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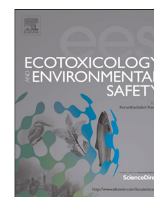

Cytotoxic and genotoxic potencies of single and combined spore extracts of airborne OTA-producing and OTA-non-producing Aspergilli in Human lung A549 cells

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ABSTRACT

Aspergillus sclerotiorum (AS) is a well-known producer of ochratoxin A (OTA) while *Aspergillus pseudoglaucus* (AP) produces a wide range of extrolites with poorly investigated toxicity. These species are frequently co-occur in grain mill aeromycota. The aim of this study was to determine OTA levels in spore extracts using HPLC and immunoaffinity columns, and to examine the cytotoxicity of pure OTA, OTA-positive (AS-OTA(+)) and OTA-negative (AS-OTA(−)) spore extracts, as well as of AP spore extract, on human lung adenocarcinoma cells A549, individually and in combination, using a colorimetric MTT test (540 nm). To establish which type of cell death predominated after treatments, a quantitative fluorescent assay with ethidium bromide and acridine orange was used, and the level of primary DNA damage in A549 cells was evaluated using the alkaline comet assay. OTA was detected in spore extracts (0.3–28 μg/mL) of 3/6 of the AS strains, while none of the tested AP strains were able to produce OTA. Taking into account the maximum detected concentration of OTA in the spores, the daily intake of OTA by inhalation was calculated to be 1 ng/kg body weight (b.w.), which is below the tolerable daily intake for OTA (17 ng/kg b.w.). Using the MTT test, the following IC_{50} values were obtained: single OTA (53 μg/mL); AS-OTA(+) (mass concentration 934 μg/mL corresponds to 10.5 μg/mL of OTA in spore extract); and 2126 μg/mL for AP. The highest applied concentration of AS-OTA(−) spore extract (4940 μg/mL) decreased cell viability by 30% and IC_{50} for the extract could not be determined. Single OTA and AS-OTA(+) and combinations (AP+AS-OTA(+) and AP+AS-OTA(−)) in subtoxic concentrations provoked significant primary DNA damage, apoptosis, and to a lesser extent, necrosis in A549 cells. Mixture of AP+AS-OTA(+) and AP+AS-OTA(−) in subtoxic concentrations showed dominant additive interactions. Despite the low calculated daily intake of OTA by inhalation, our results suggest that chronic exposure to high levels of OTA-producing airborne fungi in combination with other more or less toxic moulds pose a significant threat to human health due to their possible additive and/or synergistic interactions.

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1. Introduction

Indoor air quality is one of the most important health issues in general; we spend about 80% of our time indoors and breath between 11 and 15 m^3 of air per day (Dacarro et al., 2003; Kelman et al., 2004). Apart from chemical air pollution and tobacco smoke, microorganisms as well as airborne fungal fragments may have a role in the initiation of both acute and chronic respiratory diseases in exposed occupants (Gorny, 2004). These respiratory problems may vary from allergic inflammatory reactions mediated by IgE, systemic reactions and release of lung cytokines to non-specific inflammation provoked by cytotoxicity (Pieckova, 2012). Relationship between exposure to indoor airborne fungi and development of chronic respiratory diseases is not fully clarified but it is generally accepted that such exposure may cause allergies such as fungal rhinitis, hypersensitivity pneumonia, and/or asthma (reviewed in Cabral, 2010; Pieckova, 2012). Inhaled mycotoxins in fungal spores have also been linked to adverse health effects after chronic exposure in non-agricultural indoor environments (Kelman et al., 2004). However, epidemiologic studies and dose-

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response data concerning inhaled mycotoxins are lacking (Kelman et al., 2004). Some exposure estimations of mycotoxins inhaled through fungal spores have shown that the risk of developing respiratory toxicity is insignificant (Kelman et al., 2004).

On the other hand, agricultural facilities can be highly contaminated with fungal spores and small fragments containing mycotoxins, which might represent a serious threat to those in contact with such facilities (Hintikka and Nikulin, 1998; Adhikari et al., 2004). Several reports support that hypothesis mainly considering inhalatory exposure to aflatoxins. The link between inhalation of aflatoxin in the industrial setting and cancer incidence and mortality in oil-press workers has been suggested by Hayes et al. (1984). Dvorackova and Pichiova (1986) reported on the role of aflatoxin B_1 (AFB_1) in pulmonary interstitial fibrosis in agricultural and textile workers. According to McLaughlin et al. (1987), AFB_1 exposure contributed to the onset of primary liver cancer in grain mill workers. In one of the more recent studies (Viegas et al., 2012), the inhalation of aflatoxins in poultry farms was confirmed as a significant occupational risk factor that could contribute to the development of primary liver cancer. About 60% of the exposed workers had detectable levels of AFB_1 in serum, while AFB_1 was not detected in control subjects. Ochratoxin A (OTA) is a well-known nephrotoxic, immunotoxic and carcinogenic mycotoxin mainly considered a health hazard due to OTA-contaminated food consumption (Reddy and Bhoola, 2010). However, inhalatory exposure to OTA could also be a serious threat to health. According to Hooper et al. (2009), elevated concentrations of OTA have been detected in the urine of occupants in water-damaged buildings, while OTA was below the detection limit in non-exposed subjects. Hope and Hope (2012) reviewed two cases of focal segmental glomerulosclerosis attributed to OTA inhalation in damp dwellings. In both cases, the urine samples contained elevated levels of OTA while the aflatoxin and trichothecene mycotoxin test was negative. The most severe case that has been linked to inhalation of OTA is acute renal failure described in workers exposed to *Aspergillus*-producers of OTA in a granary (Dipaolo et al., 1994). In agricultural facilities, workers are exposed to a mixture of airborne fungi and their toxic metabolites which might interact synergistically. A recent year-round study conducted in 2012 in a grain mill, apartments, and basements (Zagreb, Croatia) showed that the calculated average concentration of airborne fungi in the grain mill was about 160 times higher (up to 250,000 cfu/m^3) than in other locations (up to 1500 cfu/m^3) (Jakšić Despot and Šegvić Klarić, 2014). Among the dominant airborne fungi (*Aspergillus* and *Penicillium*), Aspergilli from sections *Circumdati* and *Aspergillus* (formerly *Eurotium*) (McNeill et al., 2012) were constantly present in the grain mill samples. Aspergilli belonging to section *Circumdati* are well-known producers of OTA (Frisvad et al., 2004), while Aspergilli formerly known as *Eurotium* spp. produce a wide range of metabolites with poorly investigated toxicity (Nielsen, 2002; Slack et al., 2009). Since these Aspergilli were frequent in the mixture of airborne fungi in grain mill (Jakšić Despot and Šegvić Klarić, 2014), it was justified to explore the cytotoxic and genotoxic potential of single and combined spore extracts of dominant species using human lung adenocarcinoma A549 cells as an experimental model.

2. Materials and methods

2.1. Chemicals

Malt Extract Agar (MEA) and Malt Extract were obtained from Oxoid (Basingstoke, UK), Bacto Peptone was purchased from Difco (Franklin Lakes, NJ, USA) and Masterpure™ yeast DNA purification kit was from Epicentre Biotechnologies (Madison, WI, USA). Methanol (HPLC grade) was obtained from Merck (Darmstadt, Germany). RPMI 1640, foetal bovine serum (FBS), trypsin-EDTA, phosphate buffered saline (PBS; Ca^{2+} and Mg^{2+} free), penicillin and streptomycin were purchased from Gibco-Invitrogen (Paisley, UK). OTA standard, MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], ethidium bromide, acridine orange, agarose normal melting point (NMP), agarose low melting point (LMP), Triton X-100, Tris buffer, and dimethyl sulfoxide (DMSO), were purchased from Sigma-Aldrich (Deisenhofen, Germany). All other chemicals including $MgCl_2$, HCl, acetic acid, isopropanol, NaCl, Na_2EDTA, and NaOH were from Kemika (Zagreb, Croatia).

2.2. Isolation of airborne fungi

In a recently published year-round study of airborne fungi in a grain mill near Zagreb, Croatia (Jakšić Despot and Šegvić Klarić, 2014) from samples taken in November 2012 (N=20), Aspergilli were reisolated on Czapek Yeast Agar (CYA, Pitt and Hocking, 2009) and Malt Extract Agar (MEA) and incubated at 25 °C in the dark for seven days. Morphological identification was carried out according to Pitt and Hocking (2009). The strains of *Aspergillus* spp. from sections *Circumdati* and *Aspergillus* that dominated in the samples were subjected to sequence-based identification as described below.

2.3. Species identification

The cultures used for sequence analysis were grown on Malt Peptone (MP) broth using 1% (w/v) of Malt Extract and 0.1% (w/v) Bacto Peptone. The cultures were incubated at 25 °C for 3 days. DNA was extracted from the cells using the Masterpure™ yeast DNA purification kit according to the instructions of the manufacturer. Part of the calmodulin gene was amplified and sequenced as described previously (Hong et al., 2005, Varga et al., 2007, Visagie et al., 2014). Briefly, part of the calmodulin gene was amplified using primers cmd5 (5′-CCGAGTACAAGGARGCCTTC) and cmd6 (5′-CCGATRGAGGTCATRACGTGG-3P′) using 35 PCR cycles with an annealing temperature of 56 °C. Sequencing reactions were performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit and carried out for both strands. All the sequencing reactions were purified by gel filtration through Sephadex G-50 (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in double-distilled water and analysed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The complementary sequences were corrected with the MT Navigator software (Applied Biosystems). Sequence homology analyses were performed by nucleotide–nucleotide BLAST similarity search at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST), and sequences were also compared with our own sequence database. Species identification was carried out from the lowest expect value of the BLAST output (Altschul et al., 1990).

2.4. Preparation of spore extracts

Each strain of *A. sclerotiorum* (AS, N=6) and *A. pseudoglaucus* (AP, N=6) as well as *A. westerdijkiae* NRRL 3174 (OTA producing strain as positive control) were grown on 5 Dichloran 18% Glycerol agar plates (DG18, Pitt and Hocking, 2009) for 15 days at 25 °C. Spores were harvested from the surface of each plate with 5 mL of methanol/water (1:1) (HPLC grade) and transferred into weighed polystyrene centrifuge tubes. Harvested spores were counted under the microscope (magnification 400×) using a haemocytometer. Afterwards, spore extracts were prepared by sonication (3 times) at 15 kHz for 90 s, centrifuged 20 min at 6000 rpm and the extracts were evaporated to dryness under a current of nitrogen.

Prior to HPLC analysis, extracts were weighed and dissolved in methanol (HPLC grade). To collect the necessary OTA concentrations in spore extracts so that experiments on A549 cells could be performed, *A. sclerotiorum* strain that produced highest concentration of OTA was grown on 20 DG18 agar plates. Spores were harvested as described above and evaporated spore extracts of OTA-producing *A. sclerotiorum* were pooled and dissolved in 10 mL prior to HPLC analysis. The final concentration of OTA in the pooled extract was 366 μg/mL. The extract was evaporated and dry extract was weighed (400 mg) and dissolved in 1 mL DMSO. This extract (400 mg/mL) was used as stock solution for cytotoxicity and genotoxicity testing on A549 cells in which the OTA concentration was calculated to be 3660 μg/mL. Dry extracts of OTA-non-producing Aspergilli (60 mg) were dissolved in 150 μL of DMSO and the final concentration 400 mg/mL was used in cytotoxicity and genotoxicity testing.

2.5. HPLC analysis

OTA was separated from samples using an immunoaffinity column (IAC, Ochratest, Vicam, Watertown, MA, USA). One milli litre of sample was applied onto an IAC column. The column was washed with 10 mL solution of $MgCl_2$ and HCl (1:1, v/v) (0.05 M HCl and 0.05 M $MgCl_2$) and 10 mL HPLC-grade water. OTA was eluted from IAC column using 1.5 mL of methanol. Eluate was evaporated to dryness under a steam of nitrogen and dissolved in 200 μL of mobile phase. The HPLC apparatus consisted of an isocratic pump, autosampler and fluorescent detector (Shimadzu Corp., Kyoto, Japan). The separation was performed as described previously (Flajs et al., 2009) using an analytical column (125.0×4.0 mm^2) coupled with guard column (4.0×4.0 mm^2) LiChrospher RP-18 (Merck, Darmstadt, Germany) with 5 μm particles. Chromatographic results were collected and processed using the LC Solution software (Shimadzu Corp., Kyoto, Japan). The mobile phase consisted of methanol, water, and acetic acid (70:30:2) with a flow rate of 0.5 ml/min. Detector wavelengths were set at λ_{ex} 336 nm and λ_{em} 464 nm. The limit of detection was 0.05 ng/mL.

Estimation of daily inhaled dose of OTA

Using the data obtained by HPLC analysis of OTA in spore extracts, we calculated the daily inhaled dose of OTA applying the equation (Kelman et al., 2004):

$$D = \frac{COTA\ x\ N\ x\ BR\ x\ FR\ x\ BA}{WT}$$

where D is the daily dose of OTA (μg/kg), C is the concentration of OTA (μg) per spore, N is the concentration of spores (cfu/m^3), BR is the breathing rate with default value for adult male (15.2 m^3/day), FR is the fraction of retained spores and BA is OTA bioavailability both assumed to be 100% (or 1), WT is the default value of body weight of adult male (78.1 kg).

2.6. Cell culture and treatment

Human lung cancer cells A549 (European Collection of Cell Cultures, United Kingdom) were grown in 75 cm^2 flasks in RPMI supplemented with 2 mM glutamine, 10% heat-inactivated FBS, penicillin (100 IU/mL; 1 IU ≈ 67.7 μg/mL) and streptomycin (100 μg/mL) at 37 °C in 5% CO_2. A stock solution of OTA (2.5 mg/mL) was prepared in absolute ethanol, while spore extracts were dissolved in 100% DMSO. The final concentrations of OTA and spore extracts as well as vehicles (ethanol or DMSO) were obtained by dilution with the culture medium.

2.7. MTT proliferation assay

MTT proliferation test was used to test cell viability of A549 cells upon 24 h of treatment with either of OTA, spore extracts of OTA-producing AS (AS-OTA(+)), OTA-nonproducing AS (AS-OTA (−)), AP or combinations of AS and AP spore extracts (AP+AS-OTA (+) and AP+AS-OTA(−)). A549 cells were plated in a 96-well flat-bottomed microplate (10^4 cells per well). Following 36-h incubation, growth medium was replaced by the medium without FBS and cells were incubated for the 12 h. To determine the concentration that inhibits growth in 50% of cells (IC_{50}), A549 cells were treated for 24 h with OTA (from 0.2 to 80 μg/mL) and AS or AP spore extracts (92 to 4940 μg/mL). Concentrations of OTA in AS-OTA(+) spore extract dilutions (92–4940 μg/mL) were calculated to be 1–56 μg/mL. Cells in control were treated with up to 1.5% of absolute ethanol or 1.5% of DMSO, which did not alter cell viability. Following the treatment, the medium was removed and 100 μl of MTT reagent diluted in growth medium without FBS (0.5 mg/ml) was added in each well. Viable cells have active mitochondrial dehydrogenase enzymes that metabolize MTT-tetrazolium salt into purple formazan. After 3.5 h of incubation, the MTT reagent was replaced with 150 μl of DMSO to dissolve formazan, and cells were incubated at room temperature on a rotary shaker for 15 min. The absorbance was measured on a microplate reader (Labsystem iEMS, type 1404) at a wavelength of 540 nm. All tests were performed in six replicates and results are expressed as percentage of control.

Once the IC_{50} values were determined, the subcytotoxic concentrations of spore extracts (92 and 280 μg/ml of AS-OTA(+) or AS-OTA(−) and 280 μg/ml of AP) were selected for testing their combined cytotoxicity using the described MTT test procedure.

2.8. Quantitative fluorescent assay for identification of apoptotic and necrotic cells

Cells were seeded in 6-well plates (3×10^5 cells/mL) and treated for 24 h with subcytotoxic concentrations of OTA (1 and 3 μg/ml) and single or combined spore extracts (92 and 280 μg/ml of AS-OTA(+) or AS-OTA(−) and 92 μg/ml of AP), which did not reduce cell viability more than 30%. Prior to OTA and spore extracts addition, cells were incubated in serum-free media for 12 h. Control cells were exposed to 0.1% of absolute ethanol or to 0.2% DMSO for 24 h. After the treatment, the medium was removed. Using a cell scraper, the cells were gently scraped off the bottom of the microplates, resuspended in PBS, transferred into an Eppendorf tube and centrifuged at 1200 rpm for 2 min. Aliquots of cell suspension (V=10 μL) were pipetted, put on microscope slides and mixed (1:1; v/v) with ethidium bromide (100 μg/ml) and acridine orange (100 μg/ml). The preparation was covered with a coverslip and immediately examined under a fluorescence microscope (Olympus BX 51; 400× magnification; Olympus, Tokyo, Japan). Quantitative assessments were made by determination of the percentage of viable, apoptotic and necrotic cells. According to the dye exclusion method (Duke and Cohen, 1992), viable cells with intact plasma membrane excluded ethidium bromide and the appearance of their nuclei with an intact structure was bright green. Non-viable necrotic cells had orange to red coloured chromatin with organized structure, while apoptotic cells were bright green with highly condensed or fragmented nuclei. Three tests with aliquots of the same sample were performed and a total of 300 cells per sample were counted.

2.9. Alkaline comet assay

Cells were treated as described in the previous section. The comet assay was carried out according to Singh et al. (1988) with

minor modifications. After 24 h of treatment, the cells were washed with 1 mL cold phosphate buffer saline (PBS, pH 7.4), scraped with rubber, and resuspended in 300 µL of PBS. Aliquots of 20 µL of this suspension were mixed with 100 µL 0.5% low melting point agarose-LMP (in Ca- and Mg-free PBS), and 100 µL of agarose-cell suspension was spread onto a fully frosted slide pre-coated with 1% normal melting point agarose-NMP (in sterile destiled water). The slides were allowed to solidify on ice for 10 min. After 1 h of lysis at 4 °C in a mixture of 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris (pH 10) supplemented with 1% Triton-X, the slides were placed in denaturation and electrophoresis buffer (10 mM NaOH, 200 mM Na_2EDTA, pH 13), incubated for 20 min, and electrophoresed for 20 min at 25 V and 300 mA. DNA was neutralized with a neutralization solution (0.4 M Tris/ HCl, pH 7.5) three times 5 min each. The slides were kept in a humid atmosphere in a dark box at 4 °C until further analysis. For image analysis, DNA was stained with 100–250 µL ethidium bromide solution (20 µg/mL) per slide for 10 min. Slides were scored using an image analysis system (Comet assay IV, Perceptive instruments Ltd., UK) connected to a fluorescence microscope (Zeiss, Germany). All experiments were performed in duplicate, and in each experiment images of 200 randomly selected cells (100 cells from each of the two replicate slides) were measured. Only comets with a defined head were scored. As a reliable measure of genotoxicity, we used the percentage of DNA in the comet tail (or tail intensity), which is the most suitable indicator of DNA damage (Olive, 1999; Collins, 2004).

2.10. Statistics

The results of the MTT test were statistically analysed by one-way analysis of variance (ANOVA) followed by a multiple comparison procedure (Tukey's test). To obtain IC_{50} from results of MTT assay linear regression analysis was applied. Comparisons between values obtained for the cytotoxicity (apoptosis and necrosis) in the treated and control samples were made by Pearson's χ^2 test for two-by-two contingency tables. The Kolmogorov–Smirnov test was used to verify whether the results of comet parameters were normally distributed. Kruskal–Wallis followed by Dunn's multiple comparison test was used for statistical analysis of tail intensity. The level of $P<0.05$ was considered statistically significant for all statistical calculations. We also compared our measured values with the expected values that were calculated as mean and SEM values obtained after exposure to combined spore extracts; *e.g.* mean (expected for AS-OTA(+)+AP)=mean (AS-OTA (+))+mean (AP)-mean (control); SEM (expected for AS-OTA(+)+AP)=[(SEM for AS-OTA(+))^2 +(SEM for AP)^2]$^{1/2}$ (Weber et al., 2005).

The significance of difference between the expected and measured values was calculated for cell viability, comet tail intensity, apoptosis and necrosis using an unpaired *t*-test. The results were interpreted as follows: (1) an additive effect was recorded if the measured values were not significantly above or below the expected values in all tests; (2) in the MTT assay, synergism was recorded if the measured values were significantly below the expected values, while in comet assay the synergism was recorded if the measured values were significantly above the expected values; (3) in the MTT assay, an antagonistic effect was recorded if the measured values were significantly above the expected values, while in the comet assay antagonism was recorded if the measured values were significantly below the expected values.

3. Results

3.1. OTA in spore extracts of airborne Aspergilli: estimation of daily inhaled OTA

Aspergilli from the sections *Aspergillus* and *Circumdati* were were found in 100% and 35% of samples, respectively and were among the dominant airborne fungi detected in the grain mill in November. Based on the sequenced calmodulin gene region, the Aspergilli of interest were *A. pseudoglaucus* and *A. sclerotiorum* (Table 1). Three of the six isolated *A. scleorotiorum* strains produced OTA (up to 28 µg/mL) in the spores, while none of the *A. pseudoglaucus* were able to produce OTA. The OTA-positive *A. westerdijkiae* NRRL 3174, which served as positive control, produced OTA in a concentration of 0.38 µg/mL of spore extract. Using the data obtained in this study, we calculated the daily inhaled dose of OTA (D). To calculate D, we used the maximum concentration of OTA detected in the spore extracts of *A. sclerotiorum* (28 µg/mL), average spore count per one mL of methanol/water extract (2×10^6 spore/mL) and maximum concentration of airborne Aspergilli (*Circumdati*) detected in grain mill in November. Applying these values to the equation, the daily inhaled dose of OTA was calculated to be 1 ng/kg of b.w.:

$$D = \frac{COTA\ x\ N\ x\ BR\ x\ FR\ x\ BA}{WT} = \frac{\frac{28\ \mu g/mL}{2x106\ spore/mL}\ x\ 400\ cfu/m3\ \ x\ 15.2\ m3/day\ x\ 1\ x\ 1}{78.1 kg} = 1\ ng/kg$$

3.2. Cytotoxicity of OTA and Aspergillus spore extracts

Figs. 1 and 2 show the viability of A549 cells upon 24 h-treatment with OTA and Aspergilli spore extracts, respectively. OTA

Table 1
Occurrence of two OTA-producing and OTA-non-producing *Aspergillus* species in samples of airborne fungi taken in November in a grain mill (Zagreb, Croatia)

Aspergillus species	Occurrence in the samples (%)[a]	Concentration of Aspergilli (cfu/m^3)[b]			Determined Aspergilli (*n*/*N*=6)[c]	OTA in spore extracts (µg/mL)
		Min	Max	Median		
Aspergillus spp.(section *Aspergillus*)	100	100	5500	1155	*A. pseudoglaucus* (0/6)	n.d.
Aspergillus spp.(section *Circumdati*)	35	60	400	95	*A. sclerotiorum* (3/6)	0.3; 6.5; and 28

n – number of OTA positive strains;
N – number of analysed strains
; n.d. – not detected

[a] 20 samples we re collected in the grain mill.
[b] Based on traditional identification.
[c] Calmodulin gene sequencing.

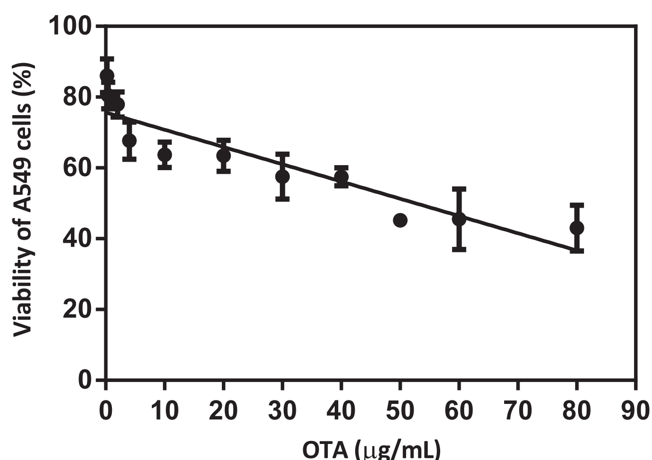

Fig. 1. Cytotoxicity of OTA in A549 cells after 24 h of exposure to concentrations ranging from 0.2 to 80 μg/mL. Data are expressed as means ± SEM% of control cells viability of six independent experiments. Regression coefficient was 0.78. Control cells were exposed to ethanol (1.5%) only, and value was taken as 100%.

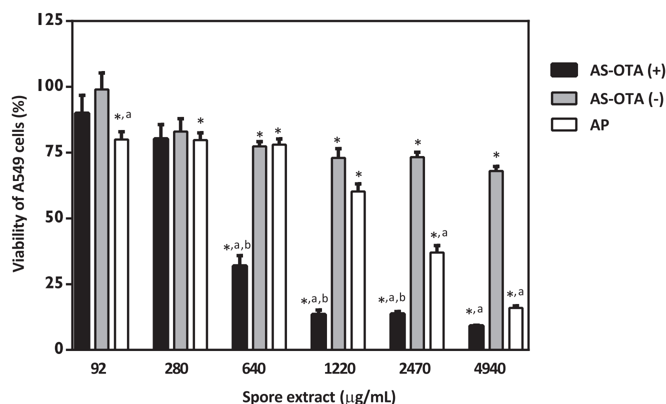

Fig. 2. Cytotoxicity of OTA-producing (AS-OTA(+)) and OTA-non-producing (AS-OTA(−)) *A. sclerotiorum*, and *A. pseudoglaucus* (AP) spore extracts in A549 cells after 24 h of exposure to mass concentrations ranging from 92 to 4940 μg/mL. Concentration of OTA in AS-OTA(+) spore extract is calculated to be 1, 3, 7, 14, 28 and 56 μg/mL. Data are expressed as means ± SEM % of control cells viability of six independent experiments. Control cells were exposed to DMSO (1.5%) only, and value was taken as 100%. Significantly different values ($P<0.05$) are marked as follows: * as compared to the control value; a- AS-OTA(+) and AP *vs* AS-OTA(−); b- AS-OTA(+) *vs* AP.

decreased cell survival in a concentration-dependant manner and viability significantly dropped (to 80%) after treatment with 0.4 μg/mL. The IC_{50} for OTA obtained by MTT was 53 μg/mL. Spore extracts of both OTA-producing and OTA-non-producing Aspergilli

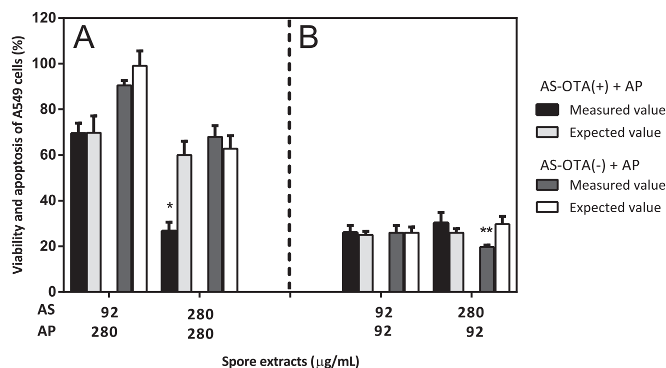

Fig. 3. Viability (A) and apoptosis (B) of A549 cells after combined treatment with AS-OTA(+) and AP as well as AS-OTA(−) and AP. * Represents significant synergistic effect and ** represents significant antagonistic effect ($P<0.05$).

Table 2
Cytotoxicity of OTA and *A. sclerotiorum* and *A. pseudoglaucus* spore extracts represented as rate of apoptotic and necrotic A549 cells.

Experimental groups	% of damaged cells (Mean ± SEM)		
	Σ	Apoptosis	Necrosis
Control	10.3 ± 0.9	7.0 ± 1.0	3.3 ± 0.3
OTA 1 μg/mL	27.7 ± 1.9[*]	16.7 ± 1.2[*]	11.0 ± 1.7[*]
OTA 3 μg/mL	33.7 ± 3.4[*]	24.7 ± 2.3[*,a]	9.0 ± 1.7[*]
AP 92 μg/mL	20.0 ± 2.1[*]	11.7 ± 1.2	8.3 ± 0.9[*,b]
AS-OTA(+) 92 μg/mL	25.0 ± 0.6[*]	20.3 ± 0.7[*,b]	4.7 ± 0.3
AS-OTA(−) 92 μg/mL	27.3 ± 2.2[*,b]	21.3 ± 2.2[*,b]	6.0 ± 0.0
AS-OTA(+) 280 μg/mL	28.0 ± 1.7[*]	21.3 ± 1.2[*]	6.7 ± 1.5
AS-OTA(−) 280 μg/mL	30.0 ± 1.5[*]	25.0 ± 3.2[*]	5.0 ± 1.7
AS-OTA(+) 92 μg/mL+AP 92 μg/mL	29.7 ± 4.5[*,b]	26.0 ± 3.1[*,b]	3.7 ± 1.5
AS-OTA(−) 92 μg/mL+AP 92 μg/mL	25.3 ± 2.7[*]	20.0 ± 3.2[*,b]	5.3 ± 1.5
AS-OTA(+) 280 μg/mL+AP 92 μg/mL	37.7 ± 5.4[*,b,c,d]	30.3 ± 4.5[*,b,c,d]	7.3 ± 0.9[*]
AS-OTA(−) 280 μg/mL+AP 92 μg/mL	25.7 ± 0.7[*]	19.7 ± 0.9[*,b]	6.0 ± 1.0

Significantly different values ($P<0.05$) are marked as follows:
[*] as compared to control.
[a] OTA 1 μg/mL *vs* OTA 3 μg/mL.
[b] AP 92 μg/mL *vs* AS-OTA(+) 92 μg/mL, AS-OTA(−) 92 μg/mL, mixtures of AS+AP (92+92 μg/mL) and AS-OTA(−)+AP (280+92 μg/mL).
[c] AS-OTA(+)+AP (280+92 μg/mL) *vs* AS-OTA(−)+AP (280+92 μg/mL).
[d] AS-OTA(+) 280 μg/mL *vs* AS-OTA(+)+AP (280+92 μg/mL)

applied in the same range of concentrations (92–4940 μg/mL) also reduced cell viability in a concentration-dependant manner (Fig. 2). The IC_{50} of AS-OTA(+) was 934 μg/mL and the calculated OTA concentration in the extract that corresponds to IC_{50} of AS-OTA(+) was 10.5 μg OTA/mL. Taking into account only the OTA concentration (10.5 μg/mL) in the extract, AS-OTA(+) was 5 times more toxic than pure OTA. Also, AS-OTA(+) was twice as toxic than AP (IC_{50}=2126 μg/mL). The highest applied concentration of AS-OTA(-) spore extract (4940 μg/mL) decreased cell viability by 30% and the IC_{50} for the extract could not be determined. Mixture of AS-OTA(+)+AP and AS-OTA(−)+AP applied in subtoxic concentrations showed dominant additive interactions (Fig. 3). Also, a synergistic effect was obtained for combination of AP and AS-OTA (+) both applied in the same mass concentration (280 μg/mL).

3.3. Apoptosis and necrosis induced by OTA and Aspergillus spore extracts

Results of the quantitative fluorescent assay for simultaneous identification of apoptotic and necrotic cells are reported in Table 2.

The fluorescent microscopic findings suggest that the cytotoxic effects of pure OTA as well as AS and AP spore extracts have been predominantly mediated by apoptosis. Single OTA, both AS extracts and AS+AP combinations significantly increased apoptosis frequency as compared to control. Also, both concentrations of pure OTA significantly increased the frequency of necrotic cells. The single AP spore extract increased apoptosis insignificantly and simultaneously evoked significantly higher cell necrosis as compared to control, AS extracts (92 μg/mL) and AS+AP combinations (92+92 μg/mL, AS-OTA(−) 280 μg/mL+AP 92 μg/mL). Mixtures of AP+AS-OTA(+) showed a concentration-dependant cytotoxicity affected by the increased frequency of both apoptotic and necrotic cells. Also, the mixture of AP and OTA positive AS (92+280 μg/mL) exerted the highest apoptotic potential as compared to single extracts and other AP+AS combinations.

The comparison between the measured and expected percent

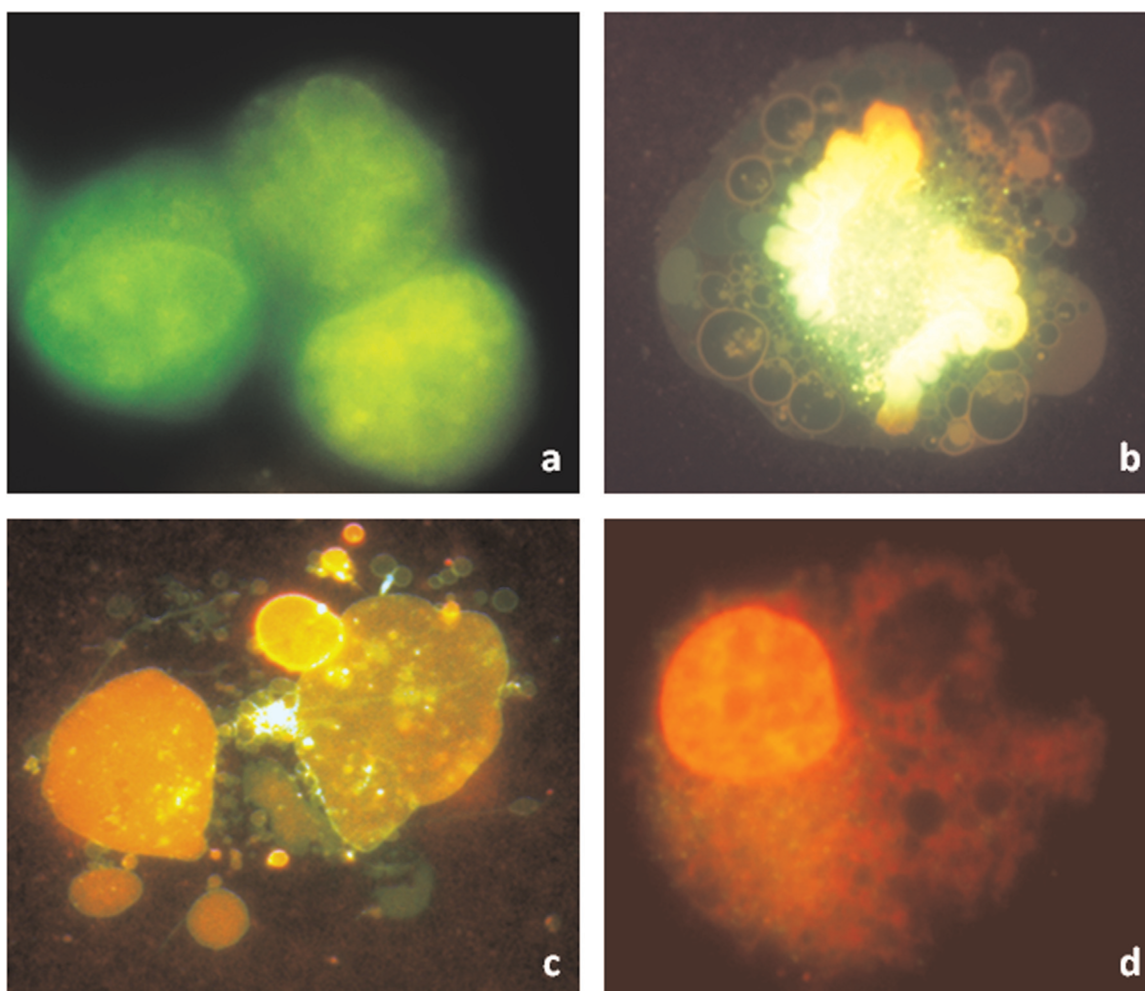

Fig. 4. Photomicrographs of A549 cells observed after simultaneous staining with fluorescence dyes acridine orange and ethidium bromide. (a) Control cells; (b) and (c) apoptotic cells; and (d) necrotic cell. Microscopic analysis was performed using fluorescence microscope Olympus BX, under magnification 400x.

of apoptosis showed additive interactions of AS+AP except in AS-OTA(−)+AP (280+92 μg/mL) where an antagonism was recorded (Fig. 4).

The rapid viability assay with fluorescence dyes acridine orange and ethidium bromide applied in this study allowed for counting fractions of viable, apoptotic and necrotic cells based on cell morphology, nuclear and chromatin disintegration. While control cells showed intact morphology and a bright green colour of chromatins (Fig. 5a), we observed fragmentation of nuclei and formation of apoptotic bodies in apoptotic cells treated with AP and AS-OTA(+) (92+280 μg/mL) (Fig. 5b,c). In necrotic cells treated with AP, the massive destruction of chromatin morphology was observed, together with vacuole formation in the cytoplasm. These cells accumulated ethidium bromide and their chromatin was thus stained bright red (Fig. 5d).

3.4. Genotoxicity of OTA and Aspergillus spore extracts

Genotoxicity obtained by comet assay is represented as tail intensity because proportion (%) of DNA in the tail is the most suitable indicator of DNA damage (Olive, 1999; Collins, 2004). Data show that single OTA as well as subtoxic concentrations of spore extracts significantly induced DNA damage in A549 cells as compared to the control (Fig. 6). Single extracts of AS-OTA(+) increased tail intensity more than both AS-OTA(−) and AP, but this genotoxic effect was not statistically significant. Extract of AS-OTA (+) exerted significantly stronger genotoxicity than the AS-OTA(−)

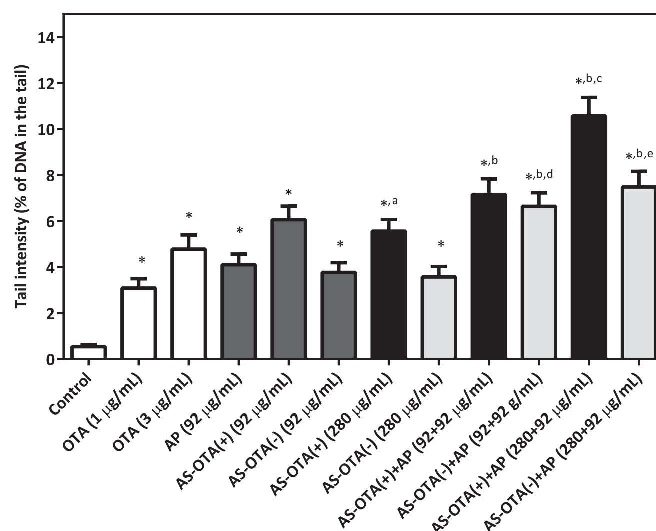

Fig. 5. Genotoxicity of subtoxic concentrations of OTA as well as single and combined spore extracts of *A. sclerotiorum* (AS-OTA(+), AS-OTA(−)) and *A. pseudoglaucus* in A549 cells determined by alkaline comet assay. Significantly different values ($P < 0.05$) are marked as follows: * – as compared to the control; a – AS-OTA (+) 280 μg/mL *vs* AS-OTA(−) 280 μg/mL; b – AP *vs* all mixtures of AS+AP (92+92 μg/mL and 280+92 μg/mL); c – AS-OTA(+) 280 μg/mL *vs* AS-OTA(+)+AP (280+92 μg/mL); d – AS-OTA(−) 92 μg/mL *vs* AS-OTA(−)+AP (92+92 μg/mL); and e – AS-OTA(−) 280 μg/mL *vs* AS-OTA(−)+AP (280+92 μg/mL).

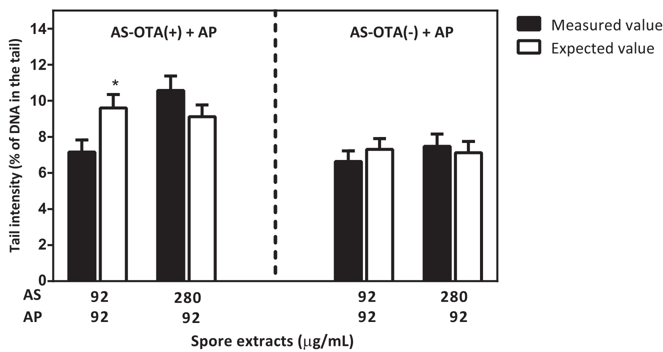

Fig. 6. Genotoxicity of combinations AS-OTA(+)+AP and AS-OTA(−)+AP represented as measured and expected tail intensity. * Represent significant antagonistic effect ($P<0.05$).

only when it was applied at 280 μg/mL. When mixtures of extracts were used, significantly higher tail intensity was obtained in cells treated with AS-OTA(−)+AP (92+92 and 280+92 μg/mL) and AS-OTA(+)+AP (280+92 μg/mL) in respect to DNA damage in cells treated with single extracts. Considering the expected and measured values of tail intensity, extract combinations exert dominant additive interactions, except for the combination of AS-OTA(+)+AP (92+92 μg/mL) which exhibited antagonism (Fig. 6).

4. Discussion

The concentration of airborne fungal particles in occupational environments such as grain mills, rice mills, agricultural facilities or sawmills, may be up to a hundred thousand times higher than outdoors (Lugauskas et al., 2004; Desai and Ghosh, 2003; Hameed and Khodr, 2001; Šegvić Klarić et al., 2012). Our one-year study conducted in a grain mill in 2012 is in agreement whit these findings. The study showed that maximum levels of airborne fungi were 25 times higher than the concentration considered as an arbitrary occupational health hazard (10^4 cfu/m^3) (Jakšić Despot and Šegvić Klarić, 2014; Opplinger et al., 2005). Aspergilli from sections *Aspergillus*, *Flavi* and *Circumdati* dominated the aeromycota in grain mill which is not surprising because these xerophilic species are common contaminants of stored grains and are able to produce vast numbers of airborne spores and small mycelial fragments (Klich, 2009). In November, the species from sections *Aspergillus* and *Circumdati* were found in 100% and 35% of samples, respectively, and constituted 36% and 2.6% of the maximum concentration of total airborne fungi counted in that sampling period. Sequence analysis of calmodulin gene region showed that isolated Aspergilli were *A. pseudoglaucus* and *A. sclerotiorum* and we can assume that *A. pseudoglaucus* and *A. sclerotiorum* were not present only in November but throughout the whole year.

A. sclerotiorum is a well-known producer of OTA from section *Circumdati*, also known as the *Aspergillus ochraceus* group (Frisvad et al., 2004; Varga et al., 1996). In our study among six isolated strains of *A. sclerotiorum* three produced OTA (up to 28 μg/mL) in the spores. Using the equation provided by Kelman et al. (2004) the daily inhaled dose of OTA was calculated to be 1 ng/kg b.w., which is 5% of tolerable daily oral intake-TDI for OTA (17 ng/kg b.w.) established by EFSA (2006). Such comparison was made because no occupational exposure limit for OTA inhalation exists so far. However, several uncertainties related to estimation of inhalatory exposure to OTA should be taken into account: (i) cultivation of viable OTA-producers but not dead spores; (ii) presence of OTA in mycelial fragments; (iii) short sampling time that might exclude other OTA-producers that constitute grain mill aeromycota. Straumfors Halstensen et al. (2004) estimated that Norwegian farmers might inhale up to 13% of OTA TDI during handling of stored grain. OTA has been detected in dust and aerosol samples (0.2–70 μg/kg) in Norwegian cowsheds (Skaug et al., 2001) and a poultry house (8.53 ng/m^3) in China (Wang et al., 2008). Wang et al. (2008) calculated that the workers daily inhaled about an amount of OTA of 68.2 ng, which is a considerable health hazard. Taking into account these calculations, occupants might be at high health risk since mycotoxin inhalation may be at least 10 times more toxic than oral intake (Creasia et al., 1987, 1990). Also, rapid systemic appearance of OTA with 98% of bioavailability has been documented in rats upon intratracheal administration (Breitholtz-Emanuelsson et al., 1995). However, this does not mean that all inhaled particles containing mycotoxins will reach the alveoli. Large particles could be deposited in the traheobronchiolar region and then transported upward by ciliary movement toward pharynx and been swallowed. Therefore, if the mycotoxin is not absorbed by lung tissue, it could be absorbed from the gastrointestinal tract (Breitholtz-Emanuelsson et al., 1995).

Besides OTA, *A. sclerotiorum* is able to produce other polyketides including penicillic acid and xanthomegnins (Samson et al., 2004) which might interact synergistically with OTA. This could be the reason why the IC_{50} of OTA in the spore extract of *A. sclerotiorum* was approximately five times lower than IC_{50} of pure OTA applied on A549 cells. Synergism between OTA and penicillic acid has been documented in kidneys of chickens, mice and pigs after oral administration (reviewed in Šegvić Klarić et al., 2013), while xanthomegnins exerted hepatotoxicity and genotoxicity (Carlton et al., 1976; Mori et al., 1983) but interactions with OTA are still unknown. Contrary to synergistic interactions of OTA and penicillic acid *in vivo*, penicillic acid in human peripheral blood mononuclear cells (PBM) increased while OTA decreased metabolic activity as measured by MTT test, whereas their mixture did not produce any kind of synergism (Stoev et al., 2009). Opposite to AS-OTA(+) spore extract, OTA-non-producing AS spore extract applied in the highest mass concentration did not produced cytotoxic effect. These results suggest that mainly OTA is responsible for the cytotoxicity of *A. sclerotiorum* spore extract but interactions with some other extrolites, which we did not analyse in this experiment, might also be involved in cytotoxicity. A contrary to OTA-producing AS, *A. pseudoglaucus* spore extract was twice less toxic to A549 cells but more toxic than AS-OTA(−). The production of various extrolites including neoechinulin A and neoechinulin B, echinulin, epiheveadride, flavoglaucin, auroglaucin, isotetrahydroauroglaucin and cladosporin have been reported in Aspergilli from section *Aspergillus* (formerly *Eurotium*) (Butinar et al., 2005; Slack et al., 2009) and some might contribute to the cytotoxicity of AP spore extract. Toxic effects of these extrolites are poorly investigated. Earlier studies revealed that echinulin evoked severe damage of alveolar organization and thickening of alveolar walls and liver damage in female mixed-breed rabbits upon intraperitoneal injection (Ali et al., 1989) and it was cytotoxic to HeLa cells at 100 μg/mL (Umeda et al., 1974). A recent study on mouse lungs *in vivo* and primary cultures of mouse alveolar macrophages showed that neuechinulin A and B and cladosporin had significant roles in the immunomodulation of toxin-induced pro-inflammatory lung responses (Miller et al., 2010). Mixture of AS-OTA(+)+AP and AS-OTA(−)+AP showed a dominant additive cytotoxic effect on A549 cells but cytotoxicity was more pronounced when cells were exposed to AS-OTA(+)+AP suggesting possible interactions of OTA and AP extrolites.

Our fluorescent microscopic findings showed that the adverse effects of OTA alone as well as of AS-OTA(+) and AS-OTA(−) spore extracts have been predominantly mediated by apoptosis. Considering previously known facts on OTA we assume that apoptosis in this experiment was triggered by oxidative stress (reviewed in

Sorrenti et al., 2013). An A549 cell possesses cytochrome P450-depending monooxygenases (Castell et al., 2005) that could transform OTA into the quinone metabolite, which is known to generate oxidative stress and can also act as direct mutagen (Akman et al., 2012; Pfohl-Leszkowicz and Manderville, 2012). A recent review on OTA implications in nephrotoxicity and carcinogenicity pointed out that OTA inhibits the nuclear factor, erythroid 2-like 2 (Nrf2) oxidative stress response pathway which in turn diminishes glutathione synthesis, recycling of oxidized glutathione, and oxidoreductase activity of (Limonciel and Jennings, 2014). Apart from single OTA, both AS-OTA(+) and AS-OTA(−) applied alone showed similar apoptotic potential suggesting that spore extracts possess some other toxic metabolites that may induce apoptosis irrespective of OTA presence. This is supported by Schulz et al. (2004), who reported on the cytotoxic synergism of spore extract fractions taken from mycotoxin non-producing airborne fungi. The same study revealed that fungal cell wall components have no or only low toxic potential (Schulz et al., 2004) suggesting that cytotoxicity could be attributed to fungal metabolites. The highest percentage of necrotic cells was found in the sample incubated with single AP. This finding suggests the ability of AP spore extracts to induce inflammation responses which could be attributed to neuechinulin A, B and cladosporin, as was recently reported by Miller et al. (2010). However, when combinations of AS+AP were applied to A549 cells, apoptosis dominated over necrosis and this effect was more pronounced upon exposure to AS-OTA(+)+AP (280+92 μg/mL) suggesting that apoptosis was enhanced by OTA. Combined spore extracts showed additive interactions except AS-OTA(−)+AP (92+280 μg/mL) where antagonism was recorded. Taken together, we can speculate that airborne fungal particles in sub-toxic concentration could trigger apoptosis and/or necrosis depending on metabolite mixtures and their interactions. Presence of mycotoxins such as OTA significantly contributes to the cytotoxicity of these mixtures.

All of the three spore extracts and OTA alone applied in sub-toxic concentrations provoked DNA damage in A549 cells, but contrary to apoptotic effects, genotoxicity was more pronounced when AS-OTA(+) was applied as a single extract or in mixture with AP. For years, the mechanisms of OTA genotoxicity have been under debate. Direct genotoxic action (DNA adduct formation), indirect oxidative DNA damage, and a network of interacting epigenetic mechanisms (inhibition of protein synthesis, oxidative stress, activation of specific signalling pathways) have been proposed (Vettorazzi et al., 2013). Akman et al. (2012) showed that the OTA transformed into hydroquinone metabolite (OTHQ) acts as a direct genotoxic mutagen in human kidney cells (Ad293). Both OTA and OTHQ can generate covalent DNA adducts in human bronchial epithelial W126 cells and human kidney HK2 cells (Hadjeba-Medjdoub et al., 2012) which speaks in favour of OTA genotoxicity. Other extrolites that might be present in spores of *A. sclerotiorum* including penicillic acid and xanthomegnins also possess some genotoxic properties but the mechanism of their genotoxicity is poorly investigated. Penicillic acid induces DNA-strand breaks in HeLa cells (Umeda et al., 1972), while xanthomegnin exerts genotoxic potential in the hepatocyte primary culture (HPC)/DNA repair test (Mori et al., 1983). Opposite to extrolites of *A. sclerotiorum*, the echinulin and flavoglaucin that might be present in *A. pseudoglaucus* spore extract did not exhibit genotoxic action in the hepatocyte primary culture (HPC)/DNA repair test (Mori et al., 1984). Since genotoxic properties of *A. pseudoglaucus* extrolites are poorly investigated or unknown, we can only speculate that such metabolites might interact synergistically in spore extracts and provoke DNA damage in A549 cells. These extrolites may also enhance OTA genotoxicity, which results in more pronounced genotoxicity of the AS-OTA(+)+AP mixture. Which mechanisms and interactions are the most relevant for the genotoxicity of studied spore extracts remains to be examined in future studies.

In conclusion, possible limitations of this study that might underestimate the inhalatory exposure of grain mill occupants to OTA should be considered including the short sampling time, cultivation of viable fungi but not dead spores that might also contain OTA as well as the OTA presence in both spores and mycelial fragments. However, despite the low calculated daily intake of OTA by inhalation, chronic exposure to elevated levels of OTA-producing airborne fungi in combination with other more or less toxic moulds poses a significant threat to human health due to possible additive and/or synergistic cytotoxic and genotoxic interactions.

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