Characterization of Exposure and Dose of Man Made Vitreous Fiber in Experimental Studies

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The use of fibrous test materials in in vivo experiments introduces a number of significant problems not associated with nonfibrinous particulates. The key to all aspects of the experiment is the accurate characterization of the test material in terms of fiber length, diameter, particulate content, and chemistry. All data related to fiber properties must be collected in a statistically sound manner to eliminate potential bias. Procedures similar to those outlined by the National Institute of Occupational Safety and Health (NIOSH) or the World Health Organization (WHO) must be the basis of any fiber characterization. The test material to which the animal is exposed must be processed to maximize the amount of respirable fiber and to minimize particulate content. The complex relationship among the characteristics of the test material, the properties of the delivery system, and the actual dose that reaches the target tissue in the lung makes verification of dose essential. In the case of man-made vitreous fibers (MMVF), dose verification through recovery of fiber from exposed animals is a complex task. The potential for high fiber solubility makes many of the conventional techniques for tissue preservation and digestion inappropriate. Processes based on the minimum use of aggressive chemicals, such as cold storage and low temperature ashing, are potentially useful for a wide range of inorganic fibers. Any processes used to assess fiber exposure and dose must be carefully validated to establish that the chemical and physical characteristics of the fibers have not been changed and that the dose to the target tissue is completely and accurately described. — Environ Health Perspect 102(Suppl 5):109-112 (1994)

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Introduction

In vivo toxicological studies of fibrous material are often complicated if not partially compromised by the problems associated with the fibrous nature of the test material. Every aspect of the study, from preparation and characterization of the test material, the mechanics of exposure and delivery, to the determination of actual dose, is subject to numerous complications that are unique to fibrous materials. In this article, we present a summary and discussion of some specific problems associated with fibrous test materials as well as our experiences on how to minimize their impact.

Fiber Counting and Measuring

Central to all aspects of experiments involving fibers is the proper characterization of the fiber. The characterization of a test fiber in terms of diameter and length distribution as well as nonfibrinous content is potentially subject to a variety of biases associated with the measuring procedure used. The two most common sources of error are related to the resolution of the measuring system and the fiber selection criteria used during counting and measuring.

The ability to determine accurately the length and diameter of individual fibers depends on the measuring system employed. Optical light microscopy (OM) has a resolution limit of about 0.25 μm. This resolution limit usually is not a problem for normal bulk materials, but with material that has been specifically processed for inhalation studies to have a mean diameter of 1 to 1.5 μm a significant portion of the fiber may fall below the 0.25 μm limit. A comparison of the scanning electron microscopy (SEM) and OM diameters in Table 1 shows the effect of limited resolution when measuring typical MMVF products. Optical microscopy, either bright field or phase contrast, should not be used for counting or measuring fibers where more than a few percent of the fibers are expected to fall below 0.5 μm in diameter. Phase contrast optical microscopy (PCOM) provides a sensitive tool for identifying fine fibers but is not a satisfactory mode in which to measure fiber diameters. Fiber edges are not always distinct in the PCOM image and diameters are frequently overestimated.

EM offers a greatly improved resolution of 0.01 μm or better; however, at the high magnifications necessary to achieve this resolution, length measurements become a problem. With SEM working magnifications of ×5000 to ×10,000, the maximum measurable fiber length is roughly 10 to 20 μm. The truncation of longer fibers by the measuring field produces a distorted length distribution possibly obscuring the true distribution.

Transmission electron microscopy (TEM) techniques employed with asbestos are generally not applicable to glass fiber because of the latter's much greater diameter and length. As fiber aspect ratios increase, accurate distributions of both length and diameter using a single technique become more difficult, and it may be necessary to employ both optical and SEM techniques.

Unless every fiber in a sample is to be measured, a bias-free technique for selecting fibers must be employed. Biases affecting both length and diameter distributions may arise from the existence of a strong correlation between length and diameter and from the failure to deal properly with partial fibers. If longer fibers are, in general, larger in diameter, the diameter distribution may be biased toward a larger mean, because the longer fibers are more likely to be encountered in any single field of view. In a similar manner, fibers only partially within the field of view can be counted more than once or their lengths only partially measured. The World Health Organization (WHO) (1) and Schneider (2) address the problems of bias in detail. Any fiber measuring and counting protocol must be free of procedural bias. National Institute of Occup-
Table 1. Characteristics of typical MMVF products.

| Company and product                      | Method | Mean diameter, μm | Standard deviation | Wt-% shot |
|-----------------------------------------|--------|-------------------|--------------------|------------|
| Certain Teed Insulsafe                  | OM     | 2.4               | 1.9                | 0          |
|                                         | SEM    | 1.2               | 1.0                | 1          |
| Knauf fiber glass insulation R-19       | OM     | 4.1               | 2.9                | 5          |
|                                         | SEM    | 4.0               | 2.4                |            |
| Schuller pot and marble insulation      | OM     | 6.1               | 4.3                |            |
|                                         | SEM    | 5.3               | 3.2                |            |
| Owens-Corning Pink R-19                | OM     | 3.6               | 2.2                |            |
|                                         | SEM    | 3.4               | 2.0                | 43         |
| Partek insulation wool                  | OM     | 3.6               | 2.9                |            |
|                                         | SEM    | 4.4               | 2.7                |            |
| Rockwool Int. insulation wool           | OM     | 4.1               | 2.6                | 24         |
|                                         | SEM    | 4.0               | 2.4                |            |
| ROXUL insulation wool                   | OM     | 3.3               | 2.4                | 45         |
|                                         | SEM    | 4.0               | 3.1                |            |
| USG Birmingham tile wool                | OM     | 4.2               | 3.0                | 49         |
|                                         | SEM    | 4.5               | 4.1                |            |

*Data extracted from Nomenclature of Man-Made Vitreous Fibers (3). Abbreviations: OM, optical microscopy; SEM, scanning electron microscopy.

![Figure 1](image_url)

**Figure 1.** The range of arithmetic mean diameters of crushed slagwool derived from a single population as a result of systematic bias in measuring.

Table 2. Dimensions of slagwool before and after processing.

| Commercial product | Size selected |
|--------------------|---------------|
| Diameter range     | 0.2-16.5 μm   |
| Mean diameter, SD  | 4.7(3.5) μm   |
| Length range       | 0.1-5 mm      |
| Mean length, SD    | 1.9-96 μm     |
| Shot content       | 20 wt-%       |
|                    | 5 wt-%        |

*Measurements by SEM.

Characterization of Test Materials

Man-made vitreous fiber (MMVF) is produced in bulk with a broad diameter distribution. The standard deviation of the mean diameter is frequently greater than or equal to the mean diameter. The mean length can range from several millimeters with blowing material to several centimeters for fiber glass insulation. Products may contain a large amount (in wt-%) of large rounded or teardrop shaped particles called “shot” as well as small flakes and glass shards (Table 1). MMVF is also commonly manufactured with a variety of coatings, binders, sizing, and dedusting agents on the surface, the presence of which may or may not be spelled out in the manufacturer’s specifications.

Material suitable for rodent inhalation studies should have a mean diameter of 1 μm or less to allow deposition in the deep lung. The mean length should approximate that of fibers found in the workplace environment, which typically have mean lengths of 20 μm or greater. As manufactured, MMVF may contain less than 1% respirable material making it unsatisfactory for inhalation studies. To differentiate clearly between the effects of fibers versus nonfibrous particles and to maximize the respirability of the particles, the test material must undergo extensive processing. Proper milling followed by fiber sizing through air or water classification, screening, or a combination of techniques can increase the respirable content of a sample from less than 1% to over 60%. The respirable fraction of rock and mineral wool may contain up to 60% particulates by weight and still appear relatively particulate-free in terms of particle number. High particulate contents invalidate any exposure and dose calculation based on gravimetric measurements. The size selection process, if properly executed, is also an effective method of removing nonfibrous particles. Table 2 illustrates the change in fiber dimensions that can be achieved with size selection processing.

As a general rule, surface agents should be removed prior to processing because of the possibility that they could interfere with the size selection process. Silicone oils, a common surface treatment, can be very tenacious; nevertheless, any fiber cleaning process that is used should not alter the fiber surface, either by leaching or deposition of new compounds.

Prior to any exposure the final test material must be completely characterized in terms of dimensions, chemistry, organic content, and homogeneity.

Exposure

In any *in vivo* experiment, exposure is the critical link between the test material and the dose delivered. Whether the exposure is *via* instillation or inhalation, there is no...
assurance the test animals receive a dose that is identical to the original test material in mass, fiber number, fiber dimension, or particle content.

Suspensions of fiber prepared for instillation are rarely homogeneous with respect to fiber size or fiber mass. Fibers in suspension occupy an effective volume related to their length and easily become tangled when concentrations are high. Inhomogeneities result from the high fiber loading needed in the solution to deliver the fiber dose in a minimum volume of liquid. Data in Table 3 show the variation associated with sampling a fiber-water suspension containing 1 mg of fiber per milliliter of water dispersed by sonication and stirring with a surfactant. The fiber was sized to have a mean length of 25 μm and sampling was done with a micropipet having a tip bore of about 1 mm.

During instillation, the suspension is easily depleted in fiber by the formation of fiber "log jams" not only at either end of the needle but also in the trachea and upper airway of the lung (4). This "bolus effect" can result in the formation of large granulomatous lesions in the upper airways of rodents (5). Thus with fiber, unlike with particles, it is difficult to deliver a known and reproducible dose to the deep lung by instillation.

With inhalation, the process of lofting the fiber to form an aerosol cloud may significantly alter the fiber size distribution. Aerosol generators can potentially shorten brittle MMVF and reduce both the diameter and length of asbestos fibers. For reliable exposure, the aerosol cloud must be monitored in terms of mass, particle count, and fiber dimensions. For optimum results, the test material should be matched with the aerosol generation system to deliver the maximum amount of material within the target range. Table 4 compares the characteristics of stock size-selected MMVF test material with those of the aerosol generated from each. In both cases every effort was made to minimize any changes associated with the lofting process.

A satisfactory lofting process producing a stable and well-characterized aerosol cloud must be established prior to exposure of the test animals. The aerosol should be monitored during the exposure on a daily basis and analyzed at a frequency that ensures that the time-weighted target values are maintained. Routine monitoring is usually accomplished by collecting the aerosol on a filter membrane and determining the loading gravimetrically. The desired exposure is, however, quantified in terms of fibers with a specific size range per unit volume of air. Consequently, it is essential that exposure be monitored using optical or electron microscopy. Figure 2 shows the results of monitoring aerosol generation for a 2-year exposure study.

### Table 3. Homogeneity of fiber suspension.

| Aliquot (μl) | Fibers/ALiquot, SD |
|--------------|-------------------|
| 50 (n = 5)   | 6.4 (0.8) × 10⁶  |
| 100 (n = 7)  | 10.6 (1.2) × 10⁶  |
| 250 (n = 5)  | 26.4 (3.4) × 10⁶  |

*Concentration = 1 g/l.

### Table 4. Comparison of bulk, aerosol, and dose fiber dimensions.

| Sample | Stock fiber, μm | Composite aerosol, μm | Fiber recovered from lung* μm |
|--------|-----------------|-----------------------|-------------------------------|
| MMVF Fiber A | Mean diameter, SD: 0.70(2.0) | 0.67(2.0) | 0.48(1.65) |
|          | Mean length, SD: 13.4(2.0) | 14.1(2.0) | 7.0(2.0) |
| MMVF Fiber B | Mean diameter, SD: 1.05(1.82) | 0.91(1.95) | 0.74(1.49) |
|          | Mean length, SD: 17.3(2.1) | 17.5(2.3) | 11.0(2.0) |

*Recovered by LTA after 5-day exposure.

### Table 5. Residual ash. *rat lungs, right lobe.*

| Sample | Lobe dry weight, mg | Lobe ash weight, mg | Residue, % |
|--------|---------------------|---------------------|------------|
| 1      | 90.3                | 0.090               | 0.099      |
| 2      | 96.3                | 0.067               | 0.069      |
| 3      | 98.3                | 0.101               | 0.103      |
| 4      | 81.1                | 0.131               | 0.162      |
| 5      | 113.4               | 0.068               | 0.060      |
| 6      | 70.2                | 0.062               | 0.074      |
| Mean, SD |                     |                     | 0.94(0.037) |

*Ashed 12 hr, dispersed in hot water on 25 mm to 0.2 μm Nuclepore filters.

### Dose

With fibrous test material, the retained dose may not be identical to the stock test material regardless of the exposure mechanism. In Table 4, the significant reduction in both length and diameter of the recovered fiber compared to the aerosol demonstrates the difference between exposure and dose, and the effectiveness of the respiratory tract of the rat at filtering out larger fibers. To understand fully the toxicological potential of fibers, the dose must be assessed by actually characterizing the material resident in the target tissue of the lung.

![Figure 2. Monthly average aerosol concentrations monitored during a 2-year exposure study from July 1989 to May 1991.](chart.png)
Numerous techniques have been employed to recover fiber from lung tissue. All of the techniques involve destruction of the tissue by the use of strong acids or bases, strong oxidizing agents such as hypochlorites and peroxides, thermal ashing, or low temperature plasma ashing. A majority of the procedures were developed to recover asbestos fiber that is very resistant to chemical attack and mechanical breakdown. MMVF is significantly less durable in all respects than asbestos (6). Furthermore, the major thrust of current MMVF research is to develop and test compositions with the lowest possible biopersistence. Techniques for recovering MMVF from tissue must be carefully designed to minimize changes in fiber chemistry or morphology. Experiments have shown that many common MMVF compositions are attacked during lung digestion. Some of the experimental compositions are even significantly etched and leached by deionized water.

The trend toward more soluble MMVF fibers presents problems not only with fiber recovery but also with tissue preservation and sample storage. Experiments have shown that MMVF has a significant solubility in a variety of tissue fixatives, including buffered formalin and Karnovsky’s solution (7). As an example of fiber loss, right accessory lobes from rats exposed to MMVF and stored for 1 year in phosphate buffered formalin contained no recoverable fiber, while those frozen immediately upon removal contained about 5 x 10^5 fibers/mg of dry tissue. Freezing the tissue at dry ice temperature (<20°C) immediately upon excision, without cryoprotectants, minimizes potential for degradation during storage.

Low temperature ashing (LTA) is the safest and most generally applicable technique for recovering a wide range of inorganic fibers. The residual ash with proper dispersal is suitable for all forms of optical and electron microscopy. The fiber surfaces are clean and suitable for electron probe analysis and the residual ash does not obscure the submicron fibers. One problem associated with LTA is the small but significant variability in the weight of the ash derived from the tissue. Table 5 illustrates the variation in ash weight derived from LTA treated lungs. A majority of the variation is thought to arise from variation in the amount of blood remaining. The blood forms ferric oxides with LTA, and any attempt to dissolve the oxides after ashing could also attack iron in the fiber and in ferruginous bodies. While the total ash is small, it may represent a significant portion of the total recovered weight from a lung and the variability may mask the mass of the fiber recovered. As a consequence, dose determinations based on gravimetric measurements and LTA are not reliable unless the blood is perfused from the tissue before ashing. This is not always possible, for example when adjacent tissue is to be used for histopathological examination.

Dosage of fibrous material should be characterized by more than one parameter. The recovered material can be characterized in terms of mass, fibers/mg of dry tissue, total fibers per animal or lung, fiber length, diameter, or aspect ratio, particulate content, and fiber chemistry. The more parameters that are obtained, the more useful the data will be and the greater the significance of the results.

Fiber chemistry, while not essential, is helpful in evaluating exposure and dose. The compositional analysis of fibers using energy dispersive spectroscopy with SEM is useful for identifying contamination or unknown fibers in the recovered material, especially in the negative control samples.

Any protocol used to recover fibers from tissue must be validated. Tests must be conducted to ensure that the recovered fiber is representative of, and as similar as possible to, the fiber in the living tissue before recovery. Validation is also critical in establishing the amount of variability to expect as a result of the processing, laboratory fiber background levels and their variability, upper and lower limits of useful concentration, and potential sources of contamination. Processing and quantifying microgram quantities of fiber is more art than science. The validation procedures also provide the necessary experience to quantitate successfully lung fiber burdens in an inhalation toxicology experiment.

Experience has taught us that laboratory blanks in the form of lungs from animals that were never part to the test group are essential with long-term inhalation studies. Even with the use of both positive and negative controls in the experimental group, instances of confusing and questionable data will arise. Data obtained from processing the tissue from totally uninvolved animals will provide a check on the entire tissue harvesting, processing, and analysis procedures. These data may be critical in the interpretation of apparently anomalous experimental results.

Conclusions

The starting point of any in vivo study is delivering an appropriate and well characterized dose to the target organs. When the test material is respirable fiber, both the characterization and the delivery become much more complicated than with nonfibrous particulates. The complex and only partially controllable relation between exposure and dose, whether delivered by instillation or inhalation, makes verification of the actual dose delivered to the deep lung essential for the interpretation of any results.

The solubility of MMVF makes many of the techniques of tissue preservation and digestion used for asbestos fiber recovery inappropriate. Tissue preservation by freezing and fiber recovery using low temperature ashing offer a general procedure that is applicable to a variety of inorganic fiber compositions. Any technique must be validated in detail to ensure the integrity of the data and experiment.

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