Investigations on Health-related Properties of Two Sepiolite Samples

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Published ip injection studies have shown different biological behavior of different sepiolite samples. There was no evidence for carcinogenic potential of sepiolite from Vicalvaro, Spain, whereas a high tumor incidence was reported for sepiolite from Finland. The low biological activity of the sepiolite from Vicalvaro, compared to the Finnish sample, could be caused by low in vivo persistence or by the short length of the fibers, or both. In this study a further sepiolite sample, obtained as a commercial sample originating from China, was investigated. This sample contained a higher fraction of fibers longer than 5 μm, comparable to the Finnish sepiolite sample. The fraction of fibers with a length >5 μm was 0.12 and 2.2% for the Vicalvaro and Chinese sepiolite, respectively. For the fiber fraction longer than 5 μm, the corresponding values were 0.0045 and 0.82%. The in vivo persistence of the sepiolite samples from China and Vicalvaro was analyzed after intratracheal instillation of 2 mg in female Wistar rats. Fiber retention in the lungs was analyzed by transmission electron microscopy at different sacrifice dates up to 12 months after application. For the Vicalvaro sepiolite, a splitting of fiber bundles was found during retention time in the lung. Therefore, no half-time of the fiber clearance could be calculated from the number of fibers. The decrease of the calculated retained fiber mass was faster for the Vicalvaro sepiolite (T1/2 = 89 days) compared to the Chinese sepiolite (T1/2 = 129 days). For 2 or 3 rats per group, at sacrifice date 12 months after ip injection, the lung was investigated by histopathology. The main difference between both treatment groups was a more pronounced fibrotic response in the Chinese sepiolite-treated rats compared to those treated with Vicalvaro sepiolite. It is concluded that both the higher fraction of long sepiolite fibers and the slower elimination rate of the fiber mass in the Chinese sample were important factors for the different biological reaction in comparison with Vicalvaro sepiolite. — Environ Health Perspect 105(Suppl 5):1049-1052 (1997)

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Introduction

Sepiolite is a natural magnesium silicate with a structure of very thin crystals that are usually combined to form either dense or spongy masses. Sepiolite is mainly used as absorbent (e.g., for pet litter). The production of Spanish sepiolite was about 375,000 tons in 1984 (1).

In ip injection studies with sepiolite samples from Vicalvaro, Spain, no evidence was found for carcinogenic potential, whereas a high tumor incidence was reported for sepiolite from Finland (2,3). Differences in the length distribution of fibers or in the biodurability of these samples could be responsible for the observed differences in biological effects.

When sepiolite mined in China became available in the European market a few years ago, this study was planned as a first step for detecting a possible health effect of this new sepiolite sample. The reported low biological activity of the Vicalvaro sepiolite could be caused by low in vivo persistence or by the short length of the fibers, or both. To investigate the impact of these various factors, the in vivo persistence of the new Chinese sepiolite sample was analyzed after intratracheal instillation in rats and compared to the Vicalvaro sepiolite.

Materials and Methods

Test Material

The Vicalvaro sepiolite sample was received from F. Pott, Düsseldorf (original source Tolsa S.A., Madrid, Spain). The same material was used by Pott for ip testing of the carcinogenic potential in rats (2,3). Commercially available sepiolite from China was supplied by Tolsa S.A. For each sample, a small fraction was suspended in double-distilled water, sonified, and filtered onto a Nuclepore filter (pore size 0.1 μm). The surface of the filter was coated with a thin carbon film and portions of the filter were placed on electron microscope grids; the polycarbonate filter was removed by an organic solvent. These samples were analyzed by transmission electron microscopy (TEM). In the Chinese sepiolite sample, no asbestos contamination could be detected by energy-dispersive X-ray analysis of 100 fibers with length >5 μm.

Size Distribution

To determine fiber size, TEM photos with two or three different magnifications were used for different fiber length fractions. Typical magnifications for analyzing sepiolite samples were approximately ×5000 (for length fraction >10 μm), ×15,000 (for length fraction 1.5–10 μm) and ×40,000 (for length fraction <1.5 μm). Because the size of the analyzed object field was different for the various length fractions, different areas of analysis at various magnifications were taken into account by weighting factors for the size distribution. For each sample the length and the diameter were measured for each of about 500 fibers. Special attention was given to the measurement of fibers longer than 5 μm, to increase the precision of number and size distribution of the fraction of long fibers. The volume of all individual fibers was calculated from length (L) and diameter (D), assuming cylindrical geometry. Using area of analysis, effective filter area, and density, the total mass of sepiolite fibers on the filter was calculated for different length fractions and combined thereafter.

The two samples of sepiolite from Vicalvaro and China were compared. Results for size distribution are presented in Table 1, which shows distinct differences in...
the length distribution of the two test materials investigated. Ninety-nine percent of the Vicalvaro sepiolite fibers are shorter than 2.8 μm; the corresponding 99th percentile for Chinese sepiolite is 7.4 μm. This means that the fraction of World Health Organization (WHO) fibers (L > 5 μm, D < 3 μm, L/D ratio > 3:1) in Chinese sepiolite is considerably higher than in Vicalvaro sepiolite. In Table 2, the number of critical fibers per mass unit is shown for four different length fractions. For the Finnish sepiolite sample mentioned above, 5500 fibers/ng (length > 5 μm) were reported (4).

**General Study Design**

Eighty-six female Wistar rats were purchased from Charles River Deutschland [Sulzfeld, Germany; strain Crl:(WI)BR]. The animals were approximately 10 weeks of age at the start of this study.

The protocol for animal treatment was used as in previous biodurability studies (5–7). In these studies a total of 2 mg was used for intratracheal instillation of natural mineral fibers and manmade vitreous fibers. Pretreatment of two extra animals per sepiolite sample revealed that a single dose of 2 mg in 0.3 ml resulted in adverse health effects in the rats. Therefore, the total dose was given in two treatments. The sepiolite samples were suspended in 0.9% NaCl solution (saline) and instilled intratracheally in two doses of 1 mg in 0.3 ml per rat on two subsequent days into the lungs of female Wistar rats with a body weight of approximately 200 g. The second instillation was study day 0. The fiber suspension used for intratracheal instillation contained no agglomerates of fibers. But bundles of fibers were present in the Vicalvaro sample.

**Table 1. Fiber size distribution of test materials.**

| Weighting parameter | Sepiolite sample | Fiber length, in μm | Fiber diameter, in μm |
|---------------------|------------------|---------------------|----------------------|
|                     |                  | 0-10%  | 0-50%  | 0-90%  | 0-99%  | 0-10%  | 0-50%  | 0-90%  | 0-99%  |
| Number of fibers    | Vicalvaro        | 0.2    | 0.3    | 0.9    | 2.8    | 0.02   | 0.04   | 0.07   | 0.13   |
|                     | China            | 0.2    | 0.6    | 2.2    | 7.4    | 0.02   | 0.05   | 0.09   | 0.17   |
| Mass of fibers      | Vicalvaro        | 0.4    | 2.6    | 6.7    | 7.9    | 0.04   | 0.13   | 0.65   | 1.60   |
|                     | China            | 0.8    | 3.7    | 14.3   | 31.3   | 0.06   | 0.13   | 0.25   | 0.36   |

L/D > 3:1; weighting by number and mass of fibers. The given length or diameter represents the upper value in the percent range.

In the treatment groups, five animals were assigned to each sacrifice date (Table 3). In the control group, only three animals were assigned for each sacrifice date. On the last date, eight rats (five for the control group) were scheduled for sacrifice so that extra animals were available as substitutes for rats that died between sacrifice dates. Two rats per group (three for the Vicalvaro sepiolite group) of the extra rats remained after 12 months of treatment. These two or three rats per group were used for histopathology.

**Fiber Retention in the Lungs**

After sacrifice, the rat lungs were dried at 110°C in an oven and subjected to low-temperature ashing. A fraction of the ashed lung was suspended in filtered water and filtered on a Nuclepore filter (Corning Costar, Acton, MA) (pore size 0.1 μm). A part of the fiber was analyzed by TEM for the characterization of the test material. For each lung ash sample, approximately 200 fibers were measured on TEM photos. Different magnifications between approximately ×5000 and ×45,000 on the TEM photos were used for analysis of different length fractions (see above). The total number of fibers per lung was calculated for each animal by using the area of analysis, the effective filter area, and the fraction of the lung ash on the filter. The total number of fibers was obtained by combination of data of the different length fractions (measured at different magnifications). The total mass of fibers per lung was calculated by the same procedure, using the mass of all individual fibers and assuming cylindrical geometry.

From these data, the kinetics of fiber elimination from the lung was calculated by regression analysis of the logarithm of the number of fibers versus time after instillation. The size distribution of the fibers was also analyzed for all sacrifice dates.

**Results**

**Lung Weights**

Lung wet weight increased after instillation of sepiolite (Table 4). For the Vicalvaro group, the increase was small and not significant. In contrast, the lung weight of animals treated with Chinese sepiolite was significantly higher for all sacrifice dates compared to control animals.

**Retention and Clearance of Test Materials**

The results of fiber retention are summarized in Table 5 for sacrifice dates up to 12 months. The evaluation of fibers in the transmission electron microscope showed

**Table 2. Calculation of number of critical fibers per nanogram test material.**

| Sepiolite sample | Critical fibers, no* |
|-----------------|----------------------|
|                 | >5 μm | >8 μm | >10 μm | >20 μm |
| Vicalvaro       | 320   | 11    | 0     | 0     |
| China           | 1478  | 560   | 318   | 48    |

*For length fractions per nanogram.

**Table 3. Study plan.**

| Sepiolite sample | Sacrifice dates and number of animals |
|-----------------|--------------------------------------|
|                 | 2 days | 1 month | 2 months | 3 months | 6 months | 12 months | Total |
| Control         | 3      | 3       | 3        | 3        | 3        | 5         | 20    |
| Vicalvaro       | 5      | 5       | 5        | 5        | 5        | 8         | 33    |
| China           | 5      | 5       | 5        | 5        | 5        | 8         | 33    |
| Sum total       | 86     |         |          |          |          |           |       |

**Table 4. Lung wet weight in grams.**

| Group | Day 2 | Day 29 | Day 59 | Day 91 | Day 181 | Day 364 |
|-------|-------|--------|--------|--------|---------|---------|
| Control | Mean: | 1.196  | 1.149  | 1.241  | 1.249   | 1.282   | 1.462   |
|        | SD:   | 0.024  | 0.068  | 0.101  | 0.060   | 0.048   | 0.044   |
|        | n:    | 5      | 3      | 3      | 3       | 3       | 3       |
| Sepiolite, | Mean: | 1.485* | 1.396  | 1.428* | 1.435*  | 1.375   | 1.469   |
| Vicalvaro | SD:   | 0.070  | 0.060  | 0.065  | 0.013   | 0.090   | 0.108   |
|        | n:    | 5      | 5      | 5      | 5       | 5       | 5       |
| Sepiolite, | Mean: | 1.896**| 1.68** | 1.784**| 1.810** | 1.714** | 1.774** |
| Vicalvaro | SD:   | 0.069  | 0.197  | 0.105  | 0.078   | 0.137   | 0.148   |
|        | n:    | 5      | 5      | 5      | 5       | 5       | 5       |

Statistics: analysis of variance + Dunnett's tests (2-sided): *p<5%, **p<1%.
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Table 5. Analysis of fibers in lung ash.

| Sacrifice     | Fibers, 10^9/lung | WHO Fibers, 10^9/lung | Mass of fibers, mg/lung |
|---------------|-------------------|-----------------------|-------------------------|
|               | Mean | SD | Mean | SD | Mean | SD |
| Sepiolite, Vicalvaro |      |     |      |     |      |     |
| 2 days        | 102  | 53 | 0.22 | 0.09 | 2.42 | 1.61 |
| 1 month       | 58   | 19 | 0.48 | 0.06 | 1.47 | 0.24 |
| 2 months      | 136  | 80 | 0.42 | 0.20 | 1.24 | 0.90 |
| 3 months      | 129  | 43 | 0.42 | 0.19 | 0.64 | 0.23 |
| 6 months      | 28   | 7  | 0.29 | 0.08 | 0.22 | 0.13 |
| 12 months     | 13   | 6  | 0.13 | 0.04 | 0.16 | 0.12 |
| Sepiolite, China |      |     |      |     |      |     |
| 2 days        | 158  | 49 | 4.28 | 1.41 | 2.80 | 1.13 |
| 1 month       | 82   | 26 | 2.54 | 1.45 | 1.87 | 0.76 |
| 2 months      | 101  | 19 | 3.20 | 0.53 | 2.36 | 1.36 |
| 3 months      | 80   | 28 | 2.60 | 0.70 | 1.19 | 0.23 |
| 6 months      | 34   | 3  | 1.82 | 0.21 | 0.79 | 0.13 |
| 12 months     | 13   | 2  | 0.88 | 0.14 | 0.36 | 0.04 |

Table 6. Clearance half time and 95% CI of test material elimination.

| Group       | Fibers, no^a | WHO fibers, no^a | Mass of fibers^b |
|-------------|---------------|------------------|------------------|
| Vicalvaro   | —             | —                | 89 (71–119)      |
| China       | 107 (92–124)  | 186 (143–257)    | 129 (106–164)    |

Half time, days.

the presence of fiber bundles, which split in the first day after treatment. This may be the case particularly for the sample of Vicalvaro sepiolite, in which the number of WHO fibers increased from sacrifice day 2 compared to the results after 1 month. The retained mass (calculated from the number of fibers, the length and diameter distribution, and a density of 2.1) decreased. Half times for the elimination of fibers were calculated from these data (Table 6). Due to the fluctuation in fiber count for the Vicalvaro sepiolite, calculation was not possible for half times based on the number of fibers.

In the control group, no fibers were detected after intratracheal instillation, even at the 12-month sacrifice date. The detection limit was approximately 0.06 × 10^6 fibers per lung, which is more than five orders of magnitude below the number of sepiolite fibers retained in the lungs of sepiolite-treated animals.

Histopathology of the Lungs

Some treatment-related morphological changes were observed in the rat lungs after intratracheal instillation of sepiolite. The main difference between both treatment groups was a more pronounced fibrotic response in the Chinese sepiolite-treated lungs compared to the lungs instilled with Vicalvaro sepiolite. In contrast, these lungs had fewer (mainly mononuclear) inflammatory cell infiltrations than the Vicalvaro sepiolite-treated lungs. The pulmonary granulomas seen in the Vicalvaro sepiolite group consisted of one or more layers of prominent histiocytic cells surrounding centrally located amorphous material, whereas the granulomas observed in Chinese sepiolite group were more fibrotic and contained a rather fibrous exogenous material.

Discussion

Size Distribution

On analysis, the Vicalvaro sepiolite contained a fraction of 0.12% of fibers (L > 5 μm) and a fraction of 0.0045% of fibers (L > 8 μm). The sample of Chinese sepiolite had a fraction of 2.2% of fibers (L > 5 μm) and a fraction of 0.82% of fibers (L > 8 μm). For the Finnish sepiolite, a fraction of 29% of fibers (L > 5 μm) and approximately 10% of fibers (L > 8 μm) can be calculated from the results of Rödelberger et al. (4). This means that the fraction of fibers (L > 5 μm) is smaller in the Chinese sepiolite sample compared to the Finnish sepiolite sample. The relative fraction of fibers longer than 5 μm that is > 8 μm in length is comparable between the Chinese and the Finnish sepiolite samples (about 35%); this relative fraction is only 3.7% for Vicalvaro sepiolite.

For the fraction of fibers longer than 10 or 20 μm, no fibers were found in the Vicalvaro sepiolite, whereas in the Chinese sepiolite a significant number was detected (Table 2).

This difference in the length distribution between Vicalvaro and Chinese sepiolite was explained by Santarén and Alvarez (8) as the different geological origins of the two types of sepiolite. Vicalvaro sepiolite is a sedimentary sepiolite that has platelike particles with low crystallinity and fibers with an average length < 2 μm. The small crystal size is attributed to the presence of a higher number of crystallization nuclei during chemical precipitation in the sedimentary environment, compared to nonsedimentary sepiolites, which are formed by hydrothermal processes.

Biodurability of Sepiolite Fibers

The biodurability of mineral fibers in the lung or in the serosa is thought to be related to their potency in inducing tumors (9,10).

Because of the fluctuation of fiber numbers in Vicalvaro sepiolite, no half time of the fiber clearance could be calculated from the number of fibers. As indicated previously, one of the reasons may be the presence of fiber bundles that split into single fibers during the retention time in the lung.

The calculated mass of fibers based on L, D, and number of fibers is shown in Table 5. The decrease in fiber mass after instillation follows approximately a first-order kinetic for the Vicalvaro sepiolite. Calculation of half time based on the calculated mass of fibers is therefore possible. The decrease in retained fiber mass is faster for Vicalvaro sepiolite (T1/2 = 89 days) than for Chinese sepiolite (T1/2 = 129 days).

An alternative analysis of the clearance kinetics by a double exponential model shows that most of the fiber mass is cleared in the first fast phase for both sepiolite samples. For Vicalvaro sepiolite, 95% of the fiber mass is eliminated with a half time of 39 days, whereas for the Chinese sepiolite, 85% were cleared with an 80-day half time. Therefore the decrease of fiber mass is about two times faster for Vicalvaro sepiolite.

Lung Wet Weight

Lung wet weight was significantly higher in the group that was treated with Chinese sepiolite compared to the group treated with Vicalvaro sepiolite (Table 4). This is in agreement with other characteristics of these two samples (e.g., fiber length distribution and fibrotic response).

Histopathology

The number of lungs investigated for histopathology is very low and provides only a rough indication of possible lesions. However, results indicate more pronounced effects after application of Chinese sepiolite compared to Vicalvaro sepiolite. This may be due to the higher fraction of long fibers and the slower elimination rate of the fiber mass in Chinese sepiolite compared to Vicalvaro sepiolite.
In an inhalation study with Madrid sepiolite (from the Vicalvaro mine), a very mild grade of fibrosis was detected at different sacrifice dates up to 12 months of exposure (11). After intratracheal instillation of a dose of 2 mg dust per rat of any poorly soluble dust, a variety of lung reactions can be expected for each of the dusts (12). Among these reactions are chronic inflammatory processes and fibrosis.

**Conclusions**

In conclusion, for new sepiolite samples in the market, size distribution should be characterized using TEM. If a new sepiolite sample contains a fraction of fibers longer than 8 μm that is higher than in the Vicalvaro sepiolite, carcinogenicity studies should be performed to analyze potential health effects.

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