INFLUENCE OF REFRACTORY CERAMIC FIBRES – ASBESTOS SUBSTITUTE – ON THE SELECTED PARAMETERS OF BRONCHOALVEOLAR LAVAGE 6 MONTHS AFTER INTRATRACHEAL INSTILLATION TO W-RATS

Marta Hurbánková^a, Silvia Černá^a, Petra Gergelová^b, Soňa Wimmerová^a

^a Research Base of the Slovak Medical University, Bratislava, Slovakia

^b Trnava University, Faculty of Health, Care and Social Work, Trnava, Slovakia

e-mail: marta.hurbankova@szu.sk

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Industrial fibrous dusts are applied in many industrial branches and represent adverse factors in occupational and environmental area. Refractory ceramic fibers (RCFs) – amorphous alumina silicates – are used as one kind of asbestos substitutes. Because RCFs are relatively durable and some RCFs are respirable, they may present a potential health hazard by inhalation. The aim of present work was to find out the subchronic effect of RCFs on selected parameters of bronchoalveolar lavage (BAL) in W-rats, confirm the biopersistence of RCFs after 6 month instillation and contribute to the understanding of the pathomechanism of lung injury after fibrous dust exposure. Wistar rats were intratracheally instilled with 4 mg/animal of RCFs – exposed group and with 0.4 ml saline solution/animal – control group. Animals were sacrificed after 6 month exposure. Bronchoalveolar lavage (BAL) was performed and selected BAL parameters (mainly inflammatory and cytotoxic) were examined. After treatment with RCFs the following changes were observed: statistically significant increase in proportion of lymphocytes and polymorphonuclears as well as in % of immature alveolar macrophages (AM) and phagocytic activity of AM; statistically significant decrease in viability of AM and proportion of AM (from the differential cell count) in comparison with the control group. The results of this study indicated that RCFs even 6 months after intratracheal instillation very significantly changed the majority of examined BAL parameters. The presence of inflammatory and cytotoxic response in lung may signalize beginning or developing disease process.

INTRODUCTION

The study of the effects of industrial fiber dusts on respiratory system has been a long lasting urgent problem. They are used in many industrial branches and represent one of the adverse factors in occupational and environmental area. The main representants are asbestos fibers, which after long time exposure cause fibrosis-asbestosis, pleural hyalinoses, lung cancer, pleural mesotelioma, eventually neoplasms of other organs. These findings have led to trends to substitute asbestos fibers with adverse effects by fibers technologically and qualitatively similar (resistant against thermal and chemical degradation) but with lower biological impact. From these facts follow important tasks on the deep and broad research activities aiming at the study of the effects of fibres substitutes. These activities are in accordance with the WHO, ILO and IARC directives. In the USA, Australia, states of E. U. and Japan the mining, production and using of asbestos is banned. Now asbestos is used only in developing and in the 3rd world countries. Industrial fibrous dusts have been used for a few decades in aviation, car production, sheaf building, building, cosmic and other industries for production of thermal and acoustic insulation materials, brake linings, fireproof textiles, products resistant to acids and alkalines,

asbestos-cement products including shingles, sheets and pipes for waste water etc.

The mechanism of action of fibrous structures during lung diseases development is not clear up to present time. It seems that type of fibres, dose, duration of exposure and individual immunodisposition play an important role in the induction of lung tissue injury. Depositional characteristics, biopersistence, chemical composition and the physical properties are among the most important determinants of the intrinsic toxicity of any inhaled fibers¹.

Solubility and clearance studies of man-made vitreous fibres (MMVFs) in animal lung tissue indicate that in general, glass fibres are more soluble than rock wool, whereas refractory ceramic fibers (RCFs) are the most durable of MMVFs (ref.²). Numerous studies suggest that the process of lung injury after asbestos exposure depends besides other factors also on the dimensions of the fibres^{3–5}. Some naturally occurring or man-made substitutes of asbestos would be judged as pathogenic according to Stanton's hypothesis, which states that long, thin particles are associated with lung diseases, the most pathogenic being fibres with diameter less than 0.25 µm and length longer than 8 µm (ref.⁶). Scientific knowledge concerning the effects of fibrous dusts on respiratory tract were obtained mainly from asbestos exposure. In the present time the research

is focused especially on the asbestos substitutes. One kind of asbestos substitutes are refractory ceramic fibers which are predominantly used in high-temperature insulation. RCFs are amorphous alumina silicates which, along with fibrous glass and mineral wool, belong to a group of materials generally referred to as synthetic vitreous fibers. They are produced from molten masses of alumina Al_2O_3 and silica SiO_2 which are fiberized through a wheel centrifuge or a steam process. RCFs are widely used to replace asbestos in applications requiring high heat resistance. They have been produced since the 1950. RCFs have numerous physical and chemical properties, i.e., low heat storage, light weight, low thermal conductivity, resistance to thermal shock, chemical/corrosion resistance, and ease of installation, all of which contribute to their utility in the industrial marketplace⁷. With high-temperature applications of up to 2800 °F, commercial production currently represents 1 to 2 % of the total production of MMVFs and mainly are used in high-temperature insulation of furnaces and kilns¹. Typically, the assessment process includes a thorough review of each step in the life cycle of an RCFs component. In most applications, these steps include RCFs manufacture, transportation, part fabrication, installation/assembly, use, maintenance/repairs, misuse scenarios, and disposal or reclamation.

Because RCFs are relatively durable and some RCFs are respirable, they may present a potential health hazard by inhalation. Most recent animal studies have found RCFs to be a rodent carcinogen, under the conditions of lifetime exposure at high dose. These studies also suggest that there may be a dose-response threshold, below which irreversible respiratory impacts do not occur⁷. It is estimated that approximately 30,000 workers are exposed to RCFs in the United States. The number of potentially exposed workers in Europe is approximately the same⁸. Recent convincing studies have demonstrated the occurrence of pleural plaques in RCF workers without concomitant interstitial fibrosis. It has yet to be determined if these pleural plaques augur a risk for human mesothelioma. Dumortier detected pseudo asbestos bodies in samples from subjects exposed to RCFs (0.5 %) (ref.⁹). Froudarakis et al. performed mineralogic study of fibers in BAL fluid from workers exposed to RCFs and found out the presence of transformed type (ferruginous body with dissolution of internal fiber) or native type of aluminosilicate fibers. Transformed type was mostly seen in nonsmokers, while native type in smokers⁷. RCFs have been classified as Group 2B, possibly carcinogenic to humans by the International Agency for Research on Cancer¹⁰. The National Toxicology Program has indicated that ceramic fibers of respirable size can reasonably be anticipated to be carcinogenic⁷. Long-term follow-up studies of the RCFs worker cohorts as well as experimental studies may provide the answers over the next decade. At the present time, significant human carcinogenic risk from any inhaled MMVFs is neither clearly established nor refuted^{1,11}.

Our present work, aimed at revealing the subchronic effects of middle experimental dose – 4 mg RCFs per

animal – on the respiratory system, focused on the understanding of the pathogenetic process in response to exposure to ceramic fibers.

MATERIAL AND METHODS

The study was conducted with the approval of the Animal Ethics Committee of Slovak Medical University, Bratislava and in accordance with the guidelines of European Convention for the Protection of Vertebrate Animals Used for Experimental Purposes. Male Wistar rats were supplied from Velaz Prague, Czech Republic. At the beginning of instillation – after two weeks quarantine – mean initial weight of animals was 153.7 g. All animals were maintained under non-infectious laboratory conditions at 22 ± 2 °C, 45 % relative humidity, under natural light and normal light/dark photoperiodicity and under air-conditioned conditions using the unit WOLF KG 100 (WOLF – Clima Technik, GmbH, Mainburg, Germany). A commercial diet ST 1 (TOP – Dovo, Horné Dubové, Slovak Republic) and tap water were available ad libitum. The fibres used in our experiments were refractory ceramic fibers (RCFs) used as a substitute of asbestos in some industrial applications.

Wistar rats (6 animals per group) were intratracheally instilled (noninvasively) under light ether anaesthesia with 4 mg of RCFs in 0.4 ml of saline solution/ animal – exposed group and with 0.4 ml saline solution/animal – control group. Dose 4 mg of RCFs suspension was instilled in two doses. Animals were dosed once a week with 2 mg during 2 weeks. Similarly we divided and instilled saline solution (2×0.2 ml/control animal).

Refractory ceramic fibres were resuspended in distilled water and examined by phase contrast microscopy at $\times 500$ magnification. The length and diameter of fibres are shown in Table 1.

Six months after the last instillation the animals were sacrificed, bronchoalveolar lavage (BAL) was performed and BAL parameters (mainly inflammatory) were examined in the bronchoalveolar lavage (BALF): the leukocyte count, the count of alveolar macrophages (AM), the differential cell count (% of AM, % of polymorphonuclears

Table 1. Distribution of RCFs

Diameter	Length
1 μm = 12 %	20–40 μm = 17 %
< 1 μm = 6 %	41–60 μm = 11 %
< 3 μm = 35 %	61–80 μm = 14 %
3 μm = 8 %	81–100 μm = 14 %
> 3 μm = 39 %	> 100 μm = 44 %

Fibre chemistry: SiO_2 45–60 %; Al_2O_3 40–55 %

– PMN, % of lymphocytes – LY), proportion of immature AM, the percentage of binucleate cells (BNC), the viability and the phagocytic activity of AM. The animals were sacrificed by severing the abdominal aorta under intra peritoneal thiopental narcosis (150 mg/kg of animal). AM were harvested using a modified method of BAL by Myrvik et al.¹². The trachea was cannulated, and the lungs were washed 3 times with 5 ml of saline solution (in situ). The BALF cell count was determined in Bürker's chamber and differential cell counts were performed on May-Grünwald-Giemsa stained preparations.

The total leukocyte count and total count of AM in BALF were determined in the Bürker's chamber (in 50 central squares $\times$ 5000) $\times$ volume of obtained BALF, the differential cell count (%AM, PMN, LY), proportion of immature form of alveolar macrophages and proportion of binucleate AM were performed on May-Grünwald Giemsa stained preparations.

The phagocytic activity of AM was investigated by the method of Fornůsek et al. using 2-hydroxyethylmetacrylate particles (HEMA) from Neosys Prague¹³. Fifty μ l of HEMA particles in PBS (phosphate buffer) are added to 100 μ l of BAL fluid and incubated for 60 min at 37 °C with shaking at frequent intervals. Staining was performed by the May-Grünwald Giemsa method. Cells were considered positive when they phagocytized three or more particles.

Viability of AM – to 200 μ l of 0.25 % erythrosine solution is added to 200 μ l aliquot of the cell suspension. The number of viable and non-viable cells is counted using a Bürker's chamber. Detail description of mentioned methods is in paper¹⁴.

The results were statistically evaluated using Wilcoxon's test.

RESULTS AND DISCUSSION

The subchronic effects of RCFs on BAL parameters are described in Table 2. Six months after the last intratracheal instillation of RCFs the total count of leukocytes in BALF, the total count of AM in BALF and proportion of binucleate cells in BALF were not influenced. In comparison with the control group the mentioned parameters were increased (Le: 12.3 %, AM: 11.3 % and BNC: 38.1 %) but not statistically significantly.

Compared with the control group, statistically significant increases were found in the following BALF parameters: proportion of PMN ($p < 0.01$), proportion of LY ($p < 0.01$), % of immature AM ($p < 0.01$) as well as phagocytic activity of AM ($p < 0.01$).

Compared with the control group, 6 month exposure to ceramic fibers statistically significantly decreased proportion of AM in BALF from the differential cell count ($p < 0.01$) and statistically significantly inhibited viability of AM in BALF ($p < 0.01$).

The number and the type of cells obtained via bronchoalveolar lavage as well as their viability and state of activation enable us to understand the extent of a possible harmful effect of inhaled noxious substances. Increased number of leukocytes after asbestos and other fibrous dust exposure as a result of inflammatory response was described by numerous authors^{4, 15, 16}.

Table 2. BAL inflammatory and cytotoxic parameters 6 month after the last instillation of RCFs (4 mg/animal)

BAL parameters	Control group (0.4 ml saline solution per animal)		Refractory ceramic fibers (4 mg RCFs in suspension per animal)
the count of leukocytes	1877.5 1762.0; 2091.0	↑	2092.5 1962.5; 2200.0
the count of AM	1406.5 1350.0; 1648.3	↑	1583.8 1329.4; 1698.4
% of AM	98.0 98.0; 98.0	↓	75.5 ** 72.0; 86.5
% of PMN	1.0 1.0; 1.0	↑	19.0 ** 9.5; 22.5
% of LY	1.0 1.0; 1.0	↑	4.0 ** 3.25; 6.25
% of AM immature forms	8.5 7.25; 9.75	↑	21.5 ** 15.25; 27.75
% of BNC	2.5 2.0; 3.0	↑	4.0 2.5; 4.75
% of AM viability	90.0 89.3; 90.8	↓	84.0 ** 83.3; 85.5
% of AM phagocytic activity	23.5 20.8; 28.5	↑	38.5 ** 37.25; 39.75

Our study indicate that six months after last instillation of RCFs, the total count of leukocytes (Le) and AM was increased in comparison with the control group (Le:12.3 %; AM:11.3 %) but these results were not statistically significant. Similar results we found out after 6 month inhalation exposure to amosite or wollastonite in our previous experimental study¹⁷. Froudarakis et al. examined workers exclusively exposed to RCFs. BAL analysis in nonsmokers revealed normal cell count, but a slight increase in neutrophils. In contrast, BAL analysis in smokers revealed increase in neutrophils. On the base of further examined parameters they concluded that lung toxicity of RCFs may be increased in smokers¹⁸. Inhaled particles and fibers deposited in the lung are usually phagocytized by alveolar macrophages. AM have been given priority in *in vivo* and *in vitro* experiments for demonstrating cytotoxicity of fibrous and non-fibrous dusts in view of the fact that these cells are the first to come into contact with the inhaled noxa. They have not only the role of phagocytes, but they are also important immunoregulatory cells, which act in both defense mechanism and pathogenesis of numerous lung diseases. AM also have an important role in inflammatory reaction – they stimulate inflammatory cells to secrete various cytokines, growth factors and proinflammatory mediators. Change in macrophage numbers or function is a factor in determining lung injury and in characterizing the pathogenesis of such a response. While a decrease in macrophage number or phagocytic capacity may result in a reduction in clearance of inhaled materials and thus an increase in the effective dose of the potentially injurious agent, an increase in AM number may also have negative effects on the lung through the release of proteases, active oxygen species, and fibroblast-regulating mediators^{1, 15, 19}. Exposure to noxious substances changes the differential cell count by increasing the proportion of lung inflammatory cell in particular that of PMN and LY. However, a reduction in the proportion of macrophages could also account for an increase in the proportion of inflammatory cells^{14, 15, 20}. In the present study we observed that % of AM was significantly decreased and on the other hand % of LY and PMN was significantly increased against the control. According to Dziedzic et al. and Morimoto and Tanaka, long – term recruitment of PMN might be an important factor in prediction of lung metaplastic processes^{15, 21}. Percentage of AM immature forms after influence of RCFs in our study was also statistically significantly increased in comparison with the control group ($p < 0,01$) (Table 2). Percentage of these monocytic AM forms can be increased as dominant pathologic reaction in the large group of cases: in inorganic dust exposure, intensive tobacco consumption, as well as in a special stage of different interstitial lung diseases (sarcoidosis, silicosis, asbestosis), etc.²². Both differential cell count and an estimate of total cell number are important when reporting the results of cell analysis based on BAL. Each gives important, unique, and complementary data. Neither alone can give the complete information necessary to asses inflammation and possible lung injury. Decreases in macrophage number, viability or phagocytic capacity

may result in impaired clearance of inhaled materials¹⁵. In our case we found out also significant suppression of viability and reduction of AM (%) in the frame of differential cell count, but 6 months after the last instillation of RCFs these fibers did not suppress phagocytic activity – in contrary, phagocytic activity was significantly increased in comparison with the control. Probably used dose (4 mg/animal) and relatively long time (6 months) after the last instillation of RCFs was the reason why AM have the sufficiency of digestion through phagocytosis retained. As regards the BNC in BAL after RCFs exposure, these cells were increased (38.1 %) in comparison with the control but not significantly. According to Beňo et al. more detailed studies with more dust types aimed at the time development of the binucleate cells and multinucleate cells pools in lungs, their turn-over rate including, are necessary for definite conclusions about their usefulness as biomarkers of inflammation²³.

Because industrial fibrous dusts are used in many industrial branches – and by reason of their harmful effect on the respiratory tract that can induce many respiratory diseases – it is necessary to test their biological impact and to choose and use only these which are the less dangerous for people and which have the less negative influence on environment.

Examination of the selected inflammatory and cytotoxic BAL parameters results in the following conclusions: (i) Relatively high biopersistence of RCFs have been confirmed; (ii) The results of this study indicated that RCFs even 6 months after intratracheal instillation very significantly changed the majority of examined BAL parameters; (iii) The presence of inflammatory and cytotoxic responses in lung may signalize beginning or developing disease process.

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