The effect of the intratracheal exposure of rats to secondary metabolites isolated from microfungi on lungs

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Abstract

The lung is a major target organ for insults induced by environmental pollutants. Following the effect of microfungal exposure relating to less studied topics, health complaints, particularly in mouldy houses, may be associated with microfungal exposure. The pulmonary health problems can be evoked both by inhalation of spores or their secondary metabolites. The fungal secondary metabolites represent a mixture of different active compounds and the mycotoxins may be a common part of them. Our study was focused on the effect of secondary metabolites produced by *Aspergillus versicolor*, a frequent indoor colonizer. The Wistar rats were intratracheally exposed to isolated metabolites produced by *A. versicolor* and after 3 days exposure the animals were sacrificed and the antioxidant status was estimated in lung tissue and bronchoalveolar lavage fluid (BALF). Alveolar macrophages and alveolar epithelial type II cells – from toxicological point of view the most important cells – were isolated and their antioxidant status and DNA damage were evaluated. The results did not show statistically significant changes of antioxidant status either in lungs or in the BALF, but the DNA damage was enhanced in both types of studied cells.
Keywords: lung, Aspergillus versicolor, metabolites, antioxidant status, DNA damage, sterigmatocystin.

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1 Introduction

During recent decades, surveys of the mould *Aspergillus versicolor* clearly show that it dominates in damp and mouldy indoor places (dwellings and public buildings) in Slovakia [1]. Since the inhalation route is the most possible route of entry into an organism, attention should be focused on the lungs. Commonly, the microfungi are recognized as a potential cause of health problems as their spores and secondary metabolites may generate inhalation problems. *Aspergillus versicolor* is able to synthesize the mycotoxin sterigmatocystin, which was detected by LC/MS-MS [2]. Inhalation exposure can evoke production of reactive oxygen species by the noxes themselves or by activated inflammatory cells. The lung has various protective mechanisms, including an antioxidant system designated to metabolize oxidants. The changes of antioxidant status or oxidative damage of DNA may point to some toxic effect of the inhaled material.

The presented study deals with the effect of intratracheal inhalation of secondary metabolites isolated from *A. versicolor.* The antioxidant status is evaluated in lung tissue, cell free bronchoalveolar lavage fluid and in isolated alveolar macrophages (AM) and alveolar epithelial type II (TII) cells, which belong to the toxicologically most important lung cells. The DNA damage was examined in the isolated cells.

2 Materials and methods

2.1 Animals

Male Albino Wistar rats (Velaz, Prague, Czech Republic) of about 240 g were used in these experiments. They were housed under standard laboratory conditions and were given a conventional laboratory diet (TOP-Dovo, Horné Dubové, Slovakia) and tap water *ad libitum.* The study was conducted with the approval of the Animal Committee of the Slovak Medical University and in accordance with the guidelines of the European Convention for the Protection of Vertebrate Animals for Experimental Purposes.

2.2 Schema of experiments

Aspergillus versicolor was isolated from mouldy houses and identified. After laboratory cultivation its metabolites were isolated: exometabolites from culture medium and endometabolites from mycelium by standard procedure [3]. The rats were randomly divided into 4 groups and intratracheally installed. The control group was installed with 0,2 ml of 0,2% DMSO in saline, other groups were installed with the same volume of 0,2% DMSO containing 4 μg exometabolites, 4 μg endometabolites and sterigmatocystine, respectively. The exposure lasted for 72 hours in the first experiment and in the second experiment the exposure was repeated in the same way and the same doses were installed for another 72 hours. After finishing the exposure the animals were exsanguinated in anaesthesia and bronchoalveolar lavage was performed [4]. The lavage fluid was

centrifuged and the cell free fraction was separated and used for analysis. The lung tissue was homogenized in PBS and the 10% homogenate was centrifuged (30 min, 10000 rpm). The analyses were done in supernatant. All samples were stored in aliquots at -80ºC till analysis. Alveolar macrophages and alveolar epithelial type II cells were isolated from intact animals by standard procedures [4] and [5], respectively.

2.3 Biochemical analysis

Superoxide dismutase (SOD) was estimated using the RANSOD kit (Randox Laboratories Ltd, UK), glutathione peroxidase activity (GSH-Px) was estimated using cumene peroxide as substrate [6], total glutathione (GSH) was determined using the GSH reductase method [7], ascorbic acid (AA) was estimated spectrophotometrically by 2,4-dinitrophenylhydrazine method [8] and protein by Lowry et al. [9].

Comet assay. For detection of genotoxic activity of fungal metabolites the alkaline single cell gel elecrophoresis was used [10] with modification [11, 12].

3 Results

The antioxidant status was evaluated by estimation of two antioxidant enzymes (GSH-Px and SOD) and two non-enzymatic antioxidants (GSH and AA) both in homogenate of lung tissue and in cell free fraction of bronchoalveolar lavage fluid (BALF). The results (means) were compared to the control group and are summarized in Figure 1 both for the single and repeated exposure. The results showed no statistically significant differences in the activity of studied enzymes either in lung tissue homogenate or in the BALF. Statistical significance was found in non-enzymatic antioxidants: GSH was enhanced after repeated exposure in lung tissue of the group exposed to exometabolites and decreased in BALF of the group exposed to sterigmatocystine. The most significant differences were seen in the level of AA both in tissue (single and repeated exposure) and BALF (only after repeated exposure).

As AA was the most affected parameter we compared its total amount (Figure 2) and distribution between tissue and BALF (Figure 3). The total amount of AA was statistically significant enhanced after both type of exposure to A. versicolor endometabolites and after repeated exposure to exometabolites ($P<0.05$ in all cases). The distribution between lung tissue and BALF was changed after single exposure to endometabolites and after repeated exposure to exometabolites.

The same parameters of antioxidant status except for AA were estimated in isolated AM and TII cells. There were not found any statistically significant differences between exposed and control groups.

The oxidative damage of DNA was followed by comet assay in both isolated cell types. The results are shown in Figure 4. The percentage of tail DNA was significantly enhanced, TII cells showed greater sensitivity compared to AM, especially to endometabolites. The enhancement after exposure to exometabolites was three times higher in TII than in AM and after exposure to endometabolites even about 30 times.

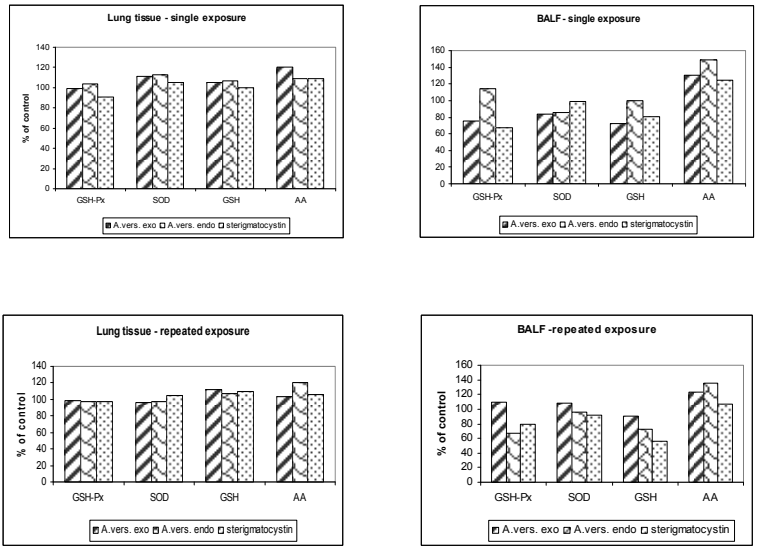

Figure 1: Antioxidant status in lung tissue and cell free bronchoalveolar lavage fluid after single and repeated intratracheal exposure to secondary metabolites produced by *Aspergillus versicolor.*

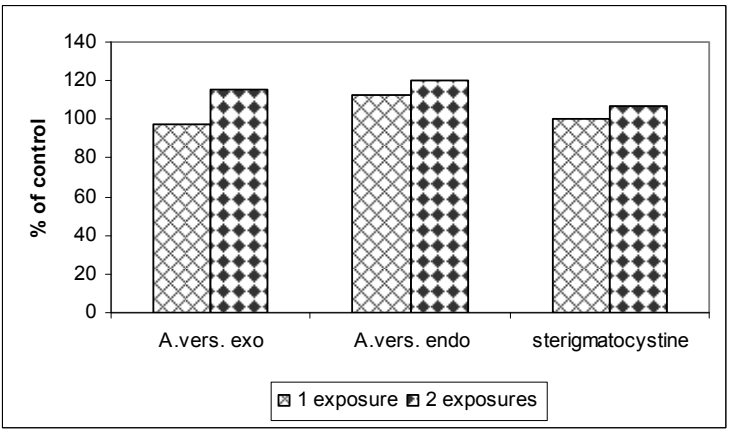

Figure 2: Total amount of ascorbic acid in lungs after intratracheal exposure to tometabolites produced by *Aspergillus versicolor.*

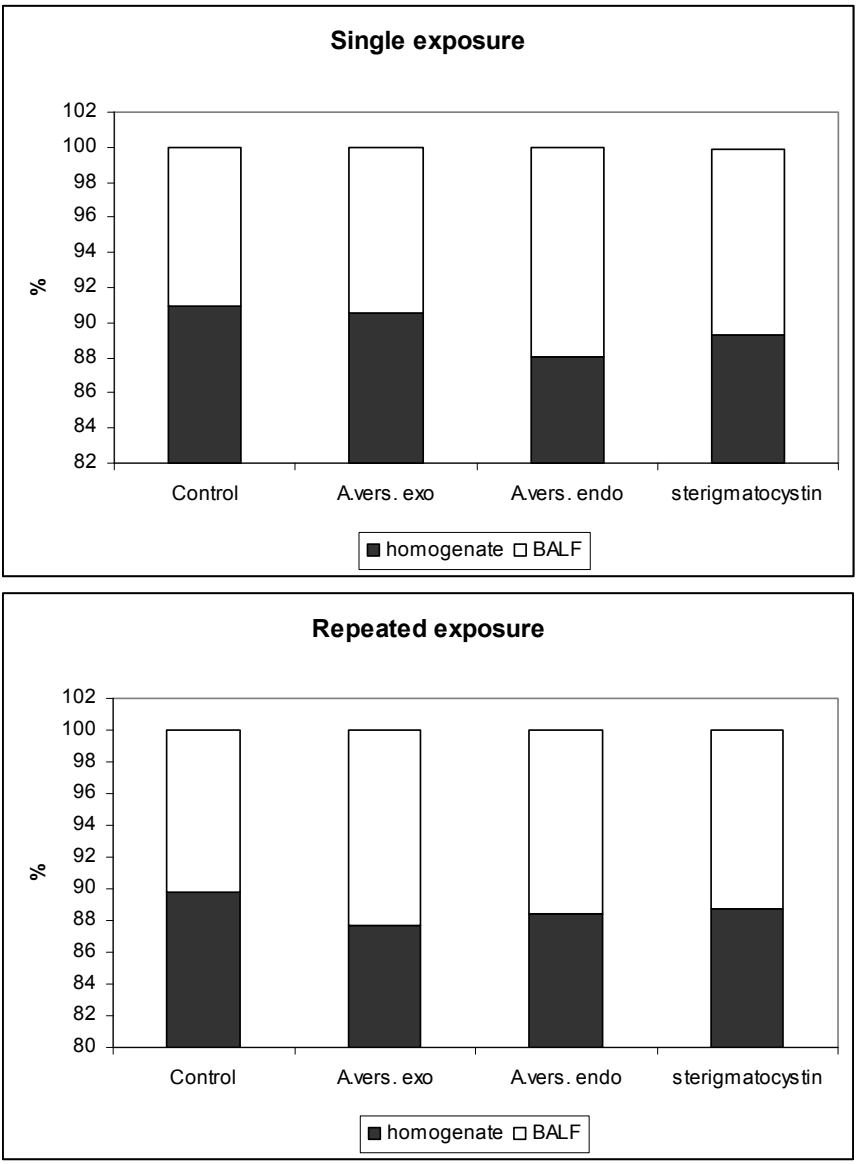

Figure 3: Distribution of ascorbic acid between lung tissue and cell free bronchoalveolar lavage fluid after exposure to metabolites of *Aspergillus versicolor*.

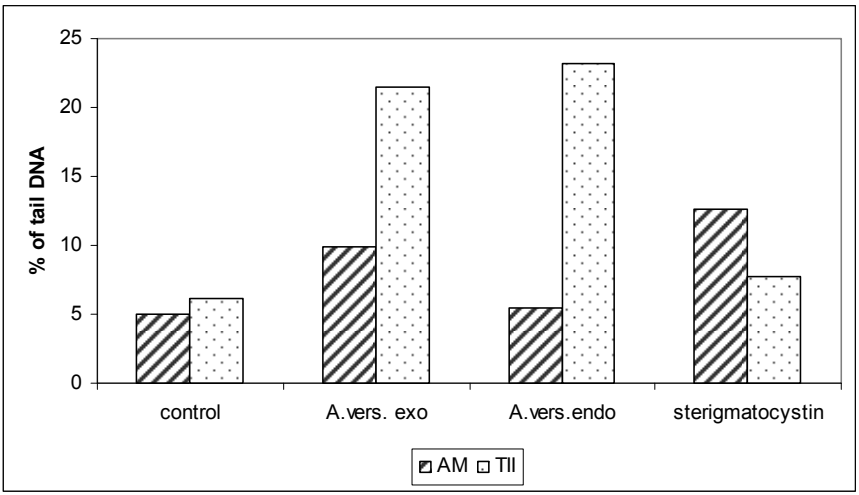

Figure 4: Comet assay in lung cells isolated from rats after exposure to metabolites produced by *Aspergillus versicolor.*

4 Discussion

Microbial growth in mouldy buildings can be associated with respiratory problems what may be caused both by spores and by produced secondary metabolites. The effect of *A. versicolor* was proved in experiments: the spores evoked acute inflammation in mouse lung [13] and the single inhalation exposure of mice to the extract provoked upper respiratory tract irritation [14].

Our previous *in vitro* studies with metabolites produced by microfungi showed toxic effects on primary culture of alveolar macrophages and alveolar epithelial type II cells isolated from rats. The aim of presented work was to study the effect of metabolites in *in vivo* experiment and compare the results. The metabolites produced by *Aspergillus versicolor* were chosen as it was the most frequently present indoor microfungi in Slovakia [1]. The rats were exposed intratracheally what is the common used model for *in vivo* inhalation exposure. The analysis of antioxidant status in lung tissue homogenate and BALF showed the most significant differences in the level of ascorbic acid and in its distribution between tissue and BALF. There was no difference in the activity of studied enzymes.

AM represent free cell population in alveolar spaces and their main role is to maintain clean and sterile alveoli. They are in direct contact with all inhaled agents. Type II cells cover about 5% of alveolar surface area and to their main role belong the re-establishing of epithelial surface after injury. They are thought to be progenitors of some types of tumours. These functions set them to the toxicologically most important lung cells.

The microfungi secondary metabolites exerted strong toxic effect of on both cell types in *in vitro* experiments, they differ only in the extent of induced

changes [15 and unpublished results]. The cells isolated from animals exposed *in vivo* showed no changes in antioxidant status in comparison to control cells isolated from control animals. The different reaction can be explained both by some protective mechanism what was not present in *in vitro* exposed system and by used concentration.

The most important result is the enhancement of DNA damage in isolated cells from exposed animals, especially in type II cells. *A. versicolor* produce a mixture of secondary metabolites and sterigmatocystin, mycotoxin with carcinogenic potency in animal models [16], is integral part of them. It was used in the experiment as positive control and enhanced the DNA damage but the enhancement evoked by endometabolites was even higher what may be caused by potentiation or additive effects of other metabolite.

Acknowledgements

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