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Scientific Paper

INFLAMMATORY AND HAEMATOTOXIC POTENTIAL OF INDOOR *STACHYBOTRYS CHARTARUM* (EHRENB.) HUGHES METABOLITES*

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Mould *Stachybotrys chartarum* (Ehrenb.) Hughes is known to pose a health risk in indoor environments. Most of its strains can produce several intra- and extracellular trichothecene mycotoxins. Complex secondary metabolites of stachybotrys isolates from mouldy dwellings/public buildings in Slovakia were intratracheally instilled in Wistar male rats (4 µg in 0.2 mL of 0.2 % dimethylsulphoxide; diacetoxyscirpenol as the positive control). After three days, haematological parameters were measured in peripheral blood and inflammatory response biomarkers in bronchoalveolar lavage fluid (BALF), and the results were statistically analysed. Exometabolites proved to suppress red blood cell (RBC), decreasing the total RBC count, haemoglobin, and haematocrit. The exposed rats showed significantly higher total BALF cell count, indicating inflammation, lower alveolar macrophage counts, and increased granulocyte count related to the BALF cells. Due to haematotoxic and inflammation-inducing properties, metabolites of *S. chartarum* can cause damage to the airways and haematological disorders in occupants of mouldy buildings.

KEY WORDS: *inflammation, intratracheal instillation, moulds, rats, red blood cells*

Indoor chemicals, viruses, bacteria (endotoxins of gram-negative in particular) or fungi can contribute to many so-called building-related illnesses, including allergic, infectious, toxic or inflammatory diseases, and may even lead to precanceroses. A combination of symptoms and general discomfort associated with staying in certain buildings is known as the sick building syndrome (SBS) (1). The role of fungi in the aetiology of SBS has not yet been fully clarified. Monitoring of exposure to indoor fungi is difficult because there are no standard methods to evaluate how indoor microclimate, outdoor ambient, and microscopic fungi affect each other. A general approach to studying how fungi affect humans includes immunosuppressive effects of beta-glucans from the fungal cell wall and since recently the toxic and irritative effects of mycotoxins and/or volatile organic compounds (2). Mycotoxins which act through food or skin can produce similar adverse effects if inhaled at one tenth of the dose taken through food (3).

Although occupational pulmonary mycotoxicoses have been reported as a consequence of inhaled microbial toxins through organic dust (4), there has been no clinical evidence of their association with inhaled indoor mycotoxins. These are usually produced by *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., *Trichoderma* spp. and cellulolytic *Stachybotrys chartarum* (5). In the fatal infant idiopathic pulmonary haemorrhage outbreaks reported in the USA in the 1990's, the moulds of *S. chartarum*

and Memnoniella echinata were isolated as possible culprits. Their toxic effects were confirmed *in vitro* and in laboratory animals. *S. chartarum* produced macrocyclic trichothecenes (stachybotryotoxins) and spirocyclic drimanes; the first were cytotoxic and immunosuppresive (*in vitro*) and the second caused inflammation and haemorrhages in the respiratory tract and intestines of laboratory animals. *M. echinata* produced griseofulvins with a genotoxic potential (6). *S. chartarum* isolated from cardboard and vinyl ceilings of Scandinavian schools and dwellings produced the cytotoxic trichothecene trichodermol (7). Exo- and endometabolites of *S. chartarum* isolated from the mouldy walls of a house in Bratislava, Slovakia, induced a pulmonary injury in rats exposed intratracheally (8). Our previous study with tracheal organ cultures of one-day-old chicks showed varying ciliostatic activity of chloroform extracts of fungal biomass obtained from pure isolates of indoor moulds *Penicillium chrysogenum, P. palitans, Trichoderma viride, Stachybotrys* sp., and *Aspergillus versicolor* from building materials (mineral wool, plasterboard, cardboard). Generally, extracts from growths on materials composed of finely divided cellulose were more active than those taken from growths on mineral wool (9).

Toxic fungal metabolites are found in micromycetal propagules (endometabolites), aerosol, detritus, or house dust (exometabolites) (10). It is highly probable that hyphal fragments, dust and small particles of ≤2 μm that can reach alveoli have the highest toxic potential. Most fungal spores do not get further than to the upper airways or bronchi at best, because of size, morphology, and the mode of propagation. This may be the reason why studies of the toxic effects of fungal spores do not reflect real exposure (11-13).

The aim of this study was to establish the short-term inflammatory potential of *S. chartarum* in the respiratory tract of rats intratracheally exposed to its metabolites. The second aim was to establish their effects on peripheral blood of these animals.

METHODS

Fungal metabolites

S. chartarum was cultivated on a liquid medium with 2 % of yeast extract and 10 % sucrose at 25 °C for 10 days. The biomass (endometabolites) and the cultivation medium (exometabolites) were extracted separately with chloroform; extracts were dried under vacuum and dissolved in 0.2 % dimethylsulphoxide (DMSO; Merck, Darmstadt, Germany) to 20 μg mL^{-1} (14).

Animal treatment and bronchoalveolar lavage

Groups of six male Wistar rats (Velaz, Prague, Czech Republic) weighing about 200 g (young adults) were exposed per toxicant. Animal treatment followed the Guidelines of the European Convention for the Protection of Vertebrate Animals for Experimental Purposes (15). Four micrograms of metabolite dissolved in 0.2 mL of 0.2 % DMSO were intratracheally instilled to each rat. Animals in the control group received 0.2 mL of 0.2 % DMSO alone. Mycotoxin diacetoxyscirpenol (DAS; Sigma, St. Louis, USA) was used as positive control. After 3-days of exposure, the animals were killed by exsanguination under thiopental anaesthesia (150 mg kg^{-1} b. w.) Bronchoalveolar lavage (BAL) was performed according to Myrvik et al. (16); the trachea was cannulated and the lungs washed with 5 mL of saline *in situ* five times. The pooled bronchoalveolar lavage fluid (BALF) was centrifuged (450 *g* at 4 °C for 10 min), and BALF cells isolated (17). Lung proteins were measured with Folin's phenol reagent according to Lowry et al. (18).

Inflammatory response biomarkers

Parameters indicating lung tissue inflammation included total BALF cell count and alveolar macrophage (AM) count (both determined in the Bürker's chamber), AM to total cell ratio, monocyte count, binucleate cell count, and polymorphonucleate (PMN) cell count. All preparations were stained with May-Grünwald-Giemsa stain (17).

The phagocytic activity of AM was determined according to Fornůsek et al. (19), using 2-hydroxyethylmetacrylate particles (HEMA; Neosys, Prague, Czech Republic). Fifty millilitres of HEMA particles in phosphate buffered saline (PBS), pH 7.2, were added to 100 mL of BALF and incubated at 37 °C for 60 min and shaken at short intervals. After May-Grünwald-Giemsa staining, the cells which phagocytised at least three particles were considered positive.

AM viability was established by fluorescent microscopy of slides stained with 1 mL of 0.25 % erythrosine per 1 mL of cell suspension (density 1x10^6

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cells mL^{-1}). Viable cells were counted in the Bürker's chamber (18). The results were statistically evaluated using the Mann-Whitney U test.

Evaluation of haematotoxicity

Before killing the animals, we collected fresh peripheral blood samples by venipuncture into heparinised vacutainers. For blood analysis, we used fully automated haematology analyser Sysmex K-4500 (Sysmex Toa Medial Electronics Co., Japan). The analysis included red and white blood cell count, platelet count, haemoglobin, hematocrit, mean corpuscular volume, and lymphocyte and neutrophil percentage. Data were statistically evaluated using Student's *t*-test.

RESULTS AND DISCUSSION

Inflammatory activity of S. chartarum metabolites in animal lungs

Intratracheal instillation is a common method to study toxic effects of different substrates in animals *in vivo*. Bronchoalveolar lavage (BAL) is the best and quickest tool to screen for pulmonary toxicants, especially complex mixtures, even if their chemical structure is unknown. The method makes it possible to quantify markers of inflammatory and/or cytotoxic response. BAL is more sensitive than histological examination, and is also used in the diagnostics of pulmonary diseases (20, 21).

We selected day 3 of exposure to measure our inflammation and haematotoxicity parameters because earlier studies of *in vitro* (22, 23) and *in vivo* (8, 13) toxicity of *S. chartarum* have shown that metabolite/spore activity drops below the detection limit after the third day, as recovery starts to take over.

Rat autopsy in our experiment showed no changes in the relative lung mass (average 9.3x10^{-3} kg to 10.2x10^{-3} kg). Neither was the increase in total proteins in cell-free BALF statistically significant (Table 1). The most common protein in BALF was albumin. Its increase is considered to be an early indicator of inflammation, but it can also reflect porcine pulmonary oedema, and can be affected by the age and a chronic disease (5, 24). Unlike us, Rao et al. found significantly elevated albumin concentrations in BALF (13) as an early effect of *S. chartarum* spores on rodent lungs.

Toxicants in the lungs can cause several immediate reactions of the epithelial cells, including type II (T2) lung cells, such as increase in capillary permeability, white blood cell chemotaxis and extravasation into BALF, or fibrin deposition in the surrounding area. Kováčiková et al. observed concentration-dependent fragmentation of the T2 cell membrane exposed to *S. chartarum* metabolites (23). In this experiment, the total inflammatory cell count in BALF significantly increased after treatment with DAS, endometabolites, and exometabolites compared to DMSO (Figure 1).
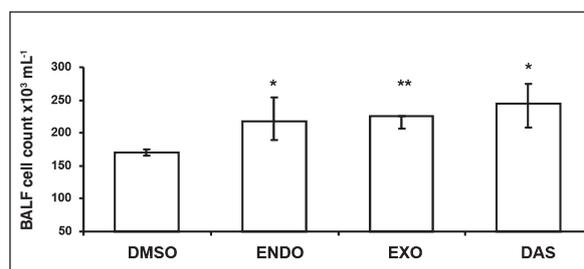

Figure 1 *Total cell count in bronchoalveolar lavage fluid (BALF) after exposure to carrier dimethysulphoxide (DMSO), endo- and exometabolites of S. chartarum, or diacetoxyscirpenol standard (DAS)*
*Values represent medians and 25th and 75th percentiles. * p< 0.05; ** p< 0.01*

Table 1 *Changes in lung weight and protein content in rats caused by S. chartarum exometabolites, endometabolites, diacetoxyscirpenol (DAS), and carrier dimethylsulphoxide (DMSO) after a 3-day intratracheal instillation*

Group	Relative lung weight g	Total amount of proteins in BALF mg
DMSO	9.7±0.9	1.95±0.16
Exometabolites	9.3±1.0	1.90±0.31
Endometabolites	9.4±1.1	1.92±0.22
DAS	10.2±1.3	1.87±0.18

BALF – bronchoalveolar lavage fluid
Values represent medians and standard deviations.

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AMs as cells of the first defence line have not only phagocytic, but also immunoregulatory properties. In addition, AMs play an important part in the inflammatory reaction; they stimulate inflammatory cells to secrete various cytokines, growth factors, and pro-inflammatory mediators. Changes in macrophage count or function can influence the lung injury and define the course of the disease. Lower macrophage count or phagocytic capacity may result in lower clearance of inhaled toxicants and thus in elevated effective dose. On the other hand, higher AM count may also increase the release of proteases, reactive oxygen species, and fibroblast-regulating mediators, which adversely affect the lung (25).

In our experiment, the number of AM in BALF was significantly lower in rats treated with DAS ($p<0.05$) and stachybotrys exometabolites ($p<0.01$) than with DMSO (Figure 2); this supports another study of the toxic effects of stachybotrys spores in rats (13). In normal rodent BALF, AMs account for more than 95 % of all white blood cells. Quite remarkably, the proportion of AMs in total BALF cells remained unchanged in our study (Figure 3). However, the significantly increased proportion of young AMs, which are an immature form of macrophages, in rats treated with exometabolites and DAS (both $p<0.01$) (Figure 4) suggest that they have replaced a number of mature AMs. The same may be true for polymorphonuclear cells (PMN), whose proportion also increased with DAS and all metabolite treatment, even though the increase was not statistically significant (Figure 5). Immature cells usually occur after exposure to inorganic dusts or due to an interstitial lung disease such as sarcoidosis (26). The significant increase in young AM proportion is probably related to significantly lower AM viability, which is either a secondary effect of inflammation or the consequence of mycotoxin cytotoxicity (27) (Figure 6).

AM phagocytic activity was not significantly affected by any of the toxicants used (Figure 7). This does not come as surprise because the toxicants were not particulate, and therefore did not induce phagocytosis.

PMNs form the second line of defence; they act as phagocytes and release vasodilators important in inflammation. Normally, they constitute less than 2 % of rodent BAL cells, but in acute inflammation their proportion may rise to over 90 %. PMNs are attracted to the lungs by chemokines secreted mostly
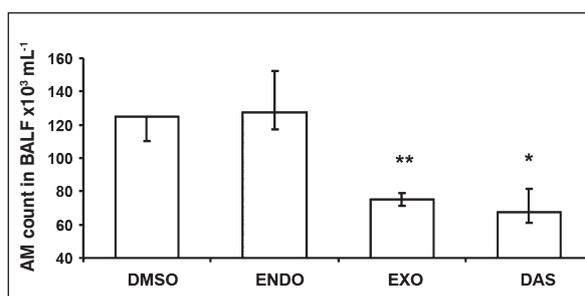

Figure 2 *Alveolar macrophage (AM) count in bronchoalveolar lavage fluid (BALF) after exposure to carrier dimethysulphoxide (DMSO), endo- and exometabolites of S. chartarum, or diacetoxyscirpenol standard (DAS)*
Values represent medians and 25th and 75th percentiles.
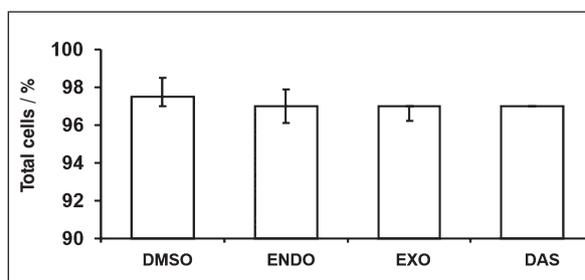** p< 0.05; ** p< 0.01*

Figure 3 *Proportion of alveolar macrophages (AM; % of total cells) in bronchoalveolar lavage fluid (BALF) after exposure to carrier dimethysulphoxide (DMSO), endo- and exometabolites of S. chartarum, or diacetoxyscirpenol standard (DAS)*
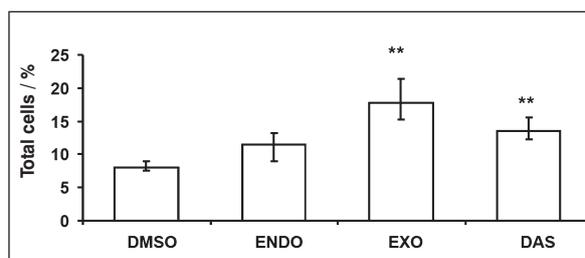*Values represent medians and 25th and 75th percentiles.*

Figure 4 *Proportion of monocytes (immature alveolar macrophages; % of total cells) in bronchoalveolar lavage fluid (BALF) after exposure to carrier dimethysulphoxide (DMSO), endo- and exometabolites of S. chartarum, or diacetoxyscirpenol standard (DAS)*
Values represent medians and 25th and 75th percentiles.
** p< 0.01

by AMs. If high levels persist for long, this may indicate a precancer (28). In our experiment, DAS and all stachybotrys metabolites increased the PMN proportion in BALF to 3 %, which this was not
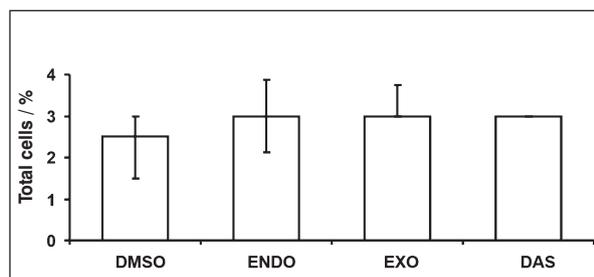
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Figure 5 *Proportion of polymorphonuclear cells (PMN; % total cells) in bronchoalveolar lavage fluid (BALF) after exposure to carrier dimethysulphoxide (DMSO), endo- and exometabolites of S. chartarum, or diacetoxyscirpenol standard (DAS)*
Values represent medians and 25th and 75th percentiles.
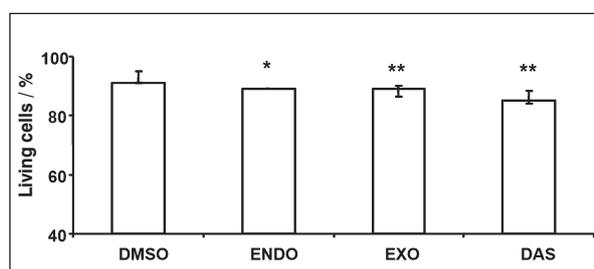

Figure 6 *Viability of alveolar macrophages (AM; % of living cells) after exposure to carrier dimethysulphoxide (DMSO), endo- and exometabolites of S. chartarum, or diacetoxyscirpenol standard (DAS))*
Values represent medians and 25th and 75th percentiles.
* p< 0.05; ** p< 0.01

Figure 7 *Phagocytic activity of alveolar macrophages (AM; % of phagocytic cells) after exposure to carrier dimethysulphoxide (DMSO), endo- and exometabolites of S. chartarum, or diacetoxyscirpenol standard(DAS)*
Values represent medians and 25th and 75th percentiles.
* p< 0.05; ** p< 0.01

Figure 8 *Proportion of binucleate cells (% of total cells) in bronchoalveolar lavage fluid (BALF) after exposure to carrier dimethysulphoxide (DMSO), endo- and exometabolites of S. chartarum, or diacetoxyscirpenol standard (DAS)*
Values represent medians and 25th and 75th percentiles.

significant in respect to DMSO (Figure 5). A similar PMN elevation was described in rats intratracheally exposed to fungal spores (13).

Binucleate cells (BNC) constitute about 1 % of a normal human lung tissue suspension and are often found in BALF. Their number may rise after long-term exposure to genotoxicants such as tobacco smoke, chemicals) (29-32) or certain dusts (33, 34). BNC and multinucleate cell (MNC) counts in lung suspension may well reflect chronic inflammation, and may serve as good semi-quantitative biomarkers (35). In our study, BNC proportion in BALF increased to almost 2 % in the rats exposed to exometabolites and DAS. However, these changes were not significant because the experiment took only three days and the inflammation was still acute (Figure 8).

Commercially available *Fusarium* spp. trichothecene mycotoxin DAS can cause inflammation and haemorrhage similar to the one caused by stachybotrys toxicants (37). The exometabolites of *S. chartarum* used in our experiments showed stronger inflammatory potential than endometabolites (Tables 2 and 3).
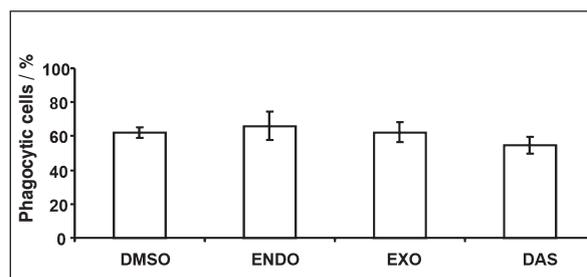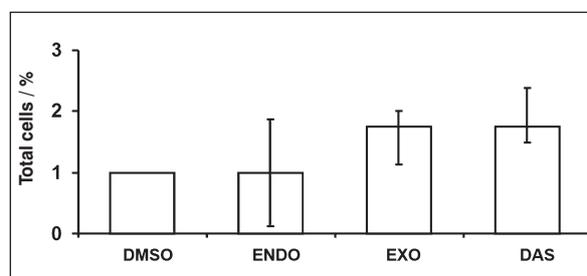
Table 2 *Comparison between groups exposed to S. chartarum endometabolites, and exometabolites*

Inflammation biomarkers	
BALF cell count	n
AM count in BALF	**↑
Proportion of AM in BALF	n
Proportion of PMN cells in BALF	n
Proportion of young monocytic AM forms in BALF	*↓
Proportion of binucleate cells in BALF	n
Viability of AM (% of living cells)	n
Phagocytic activity of AM (% of phagocytic cells)	n

BALF – bronchoalveolar lavage fluid; AM – alveolar macrophage; PMN – polymorphonuclear cells
*p< 0.05; **p<0.01; ↑ - increase against the compared group; ↓ - decrease against the compared group; n – no significant change;

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Table 3 *Comparison between groups exposed to S. chartarum endomatebolites, exometabolites, and diacetoxyscirpenol (DAS)*

Inflammation biomarkers	endome-tabolite	exome-tabolite
BALF cell count	n	n
AM count in BALF	*↑	n
Proportion of AM in BALF	n	n
Proportion of PMN cells in BALF	n	n
Proportion of young monocytiyc AM forms in BALF	n	n
Proportion of binucleate cells in BALF	n	n
Viability of AM (% of living cells)	n	n
Phagocytic activity of AM (% of phagocytic cells)	*↑	n

BALF – bronchoalveolar lavage fluid; AM – alveolar macrophage; PMN – polymorphonuclear cells
$*p< 0.05$; ↑- increase against the compared group
n – no significant change;

Haematotoxicity of S. chartarum metabolites

Rats exposed to stachybotrys exometabolites showed a statistically significant drop in RBC count ($p<0.01$), and in haemoglobin and hematocrit levels ($p<0.05$) (Figures 9 - 11). No significant changes were found in other haematological parameters in exposed rats. Our findings confirm reports in veterinary medicine, on equine stachybotryotoxicosis in particular (5). *S. chartarum* synthesises a haemolytic agent stachylysin, which lowers haemoglobin concentration, and some siderophore compounds which lower hematocrit. Stachylysin is produced by all strains of the fungus tested so far and is essentially specific to the species. It is highly immunogenic. Some scientists suggest that some flu-like and/or cold-like SBS symptoms in humans are caused by fungal haemolysins which activate histamine and cytokine producing cells. The effect of fungal haemolysins on the vascular tissue may in turn cause other symptoms such as headache, dizziness, and bleeding (37).

CONCLUSIONS

Our study has demonstrated the damaging potential of indoor *S. chartarum* metabolites to
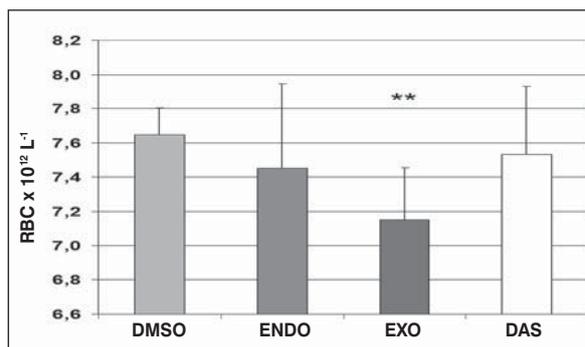
Figure 9 *Red blood cells (RBC) count in peripheral blood of rats exposed to carrier dimethysulphoxide (DMSO), endo- and exometabolites of S. chartarum, or diacetoxyscirpenol standard (DAS)*
Values represent means and standard deviations.
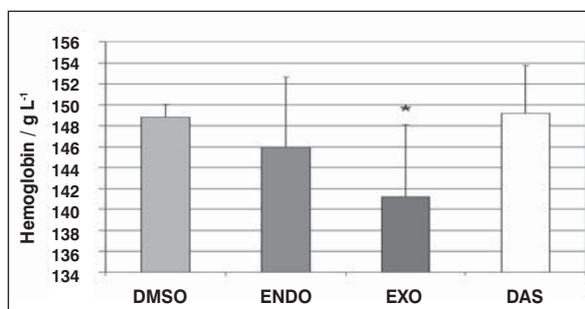*$** p< 0.01$.*

Figure 10 *Haemoglobin concentration in peripheral blood of rats exposed to carrier dimethysulphoxide (DMSO), endo- and exometabolites of S. chartarum, or diacetoxyscirpenol standard (DAS)*
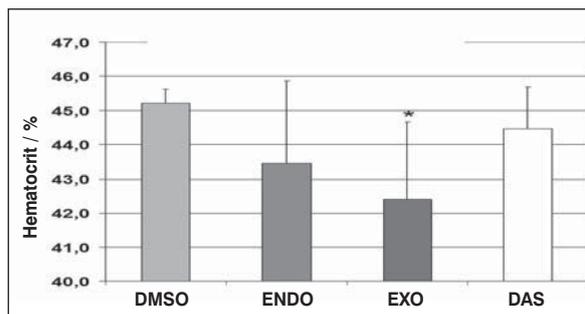*Values represent means and standard deviations.*
$* p< 0.05$

Figure 11 *Hematocrit in peripheral blood of rats exposed to carrier dimethysulphoxide (DMSO), endo- and exometabolites of S. chartarum, or diacetoxyscirpenol standard (DAS)*
Values represent means and standard deviations.
$* p< 0.05$

rodent lungs through inflammation and haematotoxic action. The toxicity was more pronounced with extracellular metabolites, which suggests that exotoxins cause greater adverse effects than fungal cell wall components. Even though data on real

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exposure to moulds in buildings are still inexact, their toxic potential should not be neglected. Future risk assessment studies should seek to assess exposure to indoor fungal toxins with as much precision as possible, especially to their real mixtures. Estimations should also include confounding factors such as endotoxins, chemicals, stress, and diet. Furthermore, knowledge of the dose-effect relationships and of fungal deposition in the lungs could help to better understand fungal contribution to the sick building syndrome.

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Sažetak

UPALNI I HEMATOTOKSIČNI POTENCIJAL METABOLITA PLIJESNI *STACHYBOTRYS CHARTARUM (*EHRENB.) HUGHES U ZATVORENIM PROSTORIJAMA

Plijesan *Stachybotrys chartarum* (Ehrenb.) Hughes poznata je kao rizični mikroorganizam u zatvorenim prostorijama. Većina njezinih sojeva može proizvesti nekoliko unutarstaničnih i izvanstaničnih trikotecenskih mikotoksina. Muškim Wistar štakorima instilirani su intratrahealno kompleksni sekundarni metaboliti stahibotrisa izolirani iz stambenih i javnih zgrada u Slovačkoj zahvaćenima plijesni (4 μg na 0,2 mL 0,2 %-tnog dimetilsulfoksida; dok se diacetoksiscirpenol rabio kao pozitivna kontrola). Tri dana kasnije izmjereni su hematološki parametri u perifernoj krvi te biopokazatelji upalnoga odgovora u bronhoalveolarnome ispirku te su rezultati obrađeni statistički. Pokazalo se da egzometaboliti suprimiraju eritrocite, smanjujući njihov ukupni broj, hemoglobin i hematokrit. Izloženi štakori imali su značajno veći broj stanica u bronhoalveolarnome ispirku, što upućuje na upalu, dok im je broj alveolarnih makrofaga bio manji, a broj granulocita povezanih sa stanicama u ispirku veći. Zbog svojih hematotoksičnih i upalnih svojstava *S. chartarum* može dovesti do oštećenja dišnih putova i poremećaja u krvotvornome sustavu osoba koje žive i/ili rade u zgradama zahvaćenima tom plijesni.

KLJUČNE RIJEČI: *eritrociti, intratrahealna instilacija, plijesni, štakori, upala*

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