ORIGINAL ARTICLES AAEM

Ann Agric Environ Med 2006, 13, 259–262

PULMONARY CYTOTOXICITY OF SECONDARY METABOLITES OF *STACHYBOTRYS CHARTARUM* (EHRENB.) HUGHES

Elena Piecková, Marta Hurbánková, Silvia Černá, Zuzana Pivovarová, Zuzana Kováčiková

Slovak Medical University, Bratislava, Slovakia

Piecková E, Hurbánková M, Černá S, Pivovarová Z, Kováčiková Z: Pulmonary cytotoxicity of secondary metabolites of *Stachybotrys chartarum* (Ehrenb.) Hughes. *Ann Agric Environ Med* 2006, **13**, 259–262.

Abstract: Damp dwellings represent suitable conditions for extended indoor moulds. A cellulolytic micromycete *Stachybotrys chartarum* (Ehrenb.) Hughes is considered to be a tertiary colonizer of surfaces in affected buildings. Known adverse health effects of *S. chartarum* result from its toxins – trichothecenes or atranones, as well as spirolactams. Mechanism of their potential pathological effects on the respiratory tract has not yet been sufficiently clarified. The cytotoxic effects of complex chloroform-extractable endo- (in biomass) and exometabolites (in cultivation medium) of an indoor *S. chartarum* isolate of an atranone chemotype, grown on a liquid medium with yeast extract and sucrose at 25ºC for 14 d, on lung tissue were evaluated in the 3-day experiment. For the purpose, 4 mg of toxicants were intratracheally instilled in 200 g Wistar male rats. A trichothecene mycotoxin diacetoxyscirpenol was used as the positive control. Bronchoalveolar lavage (BAL) parameters - viability and phagocytic activity of alveolar macrophages (AM), activity of lactate dehydrogenase, acid phosphatase and cathepsin D in cell-free BAL fluid (BALF), as well as in BAL cells, were measured. Acute exposure to the metabolites caused statistically significant changes, indicating lung tissue injury in the experimental animals. Decreased AM viability and increased activity of lysosomal enzyme cathepsin D in BAL cells after fungal exometabolite exposure were the most impressive. As toxic principles were found predominantly in the growth medium, toxins were more likely responsible for lung cell damage than e.g. fungal cell wall components. *S. chartarum* toxic metabolites can contribute to the ill health of occupants of mouldy building after inhalation of contaminated aerosol.

Address for correspondence: Ing. Elena Piecková, MPH, PhD., Slovak Medical University, Limbová 12, SK-833 03 Bratislava, Slovakia.
E-mail: elena.pieckova@szu.sk

Key words: damp indoor environment, moulds, *Stachybotrys chartarum*, respiratory toxicity, rats, intratracheal instillation, bronchoalveolar lavage parameters.

INTRODUCTION

The worldwide distributed cellulolytic micromycete *Stachybotrys chartarum* (Ehrenb.) Hughes has been known for almost more than a century as a contaminant decaying hay, straw, grains etc. that causes an animal alimentary mycotoxicosis stachybotryotoxicosis, e.g. in horses and sheep [25]. Handlers of mouldy materials overgrown with *S. chartarum* can suffer from skin and mucosal irritations or respiratory symptoms (catarrhal angina, bloody rhinitis, cough) [3]. From the indoor-mycological point of view, the mould belongs to tertiary wall colonizers, i.e. indicates long-lasting stubborn high moisture of the materials affected [9, 28]. *S. chartarum* isolates anyway are less common indoor moulds, being found in less than 3% of damp mouldy buildings, and nearly always occurring in the presence of other potentially toxic fungi, e.g. *Aspergillus versicolor* and

Received: 23 January 2006
Accepted: 1 June 2006

Penicillium spp. [19, 24, 26, 32, 33]. In the last decade, there were many reports on building-related illnesses in connection with *S. chartarum* in damp buildings/ dwellings, based on epidemiological survaillances, including fatal cases of infant idiopathic hemosiderosis. However, no clear linkage of the presence of the fungus to health damage has been found [2, 4, 10, 15, 16]. There was no evidence that the fungus itself was a pathogen. Its toxins are believed to be causative agents of health damage [7, 18]. *S. chartarum* strains are able to produce either several macrocyclic trichothecene mycotoxins stachybotryotoxins (satratoxins, roridins, verrucarins) – chemotype A, or diterpenoid atranones and simple trichothecenes (trichodermol, trichodermin) – chemotype B, both accompanied with spirocyclic drimanes, that are concentrated in the cells and diffuse into the medium, including building materials [1, 14]. The mechanism of the potential pathological effects of *S. chartarum* secondary metabolites on the respiratory tract has not been sufficiently clarified to date. However, some animal studies of the effect of intranasaly or intratracheally instilled spores yielded results indicating inflammatory and cytotoxic potential of the fungus [22, 23, 30]. Due to their morphological properties – formed in slimy heads, > 10 mm in size, stachybotrys spores are unlikely able to reach the lower airways. Therefore, spore instillations cannot reflect real human exposure conditions.

To focuse more precisely on *S. chartarum* toxic principles' role in lung injury effects of the fungus, as only that should be physiologically relevant, we used direct intratracheal instillation of complex mixtures of its endo- and exometabolites to rats. The pulmonary cytotoxicity of crude chloroform-extractable metabolites of an office *S. chartarum* isolate of the atranone chemotype [29] was evaluated in bronchoalveolar lavage fluid within the framework of this study.

MATERIALS AND METHODS

Fungal metabolites. *S. chartarum* was cultivated on the liquid medium with 2% yeast extract and 10% sucrose at 25ºC for 10 d. Separated biomass (endometabolites) and cultivation medium (exometabolites) were extracted twice with chloroform, extracts dried under vacuum and dissolved in 0.2% dimethylsulphoxide (DMSO; Merck, Darmstadt, Germany) to 20 mg/ml [27].

Treatment of animals and bronchoalveolar lavage (BAL) performance. Male Wistar rats (Velaz, Prague, Czech Republic) of about 200 g weight were used in the experiment (6 animals per toxicant). The animal treatment conformed with the Guidelines of the European Convention for the Protection of Vertebrate Animals for Experimental Purposes. After weighing and anaesthetizing of the animals with ether, an oto-rhino-laryngological mirror was set up in the correct position and the mouth cavity opened. Four mg of metabolite dissolved in 0.2 ml of 0.2% DMSO were intratracheally instilled to each rat by a special device. The animals in the control group were given 0.2 ml of 0.2% DMSO only. The mycotoxin diacetoxyscirpenol (DAS; Sigma, St. Louis, USA) was used as the positive control. After 3-days' exposure, the animals were killed by thiopental anaesthesis (150 mg/kg of animal) by exsanguination, cutting the vena cava caudalis, and bronchoalveolar lavage (BAL) performed according to Myrvik *et al.* [20]. The trachea was cannulated and the lungs washed *in situ* 5-times with 5 ml of saline. The pooled bronchoalveolar lavage fluid (BALF) was centrifuged (10 min, 450 g at 4ºC), the cell-free BALF was transferred into clean, cooled glass tubes and the BAL cell sediment adjusted to 1×10^6 cells/ml by sterile saline [13].

Evaluation of the lung cytotoxicity. The potential lung cytotoxicity of endo- and exometabolites of S. *chartarum* was evaluated by measurement of the following BALF parameters: viability and phagocytic activity of alveolar macrophages (AM) [13], activity of lactate dehydrogenase (LDH) – determined immediately in the fresh cell-free BALF by means of a test kit LD 105 UV (Lachema, Brno, Czech Republic), of acid phosphatase (ACP) - measured during the day of BAL performance (test kit AC 565, Randox Laboratories, Antrim, UK), and of cathepsin D in cell-free BALF as well as in BAL cells [21].

The phagocytic activity of AM was determinated according to Fornůsek *et al.* [8] using 2-hydroxyethylmetacrylate particles (HEMA; Neosys, Prague, Czech Republic). Fifty ml of HEMA particles in phosphate buffered saline (PBS), pH 7.2, were added to 100 ml of BALF and incubated at 37ºC for 60 min while shaken at short intervals. Afterwards, May-Grünwald Giemsa staining was performed. Cells were considered positive when phagocytized at least 3 particles.

The AM viability was detected by fluorescent microscopy with 0.25% erythrosine as a staining solution (1 ml/1 ml of the cell suspension). The number of viable cells was counted by means of Bürker's chamber.

To obtain cell-free BALF, the original BALF was centrifuged at 450 g under 4ºC for 10 min. The activities of enzymes LDH and ACP were measured in cell-free BALF spectrophotometrically at 105 and 565 nm by the means of the aforementioned kits. For the determination of cathepsin D level at the BAL cells, fresh cell sediment was resuspended at the concentration of 106 AM/ml in PBS with 0.1% Triton X100, frozen and thawed three times, and centrifuged at 4ºC, 15,000 g for 20 min, and the enzyme activity measured in the supernatant. Lowry's determination of proteins was performed, with bovine serum albumin having been used as a standard for the calibration curve [21].

The results were statistically evaluated by Mann-Whitney's test.

RESULTS

Lung cytotoxicity of *S. chartarum* metabolites. In the experiment, viability of AM strongly decreased, espe-

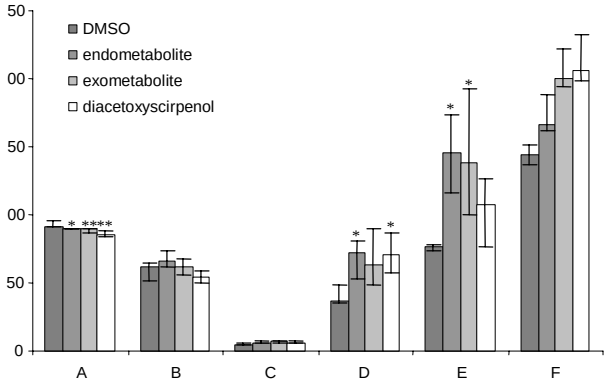

Figure 1. (A) Viability of alveolar macrophages (% of living cells); (B) phagocytic activity of alveolar macrophages (% of phagocyting cells); (C) activity of lactate dehydrogenase in cell-free bronchoalveolar lavage fluid (µkat.g protein^{-1}); (D) activity of acid phosphatase in cell-free bronchoalveolar lavage fluid (nkat.g protein^{-1}); (E) activity of cathepsin D in cell-free bronchoalveolar lavage fluid (U_{tyr}.mg protein^{-1}); (F) activity of cathepsin D in bronchoalveolar lavage cells ($U_{tyr}.10^{-6}$ cells) after exposure to carrier (DMSO), endo- and exometabolites of *Stachybotrys chartarum* or diacetoxyscirpenol. Values represent medians and 25th and 75th percentiles. (U_{tyr}: mg of thyrosine released in one hour; *p<0.05; **p<0.01.)

cially after exposure to exometabolites, p<0.01 (Fig. 1A), although the AM phagocytic activity was not significantly affected by any of the samples tested (Fig. 1B). The activity of LDH in cell-free BALF after exposure to the tested extracts was elevated, but not significantly (Fig. 1C). Activities of lysosomal enzymes - ACP and cathepsin D - in cell-free BALF increased, p<0.05, having also indicated lung tissue damage (Fig. 1D, E). However, the increase in the activity of cathepsin D in BAL cells - represented mostly by AM - after exposure to the exometabolite fungal extracts was significant expressively (Fig. 1F).

Comparison of the cytotoxic effects of endo- and exometabolites of *S. chartarum* with the effect of DAS. In our experiments, the adverse effect of DAS was more expressive than with the endometabolite extract (significantly lower phagocytic activity of AM and higher activity of cathepsin D in BALF cells in DAS group) (Fig. 1B, F). The effects of exometabolites and DAS were comparable (Fig. 1).

The differences between the effects of endo- and exometabolites on the studied cytotoxic parameters were not significant.

DISCUSSION

Intratracheal instillation is a widely-used method enabling materialization of toxic doses to experimental animals. BAL is the most suitable tool by providing estimates of relative toxicity of pulmonary toxicants, especially complex mixtures and those chemically non-defined. BAL is more sensitive than histological examination, and is also employed in pulmonary diseases diagnostics [12, 17].

Phagocytic activity of AM was not readibly affected in the study. It is improbable that phagocytosis could play a very active role in the macroorganism defence mechanisms, as the toxic principles studied were non-particulate.

Alveolar macrophages are the predominant cells present in BAL, and any changes in their number or functions determine lung injury and characterize the pathogenesis of such a response. Lowered viability and/or phagocytic capacity of AM usually result in impaired clearance of inhaled toxicants [6, 31].

Increased activity of LDH – a cytosolic enzyme – in extracellular fluids is generally considered to be a very sensitive indicator of damage to tissue cells' [5]. Rao *et al.* [30] obtained results similar to ours when the activity of LDH in cell-free BALF after exposure to the fungal extracts was slightly elevated: intratracheally instilled toxic *S. chartarum* spores (approx. 9.6 million) induced an increase of LDH activity in rats significantly (p = 0.0003).

In the case of increasing activites of ACP and cathepsin D in cell-free BALF, the effect could be caused by the enzymes' release from disturbed lung tissue and from disrupted BAL cells as well. Probably, because of the great variability of ACP activity values, its increase after exposure to the exometabolites was not highly significant (Fig. 4). The impressive increase of the activity of cathepsin D in BAL cells (AM) after exposure to exometabolites was a clear marker of cell activation burst, and reflected the higher defensive capacity of the lungs.

All enzymatic cytotoxic biomarkers in BAL were altered in our experiment. We are therefore not in full agreement with findings that the atranone-producing chemotype of *S. chartarum* does not induce toxicity to macrophages [1, 11].

The commercially available *Fusarium* spp. trichothecene mycotoxin DAS can cause inflammation and hemorrhage similar to the expected effects of complex stachybotrys-toxicants [34]. The results we obtained from the experiments proved that DAS toxic effect is similar to that of *S. chartarum* exometabolites, and more relevant than from endometabolites.

CONCLUSION

Indoor-originated *S. chartarum* is able to produce metabolites with proven lung cytotoxicity, which was apparent mainly in extracellular products. Toxins, therefore, were probably more likely to be responsible for the adverse effects of the extracts studied than e. g. fungal cell wall components. Their spreading via contaminated detritus, dust particles or liquid aerosol in mouldy buildings has to be seriously taken into account, although, there are no exact measurements on real exposure of humans staying in such spaces. The stachybotrys contribution to respiratory complications in the occupants of affected spaces, especially very young children, should not be overlooked. In the future, better assessment of the

health risk of inhalation exposure to toxic micromycetes (preferably mixtures, but the particular active compounds, too) can be achieved by more detailed dose and time dependence studies.

REFERENCES

1. Andersen B, Nielsen KF, Jarvis BB: Characterization of *Stachybotrys* from water-damaged buildings based on morphology, growth, and metabolite production. *Mycologia* 2002, **94**, 392-403.
2. Andersson MA, Nikulin M, Koljalg U, Andersson MC, Rainey F, Reijula K, Hintikka EL, Salkinoja-Salonen M: Bacteria, molds, and toxins in water-damaged building materials. *Appl Environ Microbiol* 1997, **63**, 387-393.
3. Betina V: Mycotoxins. Chemical, Biological and Environmental Aspects. Elsevier, Amsterdam 1989.
4. Dearborn DG: Pulmonary hemorrhage in infants and children. *Curr Opin Pediatr* 1997, **9**, 219-224.
5. Drent M, Cobben NAM, Henderson RF, Wounters EFM, Van Dieijen-Visser MP: Usefulness of lactate dehydrogenase and its isoenzymes as indicators of lung tissue damage or inflammation. *Eur Respir J* 1996, **9**, 1736–1742.
6. Dziedzic D, Wheeler CS, Gross KB: Broncholaveolar lavage: detecting markers of lung injury. **In:** Corn M (Ed.): *Handbook of Hazardous Materials*, 99-111. Academic Press, New York 1993.
7. Etzel R, Rylander R: Indoor mold and children's health. *Environ Health Perspect* 1999, **107**, S463.
8. Fornůsek L, Větvička V, Kopeček J: Phagocytosis of the peritoneal leukocytes – a new single method. *Immunol Zpravodaj* 1982, **13**, 67-68.
9. Gravesen S, Nielsen PA, Iversen R, Nielsen KF: Microfungal contamination of damp buildings - examples of risk constructions and risk materials. *Environ Health Perspect* 1999, **107**, S505-S508.
10. Hendry KM, Cole EC: A review of mycotoxins in indoor air. *J Toxicol Environ Health* 1993, **38**, 183-198.
11. Hinkley SF, Mazzola EP, Fettinger JC, Lam Y-F, Jarvis BB: Atranones A – G, from the toxigenic mold *Stachybotrys chartarum*. *Phytochemistry* 2000, **55**, 663-673.
12. Hurbánková M: Bronchoalveolar lavage used in pulmonary diseases induced by mineral dusts' exposition in humans and in the experiments. *Čes Prac Lék* 2005, **1**, 33-37 (in Slovakian).
13. Hurbánková M, Kaiglová A: Compared effects of asbestos and wollastonite fibrous dusts on various biological parameters measured in bronchoalveolar lavage fluid. *J Trace Microprob Techn* 1999, **17**, 233-243.
14. Jarvis B: Chemistry and toxicology of *Stachybotrys*. **In:** *Abstract Book of the International Society for Human and Animal Mycology Conference, San Antonio, 25-29 May 2003*, 253.
15. Jarvis BB, Salemme J, Morais A: *Stachybotrys* toxins. 1. *Nat. Toxins* 1995, **3**, 10-16.
16. Johanning E, Biagini R, Hull D, Morey P, Jarvis B, Landsbergis P: Health and immunology study following exposure to toxigenic fungi (*Stachybotrys chartarum*) in a water-damaged office environment. *Int Arch Occup Environ Health* 1996, **68**, 207-218.
17. Khan MF, Gupta GS: Cellular and biochemical indices of bronchoalveolar lavage for detection of lung injury following insult by airborne toxicants. *Toxicol Lett* 1991, **58**, 239-255.
18. Kuhn DM, Ghannoum MA: Indoor mold, toxigenic fungi, and *Stachybotrys chartarum*: infectious disease perspective. *Clin Microbiol Rev* 2003, **16**, 144-172.
19. Miller JD, Laflamme AM, Sobol Y, Lafontaine P, Greenalgh R: Fungi and fungal products in some Canadian houses. *Int Biodeterior* 1988, **24**, 103-120.
20. Myrvik QN, Leake ES, Farris B: Studies on pulmonary alveolar macrophages from the normal rabbit. A technique to procedure them in a high state of purity. *J Immunol* 1961, **86**, 128-132.
21. Navarová J, Nosáľová V: Effect of H2 receptor antagonists on indomethacin - induced lysosomal enzyme release from rat gastric mucosa. *Meth Fin Exp Clin Pharmacol* 1994, **16**, 119-124.
22. Nikulin M, Reijula K, Jarvis BB, Hintikka EL: Experimental lung mycotoxicoxis in mice induced by *Stachybotrys atra*. *Int J Exp Pathol* 1996, **77**, 213-218.
23. Nikulin M, Reijula K, Jarvis BB, Veijalainen P, Hintikka EL: Effects of intranasal exposure to spores of *Stachybotrys atra* in mice. *Fundam Appl Toxicol* 1997, **35**, 182-188.
24. Nolard N: Indoor moulds: a public health problem in Belgium: overview of 15 years´ experience. **In:** Johanning E (Ed.): *Bioaerosols, Fungi and Mycotoxins: Health Effects, Assessment, Prevention and Control*, 48-53. Eastern New York Occupational and Environmental Health Center, Albany 1999.
25. Ožegovič L, Pepeljnjak S: *Mycotoxicoses. Školska knjiga*. Zagreb 1995 (in Croatian).
26. Piecková E, Hurbánková M, Pivovarová Z, Černá S, Kováčiková Z, Lišková A, Wilkins K, Tátrai E: Toxic effects of *Stachybotrys chartarum*. **In:** *Trends in Medical Mycology*. *Abstract Book of the Joint Meeting of the Nineth Congress of the European Confederation of Medical Mycology and the Seventh Trends in Invasive Fungal Infections, Amsterdam, 28 September-1 October 2003*, 35-36.
27. Piecková E, Jesenská Z: Molds on house walls and the effect of their chloroform-extractable metabolites on the respiratory cilia movement of one-day-old chicks *in vitro*. *Folia Microbiol* 1998, **73**, 672-678.
28. Piecková E, Jesenská Z: Microscopic fungi in dwellings and their health implications in humans. *Ann Agric Environ Med* 1999, **6**, 1-11.
29. Piecková E, Pivovarová Z, Hurbánková M, Černá S, Kováčiková Z, Lišková A, Tátrai E, Šegvič-Klarič M: Indoor *Stachybotrys chartarum* and its toxicity. **In:** Petráš D, Feketeová M, Šabíková J (Eds): *Health, Comfort and Safety by Operation of HVAC-R Systems. Proceedings of the Fifth International Conference on Indoor Climate of Buildings' 04, Slovak Society of Environmental Technology, Štrbské Pleso, 21-24 November 2004*, 167-171. Slovak Society of Environmental Technology, Bratislava 2004.
30. Rao CY, Burge HA, Brain JD: The time course of responses to intratracheally instilled toxic *Stachybotrys chartarum* spores in rats. Mycopathol 2000, **149**, 27-34.
31. Tarkowski M, Górski P: Macrophage activity in asbestos related diseases. *Polish Occup Med Environ Health* 1991, **4**, 115-125.
32. Trout D, Bernstein J, Martinez K, Biagini R, Wallingford K: Bioaerosol lung damage in a worker with repeated exposure to fungi in a water-damaged bulding. *Environ Health Perspect* 2001, **109**, 641-644.
33. Tuomi T, Reijula K, Johnsson T, Hemminki K, Hintikka EL, Lindroos O, Kalso S, Koukila-Kahkola P, Mussalo-Rauhamaa H, Haahtela T: Mycotoxins in crude building materials from water damaged buildings. *Appl Environ Microbiol* 2000, **66**, 1899-1904.
34. Weidenbörner M: *Encyclopedia of Food Mycotoxins*. Springer-Verlag, Berlin 2001.