

# THE EFFECT OF FIBROUS DUSTS ON LUNG CELLS. *IN VITRO* STUDY

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## SUMMARY

*The mechanism of toxicity of selected asbestos substitute mineral fibres was examined and compared to that of asbestos. Alveolar macrophages and type II cells were isolated from Fischer 344 rats and after 20 h cultivation various concentration of fibres alone (amosite, wollastonite, rockwool or glass fibres) or in combination with cigarette smoke were added to cells and the cultivation continued for another 24 h. After finishing the exposure the number of alkaline phosphatase positive type II cells was counted, the comet assay was used to detect DNA damage (strand breaks) in both cell types and ultrastructural changes were evaluated by transmission electron microscopy. The decrease of the number of alkaline positive type II cells was dose dependent in all cases. The number of DNA strand breaks (SBs) in both cell types was enhanced after exposure to all types of fiber, the enhancement was dose dependent, the highest level of SBs was observed after amosite exposure. The combined exposure to mineral fibres and cigarette smoke showed synergic effect on the level of SBs. Transmission electron microscopy showed that already 1 µg.cm<sup>-2</sup> amosite caused destruction of AM while other fibres were phagocytized.*

**Key words:** fibrous dusts, alveolar macrophages, type II epithelial cells, DNA damage

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## INTRODUCTION

As to the cell types, the lung is a very heterogenous organ. It consists of more than 40 different cell types. Each cell type has an unique morphologic characteristic associated with its function. Alveolar macrophages (AM) and epithelial type II cells belong from the toxicological point of view to the most important cell types. AM play an important role in the lung defence and in inflammatory responses. Type II cells are able to proliferate and to differentiate into other cell types. They are thought to be also progenitors of some types of tumours. Isolated lung cells represent a valuable system for studying mechanism of airborne agents toxicity.

The fact that exposure to asbestos fibres causes an increased risk of pleural mesothelioma a bronchogenic carcinoma of the lungs has been documented (1). Later studies on man-made vitreous fibers and refractory ceramic fibres have shown that

these fiber types also cause pathology (2). The aim of this study was to compare the effects of asbestos (amosite - A) with three types of mineral fibres: glass fibres (GF), rockwool (RW) and wollastonite (W).

## MATERIALS AND METHODS

**Animals.** Male Fischer 344 rats (Anlab, Prague, Czech republic) weighing 200–220 g were used in these experiments. The animals were housed under standard laboratory conditions and were given a conventional laboratory diet (MOK, Velaz, Prague, Czech Republic) and tap water *ad libitum*.

**Cell isolation.** Alveolar macrophages were isolated according the method of Myrvik et al. (3) and type II pneumocytes according the method of Richards et al. (4). Briefly, after an intraperitoneal sodium pentobarbital injection (60 mg/kg), the lung was perfused *via* the pulmonary artery with sterile saline and was mechani-

cally ventilated. The lung with trachea was removed and the bronchoalveolar lavage was performed. Alveolar macrophages were isolated by centrifugation of bronchoalveolar lavage fluid. The lung tissue was partially trypsinized, chopped and the cell mixture purified on a discontinuous Percoll gradient. The interface was collected and after rewashing the cells were resuspended in the medium and plated in a Petri dish. After 1 hour incubation in an atmosphere of 95% air / 5% CO<sub>2</sub> the unattached cells (type II) were collected and sedimented.

**Cell culturing.** The cells were cultured on 24-well plates (Falcon) in DMEM (Dulbecco's modified eagle's medium) supplemented with 10% FCS (foetal calf serum) at 37 °C in an atmosphere of 95% air / 5% CO<sub>2</sub>. The plating density was 500 000 cells/well. The medium was changed after 20 h and in the fresh medium fibres were resuspended. Cigarette smoke treated medium was obtained by bubbling the cigarette smoke from one reference cigarette 1R1 (University of Kentucky) through 5 ml of DMEM supplemented with 10% FCS. The concentrated cigarette smoke treated medium was diluted with the medium, the final concentration used in the experiment was 4% of cigarette. The cultivation with tested agents continued for another 24 hours. After finishing the cultivation medium was withdrawn and cells were washed twice with PBS.

**Alkaline phosphatase activity.** Type II cells were incubated (in wells) in a mixture of AS-TR phosphate and fast red at pH 8.5 for 30 min at room temperature. Positive cells were stained bright red. Cells were counted by phase contrast microscope and only cells with four or more lamellar bodies were considered to be type II cells. In every experiment 300 cells per fiber dose were counted.

**The comet assay.** Comet assay (5) was used with small modifications. Plain glass microscope slides were precoated with 0.5% normal melting point agarose (Gibco BRL) in water. Two gels of 85 µl 1% normal melting point agarose in PBS buffer were set on each precoated slide and overlaid with 85 µl of 1% low melting point agarose in PBS in which cells were resuspended at 37 °C. The two gels served as duplicates. The cells were lysed by immersing the slides in 2.5 M NaCl, 0.1 M Na<sub>2</sub>EDTA, 10 mM TRIS-HCl pH 10, 1% Triton X-100 for 1 h, then placed in an electrophoresis tank in 0.3 M NaOH, 1 mM Na<sub>2</sub>EDTA for 40 min. Electrophoresis was carried out at 25 V and 300 mA for 30 min. Strand breaks allow DNA to extend from the nucleoids towards the anode,

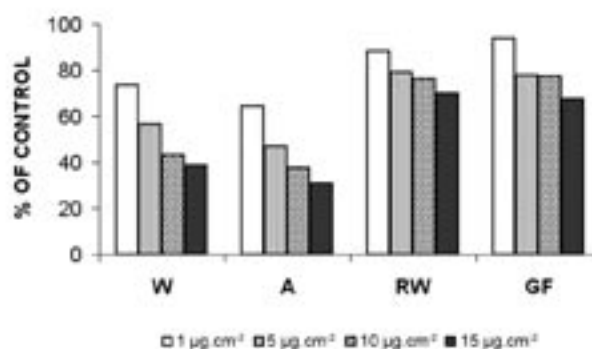
forming a "comet tail". Comets were visualised by fluorescence microscopy after staining with 4,6-diamidino-2-phenylindole.

**Electron microscopy.** 2x10<sup>6</sup> cells were fixed in 2% glutaraldehyde in 0.1% cacodylate buffer at pH 7.4. Cells were sedimented and postfixed with cacodylate buffered OsO<sub>4</sub>, dehydrated through a graded ethanol series and embedded in Durcupan ACM. Sections were cut with a Reichert Jung ultramicrotome, stained by uranyl acetate-lead citrate and examined using a JEOL JEM 100C electron microscope.

## RESULTS

Freshly isolated type II cells exhibit intense alkaline phosphatase activity. After terminating the cultivation the alkaline phosphatase activity was evaluated histochemically. The number of cells with positive alkaline phosphatase reaction decreased with increasing dose of all fibres. The lowest dose which decreased the number of positive cells to less than 50% of control ones was 5 µg.cm<sup>-2</sup> of amosite (Fig. 1).

Alveolar macrophages and type II cells were analysed for DNA damage by comet assay. Every fiber was tested in 3 separate experiments and in every experiment two gels served as duplicates, it means that each data represents the average of 6 determinations.



**Fig.1.** The effect of exposure to various types and doses of fibers on the alkaline positivity of lung epithelial type II cells.

**Table 1.** DNA damage induced by fibres in alveolar macrophages and type II cells

Cell type	Fibre	Dose (µg.cm <sup>-2</sup> )					
		0	1	5	10	15	Δ (15-0)
AM	W	135	180.8	194.3	202.7	213.3	78.3
	A	183	230	256.8	287	305.2	122.2
	RW	208.8	234.2	272	294.3	305.7	96.9
	GF	180.5	197.5	213.5	226.3	258	77.5
Type II	W	85.4	148	159	197	190	104.6
	A	163	192.8	209.7	243.3	260.5	97.5
	RW	192	224	238.5	251.3	288.5	96.5
	GF	154.5	174.2	189.2	197.8	216.5	62

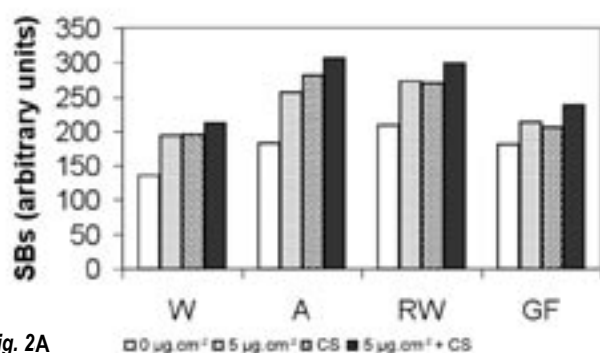


Fig. 2A

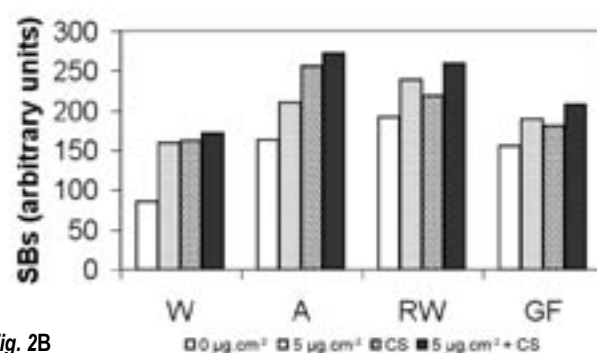


Fig. 2B

**Fig. 2.** DNA damage in alveolar macrophages (A) and type II cells (B) treated with fibres, cigarette smoke and their combination. CS - cigarette smoke. For other acronyms see text.

Both cell types respond to the exposure to all studied fibres with dose-dependent increase in single strand DNA breaks (Table 1). Evaluation of the difference between the value after exposure to the highest fiber concentration ( $15 \mu\text{g.cm}^{-2}$ ) and the control one showed that in AM the enhancement of SBs was the same after exposure to wollastonite and glass fibres and the highest after exposure to amosite. In type II cells rockwool, amosite and wollastonite evoked the same enhancement of DNA damage while the exposure to glass fibres showed smaller extent of damage. The cigarette smoke alone enhanced the number of SBs and the combination with  $5 \mu\text{g.cm}^{-2}$  fibres showed synergic effect in all cases (Fig. 2A and 2B).

The electron microscopy revealed the changes in AM after phagocytosis of studied agents. Figure 3A shows the intact control cell. Lots of particles can be found in AM after exposure to wollastonite (3B), electrondense rectangular rockwool particles are seen after exposure to rockwool (3C), amosite strongly da-

maged AM (3D) and after exposure to cigarette smoke some electrondense bodies can be visualized in the cells (3E). The concentration of wollastonite and rockwool was  $5 \mu\text{g.cm}^{-2}$  and of amosite  $1 \mu\text{g.cm}^{-2}$ .

## DISCUSSION

Our knowledge about possible effect of man made mineral fibres in the lung is limited and that was the cause of studying 3 types of fibres in in vitro system of lung target cells: AM and type II cells. The effect was compared with that of amosite (asbestos). The fibre size distribution of all studied fibres was similar (unpublished data).

The transmission electron microscopy showed different reaction of cultured AM to fibres: amosite in the lowest tested dose ( $1 \mu\text{g.cm}^{-2}$ ) caused destruction of macrophages while other fibres

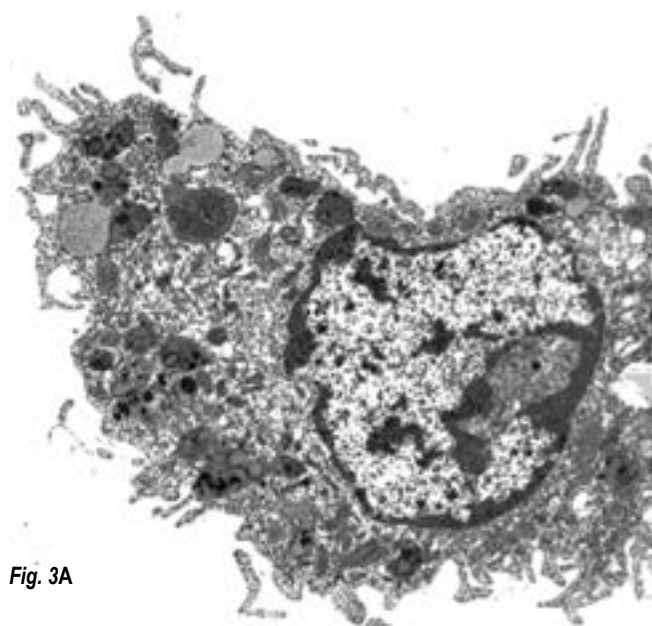


Fig. 3A

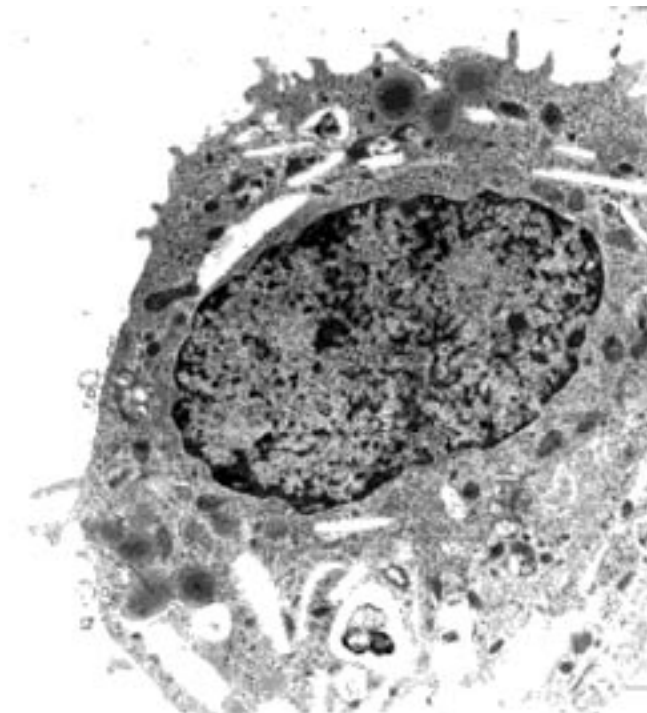


Fig. 3B

**Fig. 3.** Alveolar macrophages. (A) control cells, (B) cells after exposure to wollastonite, (C) cells after exposure to rockwool, (D) cells after exposure to amosite, (E) cells after exposure to cigarette smoke. Original magnification:  $\times 8300$ .

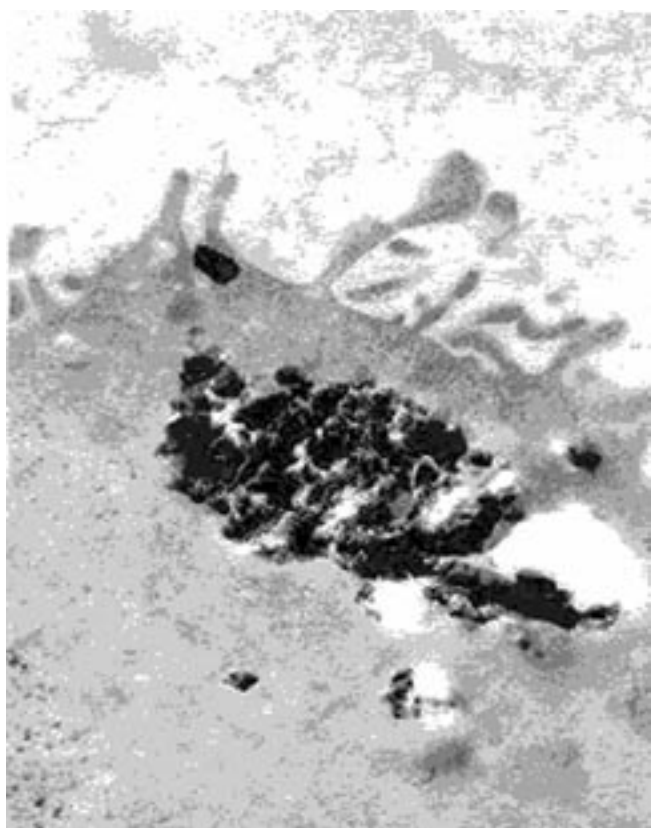


Fig. 3C

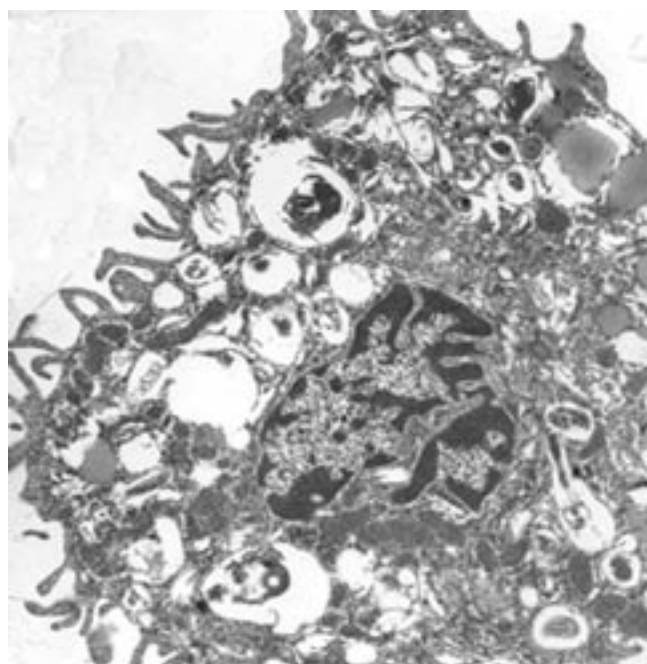


Fig. 3D

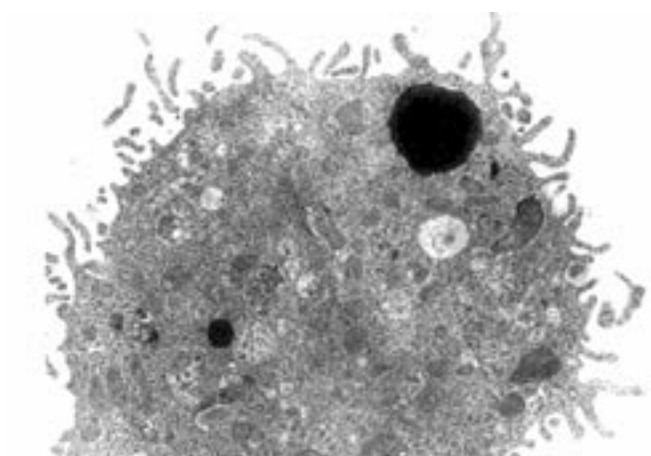


Fig. 3E

at five times higher dose were phagocytized without destruction of the cell. As AM represent the first line of lung defence mechanism their destruction can start a cascade of events in the lung (6).

Alkaline phosphatase is a functional marker enzyme of type II cells (7) but the significance of this remains unclear. The decrease in activity may be the consequence of the toxic effects of tested fibres.

There are reports in literature about DNA damage in various cell types after exposure to both asbestos (crocidolite-8,9, chrysotile-9,10) and non-asbestos fibres (glass fibres-11). Crocidolite evoked in transformed human mesothelial cells only small effect which was neither concentration ( $1-4 \mu\text{g.cm}^{-2}$ ) nor time (0.5-48 h exposure) dependent (8). Studies on SV40-transformed rat pleural mesothelial cells with chrysotile and crocidolite showed concen-

tration (to  $10 \mu\text{g.cm}^{-2}$ ) related DNA damage (9). DNA damage was seen also in A549 cells (human lung epithelial cells) after 1 h exposure to  $40 \mu\text{g.cm}^{-2}$  of chrysotile (10). Glass fibres evoked dose dependent ( $0-13.8 \mu\text{g.cm}^{-2}$ ) DNA damage of Hel 299 (human embryonic lung fibroblast) and V 79 (chinese hamster lung fibroblasts) after 3 h exposure (11). Our experiments with primary cells give evidence of DNA damage after 24 h exposure to both asbestos (amosite) and non-asbestos fibres. The damage was in all cases dose dependent. The extent of type II damage was the same after exposure to amosite, rockwool and wollastonite. In cultured AM the damage after exposure to amosite was higher than with other fibres. By comparison of our results and those from above cited papers we can conclude that the effect of fibres (as concerning DNA damage) may be different in various cell types. The novelty of our experiment was that we were testing the cell types which are the real target cells for the fibres. Some discrepancies between authors can be caused also by different experimental conditions as for instance dose and time. Positive comet assay results indicate direct or indirect interaction of the test material with the genome. Because the comet assay is an indicator test, effects need not be directly related to mutagenesis (11).

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#### REFERENCES

1. Mossman BT, Gee JBL: Medical progress: asbestos-related diseases. *New Engl J Med* 1989; 320: 1721-1730.
2. Bunn WB, Bender RJ, Hesterberg TW, Chase GR, Konzen JL: Recent studies of man-made vitreous fibres. *J Occup Med* 1993; 35: 101-113.
3. Myrvik QN, Leake ES, Farris B: Studies on pulmonary alveolar macrophages from the normal rabbit: A technique to procure them in a high state of purity. *J Immunol* 1961; 86: 128-132.

4. **Richards RJ, Davies N, Atkins J, Oreffo VIC:** Isolation, biochemical characterization and culture of lung type II cells of the rat. *Lung* 1987; 165: 143-158.
  5. **Collins AR, Dušinská M, Gedik CM, Štetina R:** Oxidative damage to DNA: do we have a reliable biomarker? *Environ Hlth Perspect* 1996; 104, Suppl 3: 465-469.
  6. **Nicod LP:** Pulmonary defence mechanism. *Respiration* 1999; 66: 2-11.
  7. **Edelson JD, Shannon JM, Mason RJ:** Alkaline phosphatase: a marker of alveolar type II cell differentiation. *Am Rev Respir Dis* 1988; 138: 1268-1275.
  8. **Ollikainen T, Linnainmaa K, Kinnula VL:** DNA single strand breaks induced by asbestos fibres in human pleural mesothelial cells in vitro. *Environ Mol Mutagen* 1999; 33: 153-160.
  9. **Levresse V, Renier A, Levy F, Broaddus VC, Jaurand MC:** DNA breakage in asbestos-treated normal and transformed (TSV40) rat pleural mesothelial cells. *Mutagenesis* 2000; 15: 239-244.
  10. **Wang Q, Han C, Yang Y, Wang H, Wu W, Liu S, Kohyama N:** Biological effects of man-made mineral fibres (II). *Ind Health* 1999; 37: 342-347.
  11. **Zhong B, Whong W, Ong T:** Detection of mineral-dust-induced DNA damage in two mammalian cell lines using the alkaline single cell gel / comet assay. *Mutat Res* 1997; 393: 181-187.
  12. **Speit G:** Appropriate in vitro test conditions for genotoxicity testing of fibres. *Inhalation Toxicol* 2002; 14: 79-90.
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