of 1 is considered normal, grades 2–3 are evidence of focal cellular change, while grades 4–8 represent the former lesion plus increasing degrees of fibrosis. A quantitative evaluation of fibrosis using a morphometric method according to the criteria of McConnell and Davis (2002) was also done. By this method the combined area of fibrotic lesions was calculated using an eyepiece graticule in the microscope.

Formalin fixed tissue of the terminal bronchioles, pleural cells and lung parenchyma cells were examined for cell proliferation using the sensitive S phase response method. Proliferating cells were labelled with BrdU, which was administered to the animals by a minipump prior to death. The lung section slides were prepared according to routine histological procedures and stained immunohistochemically

following denaturation of the DNA (antibody technique). The slides were evaluated by analysing an appropriate number of airway cells [unit length proliferation index (ULLI) given as BrdU-positive cells per mm of airway] and cells of the proximal regions of the pulmonary parenchyma per rat (a minimum of 2000 cells were counted and the percentage of positive cells given). In addition, pleural cells were analysed and the ULLI estimated (given as BrdU-positive cells per cm of pleura per lung section, at least 1 cm evaluated). The same method was used for MMVF21 in the previous study. However, as different batches of BrdU and especially of the immunochemicals were used for staining, the absolute values cannot be compared. Only the relative effects in comparison with the control group can be compared between the different studies.

For bronchoalveolar lavage the method of Henderson *et al.* (1987) was used with minor modifications. Following preparation of the lungs, they were lavaged with 2 × 5 ml of saline without massage. Further lavages would dilute the non-cellular constituents like lactic dehydrogenase (LDH). For this reason two lavages were optimal. The lavage fluid was collected in calibrated tubes and the harvested volume was recorded. Until processing the lavage fluid was kept on ice. Using a 20 µl aliquot, the cell concentration was determined with a Fuchs-Rosenthal counting chamber under a light microscope at 125× magnification. From each lavage sample two cytoslides were prepared. An optimum coating of cytoslides is achieved using ~80000 cells/slide. Cells were centrifuged onto the slides in a cytocentrifuge running at 1500 r.p.m. (250 *g*) for 10 min. Cytoslides were stained with May-Grünwald and Giemsa solution. Under a light microscope at 625× magnification, 2 × 100 cells were differentiated per cytoslide. Differentiation included macrophages, neutrophils, eosinophils, epithelial cells and other cells. The lavagate was centrifuged in a cooled centrifuge for 10 min at 1000 r.p.m. (209 *g*). One millilitre of the cell-free supernatant was taken for the determination of the biochemical parameters (LDH, β-glucuronidase and total protein). These parameters were analysed according to routine clinical chemistry protocols using a Cobas Fara device (Roche Co., Grenzach, Germany).

For lung burden analysis, at necropsy the right lung lobes from each animal were removed, weighed and kept deep frozen.

The right lung lobes were then freeze-dried and subjected to low temperature ashing at an energy of 400 W for at least 6 h. A fraction of the ashed lung was suspended in filtered water using ultrasonic treatment for 1 min. The homogeneity of the suspension was checked through the bottle in front of a light. If clusters remained, sonification for 1 min was repeated until no clusters remained evident. The homogeneous suspension was filtered on a nuclepore filter. The fibre and particle concentration and the size distribution of fibres and particles were analysed by SEM as described above. From these data the total number of fibres per right lung lobe was determined for each animal.

Total lung burdens were calculated, using three measured data: total lung weight (measured)/right lobe weight (measured) = total lung burden (calculated)/right lobe lung burden (measured).

It was assumed that there was a homogeneous distribution of fibres in lungs after inhalative uptake.

Evaluation of data

The individual toxicological data were compared with the clean air control group. For numeric parameters the statistics were done using Dunnett's test. For comparison of pathological findings a pairwise Fischer's test between control and treatment groups was performed.

RESULTS

Exposure atmosphere and fibre characterization

Table 3 shows the mean aerosol concentrations over the 3 month exposure phase for total fibres, WHO fibres, fibres with a length >20 µm and gravimetric concentrations. In addition, the mean dimensions of the fibres are given. As can be seen, the differences in the dimensions of the stock fibres given in Table 2 are reflected in the aerosols. As indicated by the data, thinner fibres produced higher concentrations of airborne fibres, as seen previously in studies of naturally occurring fibres and MMVFs.

The total number of fibres in the HT groups was lower than in the MMVF21 group, while the number

Table 3. Mean exposure aerosol concentrations (± SD) and dimensions [geometric mean (µm) ± SD]

Fibre	Fibres (f/cm^3)	WHO (f/cm^3)	>20 µm (f/cm^3)	Gravimetric fibre concentration (mg/m^3)	Geometric mean (µm)			
					All fibres ($L/D > 3$)		$L > 20$ µm	
					Diameter	Length	Diameter	Length
RIF41001	529 (110)	408 (88)	149 (40)	15.9 (3.3)	0.55 (1.7)	11.3 (2.7)	0.66 (1.7)	36.8 (1.6)
RIF42020-6	605 (113)	461 (87)	157 (33)	14.6 (2.4)	0.45 (1.8)	10.1 (2.6)	0.64 (1.8)	32.4 (1.5)
RIF43006-1	689 (114)	524 (89)	157 (35)	14.0 (1.9)	0.44 (1.8)	9.9 (2.5)	0.58 (1.8)	31.4 (1.4)
MMVF21	959 (249)	705 (181)	174 (47)	37.0 (10.9)	0.83 (1.7)	9.1 (2.3)	0.93 (1.7)	33.1 (1.5)

of long fibres (L > 20 µm) was comparable. The calculated gravimetric concentration was much higher in the MMVF21 group than in the HT groups.

For all fibres, there was a reduction in diameter and length values from the stock to the aerosol fibres, indicating a minor influence of the aerosolization procedure on the fibre characteristics, probably by sedimentation of long and thick fibres.

The mean value of the long fibre concentration was within ±10% of the target value.

Lung burden analyses

The lung fibre burden gives the retained amount of the administered dose present in the lungs, i.e. retention = deposition – clearance. The number of total fibres, WHO and long (L > 20 µm) fibres per total lung at the kills after 3 months exposure and 1.5 and 3 months non-exposure are shown in Table 4.

In both stock fibres and aerosol fibres the diameter of the HT fibres was smaller than that of the MMVF21 fibres. However, when the lung fibre burden was examined at the first time point, the dimension of the retained fibres was very similar for all fibre types. This probably indicates that the lung filters out the thicker fibres, so that the lung tissue is exposed to very similar fibre dimensions regardless of the fibre type.

The normalized number of long fibres per total lung is also shown in Figure 1 and illustrates the clearance of long fibres with time and illustrates the higher biopersistence of the long MMVF21 fibres.

The maximum lung burden achieved was considerably lower in the HT groups than in the MMVF21 group, except in the RIF41001 group, which showed comparable numbers at the end of exposure. Even with this HT type the long fibres cleared much faster in the recovery period than MMVF21.

Clinical signs and mortality

In both studies, clinical signs were recorded in isolated animals but were not attributed to treatment with the test articles.

Body weights

In the study with HT, body weight gains were comparable between the exposed and the air control group. In the study with MMVF21 a similar picture was seen.

Lung weights and lung:body weight ratios

At the 3 months kill, the mean lung weights in both the HT and MMVF21 exposed groups showed statistically significant increases compared with the control values. The significant increase in lung weights was still present in the MMVF21 group but not in the HT groups at the 3 month post-exposure kill.

The actual lung weights and the lung/body weight ratios are shown in Table 5.

Bronchoalveolar lavage

At the end of exposure and during the post-exposure recovery period bronchoalveolar lavages were performed on five rats per group and kill date. The results of the differential cell count are presented in Table 6 and the results of the biochemical analyses are presented in Table 7.

For all biosoluble fibre groups and MMVF21 a significant increase in PMN and a reduction in the percentage of macrophages in the BALF was observed.

There was no clear difference in the BALF cell concentration and percentage of cells between MMVF21 and the HT groups, although there was a

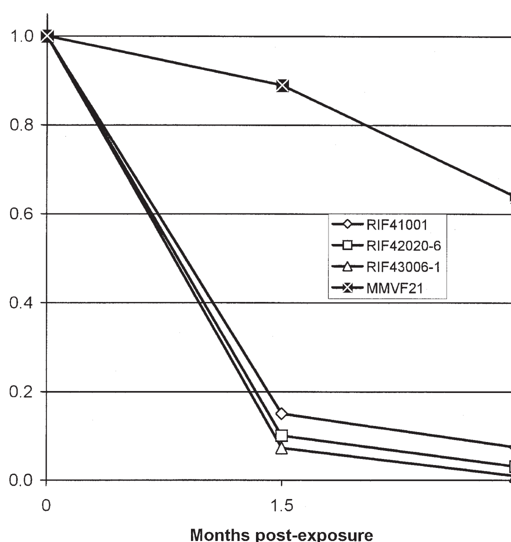

Fig. 1. Normalized lung burdens (long fibres, L > 20 µm) after different post-exposure periods.

Table 4. Mean total lung burdens (fibres × 10^6 ± SD) after different post-exposure periods

Time (months)	RIF41001			RIF42020-6			RIF43006-1			MMVF21		
	Fibres	WHO	L > 20 µm	Fibres	WHO	L > 20 µm	Fibres	WHO	L > 20 µm	Fibres	WHO	L > 20 µm
EO	123 (61)	74 (40)	5.2 (2.2)	77 (25)	35 (12)	0.4 (0.1)	107 (36)	47 (14)	1.1 (0.6)	124 (12)	66 (6)	5.7 (2.1)
1.5	73 (14)	41 (9)	0.8 (0.7)	28 (5)	9 (2)	0.0 (0.0)	52 (10)	17 (3)	0.1 (0.0)	106 (37)	57 (17)	5.1 (1.0)
3	38 (8)	24 (6)	0.4 (0.1)	11 (3)	3 (2)	0.0 (0.0)	16 (5)	4 (1)	0.0 (0.0)	109 (41)	62 (18)	3.6 (1.4)

EO, end of exposure.

Table 5. Mean lung weights (g) and lung:body weight ratios (%) after different post-exposure periods

Group/exposure	End of exposure		1.5 months		3 months		*n*
	LW	LW/BW	LW	LW/BW	LW	LW/BW	
RIF41001	1.48***	0.40***	1.55**	0.36*	1.52	0.34	10
RIF42020-6	1.51***	0.39***	1.52*	0.36**	1.53	0.34	10
RIF43006-1	1.47***	0.38***	1.57**	0.36**	1.52	0.32	10
Control[a]	1.24	0.32	1.38	0.32	1.38	0.31	10
MMVF21	1.40**	0.38**	1.55**	0.37	1.62*	0.37*	10
Control[b]	1.25	0.34	1.37	0.34	1.37	0.31	13

ANOVA + Dunnett's test (two-sided): $*P \leq 5\%$, $**P \leq 1\%$, $***P \leq 0.1\%$.
[a]HT study control.
[b]MMVF21 study control.

tendency towards a higher percentage of PMNs in the HT groups.

LDH and total protein were significantly elevated in all groups at the end of exposure kill and remained elevated at the 3 month post-exposure kill.

Cell proliferation test

The results of the BrdU proliferation test are summarized in Table 8. Cell proliferation was enhanced at the end of exposure for MMVF21 for all three labelling indices, but only for the bronchiolar epithelium in the RIF41001 group and for alveolar parenchymal cells in the RIF43006-1 group. No significant increase in cell proliferation was found at the later kill dates.

Histopathology

Table 9 shows the histological results according to the EPS grading system for alveolar bronchiolization (replacement of epithelial cells in some alveoli with cells of bronchiolar epithelial type), microgranulomas and collagen deposition at the bronchiolar–alveolar junction, pleural collagen deposition and macrophages in the alveolar lumina. As can be seen from Table 9, after 3 months exposure differences between HT and MMVF21 were seen for all the parameters. In the control group there were no lesions (grade 0).

Table 10 summarizes the mean (Wagner) pulmonary change findings. At the corresponding kill points, all the control animals were graded 1 according to the Wagner scale. As indicated in Table 10, examinations of lung tissue from rats exposed to the HT fibre types had lower Wagner scores than animals exposed in the study of MMVF21.

Throughout the observation period, only slight macrophage reaction was seen in the lungs of rats exposed to HT fibre types (Wagner grade 1–2). At the end of exposure kill, Wagner grade 1 was noted in all animals exposed to RIF41001 and RIF43006-1. In the rats exposed to RIF42020-6, Wagner grade 1 was noted in two rats and grade 2 was noted in three rats. The Wagner grade 2 was characterized by focal minimal microgranulomas at the bronchiolar–alveolar junction. In one of these rats, minimal focal collagen deposition was noted in a microgranuloma. Since this collagen deposition was restricted to one small focus, it was considered not sufficient for the Wagner score grade 4.

In contrast, in the MMVF21 group, Wagner grade 3 was noted in one rat and grade 4 was noted in four rats by 3 months.

Table 11 presents the mean morphometric fibrosis scores after different post-exposure periods. At the end of exposure kill marked differences between MMVF21 and the HT fibre types were seen. In the HT fibre groups there was literally nothing to measure. However, in the MMVF21 group the amount of interstitial collagen was diminished at the 1.5 month post-exposure kill and nearly disappeared at the 3 month post-exposure kill.

Table 12 summarizes the main pathology findings from the study

DISCUSSION

The primary objective of the present study was to assess the pathological effects of three HT type fibres, a newly developed stone wool with increased biosolubility, in a subchronic inhalation study. The HT type fibre was developed recognizing that the potential pathogenicity of a given fibre type is mainly dependent on the extent to which the fibres can be inhaled and persist in the lung (Davis, 1991). Since only one dose was used in the study with the HT fibre types, any dose-dependent effects could not be evaluated.

Long fibres are thought to be of greatest biological activity and more pathogenic than shorter ones (Davis, 1991) and the draft EU guidelines on subchronic inhalation toxicity studies with mineral fibres in rats specify that the aerosol concentration to which the animals are exposed at the highest dose should be 150 fibres/cm^3 of those >20 μm in length (European Joint Research Centre, 1999). This

Table 6. Bronchoalveolar lavage fluid cell concentration and percentage of cells

Group		Post-exposure period (months)											
		End of exposure				1.5				3			
		Cell conc. (cells/µl)	Macrophages (%)	PMNs (%)	Lymphocytes (%)	Cell conc. (cells/µl)	Macrophages (%)	PMNs (%)	Lymphocytes (%)	Cell conc. (cells/µl)	Macrophages (%)	PMNs (%)	Lymphocytes (%)
Control[a]	Mean	93.8	98.1	0.4	1.6	84.5	99.2	0.7	0.2	126.4	99.4	0.4	0.2
	SD	16.8	2.7	0.4	2.4	16.6	1.0	0.9	0.2	21.6	0.6	0.3	0.3
	n	5	5	5	5	5	5	5	5	6	6	6	6
RIF41001	Mean	121.8	76.1***	18.8***	5.2	71.3	84.4*	13.1	2.5**	111.7	87.3***	10.7***	2.0*
	SD	53.5	9.4	7.2	3.1	11.6	2.3	3.2	1.1	34.4	6.4	7.5	1.8
	n	5	5	5	5	5	5	5	5	6	6	6	6
RIF42020-6	Mean	126.0	63.3***	32.3***	4.5	69.1	74.2*	24.2***	1.6*	105.3	83.6***	14.5***	1.9
	SD	30.0	6.7	6.7	2.1	16.2	14.5	14.4	1.2	39.1	2.0	1.0	1.5
	n	5	5	5	5	5	5	5	5	6	6	6	6
RIF43006-1	Mean	139.8	72.0***	22.7***	5.4	82.8	75.3***	22.3**	2.5**	117.6	86.5***	12.0***	1.5
	SD	38.6	5.5	5.3	4.0	17.9	3.7	3.9	0.5	23.8	3.3	2.6	0.8
	n	5	5	5	5	5	5	5	5	6	61	6	6
Control[b]	Mean	99.4	99.3	0.2	0.5	106.5	99.1	0.6	0.3	110.1	99.3	0.3	0.4
	SD	15.8	0.5	0.2	0.3	28.2	0.8	0.6	0.3	14.5	0.7	0.3	0.5
	n	8	8	8	8	8	8	8	8	8	8	8	8
MMVF21	Mean	114.1	83.7***	10.9***	2.7*	93.1	92.8***	5.6***	1.7	109.4	93.2**	5.4***	1.5
	SD	23.6	8.7	9.0	1.2	8.9	2.6	1.9	1.1	39.2	3.2	2.5	1.0
	n	5	5	5	5	5	5	5	5	5	5	5	5

SD, standard deviation; *n*, number of animals.
ANOVA + Dunnett's test (two-sided): $^{*}P \leq 5\%$, $^{**}P \leq 1\%$, $^{***}P \leq 0.1\%$.
[a]HT study control.
[b]MMVF21 study control.

Table 7. Bronchoalveolar lavage fluid biochemical parameters

Group		Post-exposure period (months)								
		End of exposure			1.5			3		
		LDH (U/l)	βGL	Protein (mg/l)	LDH (U/l)	βGL	Protein (mg/l)	LDH (U/l)	βGL	Protein (mg/l)
Control[a]	Mean	27.2	0.2	98.0	35.6	0.1	109.2	33.3	0.2	103.8
	SD	5.0	0.1	19.8	18.2	0.0	34.0	15.8	0.1	19.3
	n	5	5	5	5	5	5	6	6	6
RIF41001	Mean	94.6***	0.3*	201.2***	51.0	0.2	141.4	80.5	0.2	161.8
	SD	25.7	0.1	34.2	11.6	0.1	18.8	83.6	0.1	73.4
	n	5	5	5	5	5	5	6	6	6
RIF42020-6	Mean	60.4*	0.2	179.6***	46.8	0.1	142.6	45.5	0.3	134.3
	SD	12.1	0.1	23.5	7.5	0.0	13.7	11.0	0.3	25.2
	n	5	5	5	5	5	5	6	6	6
RIF43006-1	Mean	86.6***	0.3	215.0***	61.6	0.1	154.2*	50.3	0.2	154.7
	SD	13.6	0.0	25.3	18.4	0.1	17.9	13.5	0.1	31.8
	n	5	5	5	5	5	5	6	6	6
Control[b]	Mean	39	0.2	105.0	30.0	0.2	100	31	0.1	116
	SD	24	0.1	38	9.0	0.0	15	18	0.1	25
	n	8	8	8	8	8	8	8	8	8
MMVF21	Mean	104***	0.5**	205***	51*	0.3	146**	50.0	0.2*	162**
	SD	42.0	0.1	36.0	11.0	0.1	22.0	16.0	0.1	17.0
	n	5	5	5	5	5	5	5	5	5

SD, standard deviation; *n*, number of animals.
ANOVA + Dunnett's test (two-sided): $*P \leq 5\%$, $**P \leq 1\%$, $***P \leq 0.1\%$.
[a]HT study control.
[b]MMVF21 study control.

concentration of long fibres was achieved for all fibres in the studies.

In the present study, the lung burdens of long fibres observed demonstrate the higher biosolubility of the HT fibre compared to traditional stone wool (MMVF21), as lower lung burdens after 3 months of recovery were found with the HT fibre than in the MMVF21 group. No elimination half-times are reported because there were only three kill points available for the calculation. A previous short-term (5 days) inhalation biopersistence study with the HT and MMVF21 fibres showed elimination half-times, calculated from the decrease in WHO fibres and long fibres ($L > 20$ μm), of 25 and 6 days, respectively, for the HT fibre. Elimination of the traditional stone wool (MMVF21) was much slower, with elimination half-times of 65 days (WHO fibres) and 92 days (long fibres). After 3 months inhalation of HT fibres, an elimination half-time of 17 days (long fibres) was shown (Kamstrup *et al.*, 1998). This is more consistent with the lung burdens shown in Fig. 1 than the elimination half-time of 6 days after 5 days inhalation, and may indicate that longer exposure periods can increase the elimination half-time.

It is important that the biological testing of fibrous materials is undertaken at a dose sufficient to cause pathological changes when the fibres have this potential. The maximum tolerated dose (MTD) is recommended (McConnell, 1996) and this is recognized using a number of parameters, including an increase in the lung/body weight ratio. In the present study, this ratio was significantly increased in both the HT and MMVF21 experimental groups, indicating that an appropriate MTD had been achieved.

The effects on biochemical parameters in the BALF were significant at the end of the 3 months exposure for all fibre groups. Full recovery was found at 3 months post-exposure for all HT groups. In the MMVF21 group there was still an effect in two out of three biochemical parameters at 3 months post-exposure.

For all fibre groups a significant increase in PMN and a reduction in the percentage of macrophages in the BALF was still observed at 3 months post-exposure. The significant increase in PMNs in the very biosoluble HT groups as well as in the less biosoluble MMVF21 group should be noted. Furthermore, the study with MMVF21 included both a biosoluble calcium magnesium silicate fibre and a durable special purpose glass microfibre (E-glass). With both of these fibres there was a significant

Table 8. Proliferation index of lung tissue cells

Group	End of exposure			1.5 months			3 months[a]		
	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*
Unit length labelling index (%) of terminal bronchiolar epithelium post-exposure (positive cells/mm)									
Control[b]	2.85	1.1	5	1.95	0.9	5	3.56	1.0	4
RIF41001	11.05**	5.0	5	5.05	3.9	5	4.60	2.7	5
RIF42020-6	5.70	2.5	5	3.85	1.8	5	3.05	0.8	5
RIF43006-1	7.30	3.2	5	4.3	2.5	5	3.65	2.3	5
Control[c]	3.85	1.7	5	4.87	2.3	4	3.03	1.7	4
MMVF21	22.48**	11.0	5	5.62	1.8	5	3.84	1.4	4
Labelling index of alveolar parenchymal cells post-exposure (% positive cells)									
Control[b]	0.65	0.2	5	0.89	0.3	5	1.65	0.5	4
RIF41001	1.45	0.4	5	1.69	0.8	5	2.23	0.7	5
RIF42020-6	1.74	1.0	5	1.92	0.3	5	1.32	0.3	5
RIF43006-1	2.87**	1.2	5	1.77	1.1	5	1.24	0.5	5
Control[c]	2.17	0.8	5	2.73	1.2	4	1.76	0.6	4
MMVF21	4.59	2.1	5	4.93	2.5	5	2.03	0.3	4
Unit length labelling index (%) of pleural cells at post-exposure (positive cells/cm)									
Control[b]	4.0	1.7	5	1.4	0.9	5	8.3	3.6	4
RIF41001	2.2	0.8	5	3.2	1.4	5	4.9	4.6	5
RIF42020-6	1.9	1.0	5	1.9	1.2	5	2.4*	1.9	5
RIF43006-1	5.8	4.2	5	2.0	1.1	5	1.9*	1.5	5
Control[c]	26.4	11.8	5	27.0	6.1	4	16.2	8.6	4
MMVF21	56.8*	31.4	5	34.2	8.3	5	39.2	37.7	4

SD, standard deviation; *n*, number of animals.
ANOVA + Dunnett's test (two-sided): $*P \leq 5\%$, $**P \leq 1\%$.
[a]Animals were excluded from analysis because of the presence of a foreign body granuloma.
[b]HT study control.
[c]MMVF21 study control.

increase in PMNs at 3 months post-exposure (Bellmann, 2003).

The histological scoring system according to the criteria given by Wagner *et al.* (1974), and further defined by detailed description of each grade (McConnell *et al.*, 1984), was used to evaluate the fibrosis in the studies. Also, an EPS scoring system for collagen deposition at the bronchiolar–alveolar junction was used. In this system it is recognized that the degree of collagen deposition presents a morphological continuum from grade 0, with no remarkable changes, to grade 1, with very few, very small foci of collagen deposition not considered to be sufficient to apply grade 4 in the Wagner scoring system, to grade 2, with slight, fairly easily detected, few, small foci of collagen deposition, representing the lowest level of grade 4 in the Wagner scoring system. Grades 3 and 4 and, partly, 5 represent increasing degrees of collagen deposition but still within the Wagner grade 4 class (European Joint Research Centre, 1999).

A quantitative morphometric method for calculating collagen deposition in the lung parenchyma was also used. This method has been shown to distinguish between levels of fibrosis in a way not possible with the Wagner scale. In the present state of development, differences between measurements from different readers appear too great to allow comparisons, but single readers have been shown to produce consistent results from separate reading exercises (McConnell and Davis, 2002). In the present study, the same reader was used for all the exposure groups.

In agreement with the increased biosolubility of the HT type fibres, the pathology after 3 months exposure showed minor histopathological changes with HT type fibres compared to MMVF21.

At the termination of the 3 month exposure period, as well as after 1.5 and 3 month recovery periods, minimal morphological changes were diagnosed in the HT type fibres. These changes included alveolar macrophage aggregation and/or microgranulomas at the bronchiolar–alveolar junction in the few rats affected. In two of these rats, minimal focal collagen deposition in a microgranuloma was noted. No fibrogenic potential was noted in any of the three HT fibre types. No clear-cut difference between the HT fibre types was noted. At the termination of the exposure

Table 9. Histological results (mean scores) according to EPS grading system after different post-exposure periods

	Fibre group	Post-exposure death (months)		
		End of exposure	1.5	3
Macrophages in alveolar lumina	RIF41001	0.0	0.8	0.2
	RIF42020-6	0.0	0.6	0.0
	RIF43006-1	0.0	0.0	0.0
	MMVF21	1.8	1.2	1.0
Alveolar bronchiolization	RIF41001	0.0	0.0	0.0
	RIF42020-6	0.0	0.0	0.0
	RIF43006-1	0.0	0.0	0.0
	MMVF21	1.6	0.8	0.6
Microgranulomas at bronchoalveolar junction	RIF41001	0.0	0.8	0.2
	RIF42020-6	0.4	0.8	0.0
	RIF43006-1	0.0	0.0	0.0
	MMVF21	1.4	1.0	0.4
Collagen deposition at bronchoalveolar junction	RIF41001	0.0	0.2	0.0
	RIF42020-6	0.2	0.0	0.0
	RIF43006-1	0.0	0.0	0.0
	MMVF21	1.8	1.0	0.4

Histological changes were described according to distribution, severity and morphological character and scored no lesions, minimal, slight, moderate, marked or massive (grades 0–5).

Table 10. Pulmonary changes (mean Wagner scores) after 3 months exposure and after different post-exposure periods

	Wagner scores			*n*
	End of exposure	1.5 months	3 months	
RIF41001	1.0	1.6	1.2	5
RIF42020-6	1.4	1.6	1.0	5
RIF43006-1	1.0	1.0	1.0	5
MMVF21	3.8	3.0	2.4	5
Controls	1.0	1.0	1.0	10

Wagner score. Cellular change: 1, normal; 2, minimal; 3, mild. Fibrosis: 4, minimal; 5, mild; 6, moderate; 7 and 8, severe.

Table 11. Pulmonary changes (mean morphometric scores – percentage of lung parenchyma affected, ± SD) after different post-exposure periods

	Interstitial collagen/fibrosis					
	Morphometric method (mean %)					
	End of exposure		1.5 months		3 months	
RIF41001	–	–	–	–	–	–
RIF42020-6	–	–	–	–	–	–
RIF43006-1	–	–	–	–	–	–
MMVF21	0.13	(0.11)	0.05	(0.06)	0.02	(0.04)

–, not measurable.

period, exposure related changes were only noted in rats exposed to RIF42020-6. After the 1.5 month recovery period, changes were noted in rats exposed to RIF 41001 and RIF 42020-6. After the 3 month recovery period, changes were only noted in rats exposed to RIF 41001. The fact that both PMN and LDH levels remain raised in all the dusted groups of animals at 3 months probably indicates that these changes are a general response of lung tissue to heavy dust exposure and not a specific response to pathogenic fibres.

The quantitative morphometric method for calculating collagen deposition in the lung parenchyma showed marked differences between the MMVF21 and HT fibre types in favour of the latter after 3 months exposure. Both this method and the Wagner scoring system showed a reduction in fibrotic lesions during the recovery period.

CONCLUSION

This subchronic inhalation study showed a clear difference between MMVF21 and HT with regard to fibrogenic potential (Table 11). This is consistent with the results from long-term chronic inhalation studies, where MMVF21 induced lung fibrosis and HT did not (Kamstrup *et al.*, 2001), and with the increased biosolubility of the HT fibre type compared with MMVF21 reported in connection with short-term biopersistence studies.

In the present 90 day study, the pathological changes found in the lungs of exposed rats were in accordance with pathology previously reported from full lifespan inhalation studies. Thus, traditional

Table 12. Summary of main pathology findings at different post-exposure death dates

Parameter	MMVF21			RIF41001			RIF42020-6			RIF43006-1		
	EO	1.5 months	3 months	EO	1.5 months	3 months	EO	1.5 months	3 months	EO	1.5 months	3 months
Increase in lung weight	**	**	**	***	**		***	*		***	**	
Biochemical parameters in BALF (LDH, R-glucuronidase, protein)	***	*	*	***			**			***	*	
PMN increase in BALF	***	***	***	***		***	***	***	***	***	**	***
Proliferation of terminal bronchiolar epithelium	**			**								
Proliferation of alveolar parenchymal cells	*									**		
Proliferation of pleural cells	*											

EO, end of exposure.
Significance compared with controls (two-sided Dunnett test): $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

stone wool (MMVF21) caused significant pathology, including pulmonary fibrosis, while the soluble HT fibres produced little recognizable pathological change. These findings support the suggestion that a 90 day study that includes evaluation of biopersistence may be all that is necessary for the testing of new fibre formulations. The findings of the present study, however, go further than this. It is evident that such pathology as was found over 90 days correlated extremely well with the biopersistence of the fibres. It may be that our knowledge of fibre pathology has reached the stage where only biopersistence data are needed to predict long-term harmful effects of new man-made vitreous fibre types.

The significance of biopersistence was confirmed in this study and shows that the introduction of fibres with higher biosolubility has increased the safety margins in manufacturing and use of fibrous insulation material.

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