Study of health risks associated with *Aspergillus amstelodami* and its mycotoxic effects

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Fungi, as a result of fungal infection, may colonise many kinds of feed and food on their route from the field to the table. The aim of this study was to evaluate health risks associated with the exposure of living beings to *Aspergillus amstelodami.*

A. amstelodami 724 strain was isolated from wheat grains stored in bins of a food processing company. The inhibition zone of bacteria growth (3–5 mm) under the action of fungus filtrate proved that the fungus is toxic. Five mycotoxins were found in the mycelium of *A. amstelodami* 724 and two in the medium. There were identified patulin, ochratoxin A (OTA) and sterigmatocystin. The ability of *A. amstelodami* 724 to produce one of these mycotoxins – OTA – was confirmed quantitatively growing the fungi on grains. The greatest amount of OTA was estimated after 21 days of growing.

The study of health risks associated with *A. amstelodami* and its mycotoxins were performed *in vivo* using laboratory mice and *in vitro* using cells derived from potent mycotoxin-target organs. Our results prove that after 12 days of exposure to the test micromycetes, mice showed a 20% lower weight gain.

The basic hematological data of mice after exposure to *A. amstelodami* were in the physiological range; nevertheless, they showed a slight increase in the number of all white blood cells. Meanwhile, the percentage of eosinophyls was more than two times higher in the blood of test animals in comparison to control group.

To study the possible role of *A. amstelodami* and its secondary metabolites *in vivo*, cell toxicity mechanisms were studied in the cell cultures derived from potentially OTA-targeted organs. The data have revealed that OTA acts as a genotoxic agent in the Jurkat, blood-derived cell line, and evokes retardation of cell proliferation in the hepatic cell line MH-22A.

The data of the present investigation showed that the test strain *A. amstelodami* 724 isolated from wheat grains stored in bins might be evaluated as a toxin producer. Health risks associated with *A. amstelodami* and its mycotoxins were proved using laboratory mice *in vivo* and cells in the culture *in vitro*.

Key words: *Aspergillus amstelodami*, ochratoxin A, toxicity, proliferation, apoptosis

INTRODUCTION

The occurrence of mycotoxins is global. It is estimated that one quarter of the world's crops are contaminated to some extent with mycotoxins, especially prevalent in developing countries. 77 countries have specific regulations for mycotoxins in different foods and feeds and 13 countries have general provisions, while about 50 countries have no data available (FAO, 2004). The number of countries with specific regulations for mycotoxins has increased over the recent years. Foods are no longer appreciated by consumers only in terms of their immediate nutritional needs, but also in terms of their ability to provide specific health benefits.

Aspergillus fungi are found everywhere world-wide. The genus includes over 150 species, but only a few of them are harmful to man and animals. The type of diseases caused by *Aspergillus* varies, ranging from an "allergy"-type illness to life-threatening generalized infections.

Aspergillus amstelodami Thom et Church is an anamorph of *Eurotium amstelodami* L. Mangin. *A. amstelodami* is more common in tropical and subtropical regions, but it is detected in high latitute countries as well. It was isolated from soils, indoor environments (Klich, 2002; Lugauskas et al., 2003), synthetic polymeric materials (Lugauskas et al., 1987), dried food products, stored grains, nuts (Kozakiewicz, 1989). *A.*

amstelodami is one of the common stored product fungi, which is growing at 14% of moisture. This fungus produces echinulin, preechinulin, neoechinulins, cryptoechinulin (Cole, Cox, 1981). There is some evidence that the fungus is toxic to rabbits, it was isolated from human organs (Lugauskas et al., 2002).

Circumdati and *Nigri* fungi from the *Aspergillus* section had been reported as ochratoxin producers; however, other researchers found this mycotoxin in 12 species of *Aspergillus* (Bragulat et al., 2001). Ochratoxin A (OTA) occurs predominantly in plant products, mainly in corn, coffee, barley, oats, wheat and in their products (Klich, 2002). OTA exhibited carcinogenic, nephrotoxic, teratogenic, neurotoxic and immunotoxic effects in experimental rodents and other animals (IPCS, 2001). OTA has been identified as a causative factor in the human disease called Balkan endemic nephropathy. It evokes an acute hepatic injury, fatty infiltration, and focal necrosis in livers of laboratory animals. Beagle dogs dosed with OTA *per os* developed anorexia, weight loss, dehydratation. Toxin is teratogenic in mice and acts as an inhibitor of mitochondrial transport systems (Cole, Cox, 1981). It is well established that OTA is a potent carcinogen in rodents (Bendele et al., 1985). In the rat kidney, tumour induction was seen at a very low dose level (70 µg/kg) (Boorman, 1989). Although the carcinogenicity of OTA has been described, it is not clear yet whether OTA acts as a direct genotoxic carcinogen or whether its carcinogenicity is related to indirect mechanisms, such as induction of cellular proliferation or cytotoxicity. OTA-dependent induction of apoptosis has been reported *in vivo* (kidney of rats and mice) and *in vitro* (Seegers et al., 1994; Gekle et al., 2000; Petrik et al., 2003).

The aim of our examination was to estimate health risk associated with *A. amstelodami* 724 strain effects. Our goal was to evaluate the toxicity of *A. amstelodami* using laboratory animals *in vivo* and to compare the OTA-dependent effect on blood-derived and hepatic cell proliferation and viability *in vitro*.

METHODS

Isolation of fungi. Dilution plating technique was applied for the isolation of fungi from grains. One millilitre of suspension was drawn into a Petri dish and poured over malt agar medium with chloramphenicol (50 mg/l). Fungi were cultivated for 7–10 days at 28 °C. Pure micromycete strains were isolated on standard Czapek, malt and corn extract media and identified according to manuals (Kozakiewich, 1989; Klich, 2002).

Toxicity to bacteria. Primary toxicity of *A. amstelodami* was defined with the aid of the test organisms *Bacillus stearothermophilus* and *Bacillus megaterium*. Fungi were grown for 7, 14 and 21 days in Czapek yeast extract media, 0.1 ml of filtrates was poured in wheels made on agar with test organisms. After three days the growth inhibition zone of bacteria was measured (Diğrak, Özçelik, 2001; Loiveke et al., 2003).

Analysis of toxins by chromatography. The thin-layer chromatography method was used for the estimation of fungi-produced toxins. Silica gel 60 with the fluorescent indicator UV_{254} (Mackerey-Nagel) was used. Selected fungal strains were grown in Czapek yeast extract medium for 21 days. The biomass was collected and extracted with ethanol (96°). The medium was evaporated and the residue dissolved in ethanol. 0.05–0.1 ml of extract and standard toxins were put to chromatogram. The separation of compounds was carried out in the system of solvents toluol – etylacetate – formic acid (5:4:1). The obtained mycotoxins were identified according to Rf (distance of compound / distance of solvent) and to fluorescence in the UV, as compared with standards (Cole, Cox, 1981).

Quantitative evaluation of mycotoxins. For the estimation of mycotoxins produced by *A. amstelodami* 724, the strain was grown on sterile wheat grains for 7, 14 and 21 days. The amount of patulin were determined using the international standard (ISO, 1993), and the amount of aflatoxins and ochratoxins was established employing the ELISA (enzyme-linked immunosorbent assay) method (Egmond van, 1988).

Preparation of specimen for toxicity test. For the evaluation of toxicity, the fungi were grown for 14 days on Czapek yeast extract agar (CYA). The biomass was collected and a suspension was prepared in physiological solution.

Repeated dose (28 days) toxicity test *in vivo*. Young (12 weeks old) male and female Balb/c mice (Vivarium of the Institute of Biochemistry, Vilnius) weighing 20–22 g were used for the toxicity test. Two doses of fungi, 1000 and 2000 mg/kg body weight, were applied to a group of five mice males and five females. The test substance of *A. amstelodami* 724 fungi was administered *per os* according to EU Commission Directive 92/69/EEC, 0.5 ml of suspension per animal daily. During the experiment, pure tap water was available *ad libitum* for the experimental as well as for the control groups. The animals were observed daily to detect signs of toxicity. The weight of mice was recorded once a week. At the end of the test period, blood analysis was performed for all mice including the control group. Applying the HEMAVET Multispecies Hematology Analyzer (Model 800, MascotTM for Multispecies Hematology Instruments, CDC Technologies, Inc., USA) the following parameters of blood cells were estimated: leukocyte, erythrocyte, lymphocyte, eosinophil, monocyte and basophil count, hemoglobin concentration and hematocrit value.

All experimental procedures were approved by Laboratory Animal Ethics Committee at the Lithuanian State Veterinary and Food Service, Licence Nr. 0107, 13-01-2004.

Cell culture and treatment. Mice-derived hepatoma MH-22A and human T-lymphocyte precursor Jurkat cell lines were used as a model system for the study of

OTA effect *in vitro*. Both cell lines were grown in Iscove's modified Dulbecco's (Sigma-Aldrich) essential medium supplemented with 10% fetal calf serum (Biochrom), 100 U/ml penicillin and 100 µg/ml streptomycin (Biological Industries) at 37 ˚C with 5% CO_2. OTA was purchased from Sigma-Aldrich. The mycotoxin was dissolved in ethanol as a 2 mg/ml stock solution. The final concentrations of mycotoxin were prepared in the complete cell culture medium.

Cell proliferation analysis. Mycotoxin action in cultures of MH-22A and Jurkat cells was tested by counting viable cells in a Goriaev chamber. Cell viability was measured using the trypan blue dye (Sigma) exclusion method. Trypan blue stain (0.4%) was mixed with an equal volume of cells. Viable cells, given their intact membranes, exclude the trypan blue stain; nonviable cells, membrane-permeable, stain dark blue.

Apoptosis assay. The viability of cell population was determined using the acridine orange / ethidium bromide staining technique and fluorescent microscopy. Acridine orange (Molecular Probes) was used to characterize chromatin condensation, and ethidium bromide (Molecular Probes) was employed to characterize membrane integrity. Cells were categorized as follows: – viable non-apoptotic, apoptotic, necrotic and chromatin-free (Mercille et al., 1994).

Statistical analysis. The results are expressed as the mean ± standard error of the mean (SEM). Statistical significance was set at $p < 0,05$. Analysis was carried out using the statistical package of MS Excel 2002 program.

RESULTS AND DISCUSSION

The *A. amstelodami* 724 strain was isolated from wheat grains stored in bins of a food processing company.

The diameter of *A. amstelodami* colonies on Czapek agar was 17–20 mm, on malt extract agar 18–22 mm at 7 days (Fig. 1, A). The colony was low, plane to sulcate. The mycelium was white, later yellow or yellow grey, conidia grey green, reverse uncolored or yellow. Conidial heads radiate, stipes smooth-walled, vesicles globose to spahtulate, uniseriate (Fig. 1, B). Phialides covering the upper two-thirds of the vesicle. Conidia subglobose or broadly ellipsoidal, finely roughened 5–6.5×2.5–3.5 mm.

Preliminary data on the toxicity of *A. amstelodami* 724 was received using bacteria *B. stearothermophilus* and *B. megaterium* as test-organisms. The action of fungus filtrates on bacteria depended on the growth time (Table 1). The inhibition zone of bacterium growth was greater under the action of fungus filtrate after 21 days of cultivation. The inhibition zone 2–5 mm in diameter shows that fungi are toxic (Loiveke et al., 2003).

Table 1. **Growth inhibition (mm) of *B. stearothermophilus* and *B. megaterium* by metabolites of *A. amstelodami* 724**

Growth inhibition (mm) Test organism	Days of growth		
	7	14	21
B. stearothermophilus	2	3	3
B. megaterium	2	4	5

Our data have confirmed that cultivation time is significant for fungus toxin-producing activity. For example, extracts from *Penicillium italicum, Aspergillus candidus* and *Aspergillus parasiticus* were not toxic to *B. megaterium* after two weeks but produced toxic metabolites after four weeks of incubation (Diğrak, Özçelik, 2001).

Thin-layer chromatography allows a qualitative estimation of toxin production and identification of some of them according to Rf comparing with standards and to scintillation in UV. After 21 days of growth on CYA, five metabolites were found in the mycelium of *A. amstelodami* 724 and two in the medium (Table 2). According to Rf such toxins as patulin, OTA and sterigmatocystin were identified. The other compounds were not
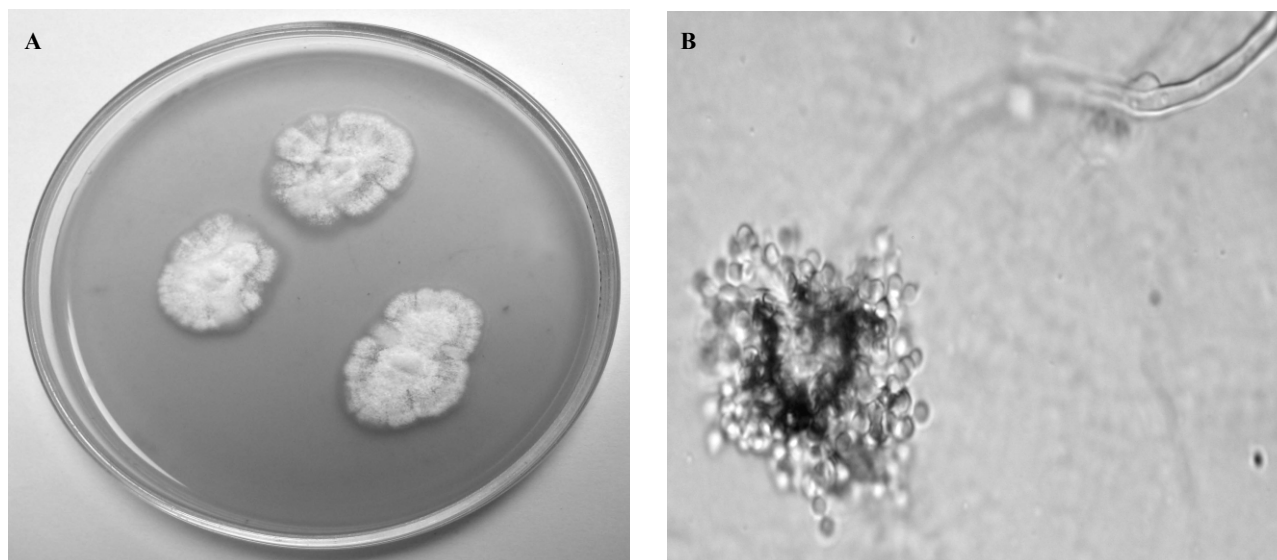

Fig. 1. **A** − *A. amstelodami* colonies on malt extract agar; **B** − *A. amstelodami* conidial head, ×400

identified because of the absence of standard toxins. Our research showed that distinct strains of *A. amstelodami* may synthesize sets of secondary metabolites different from those known for these species in the literature (Cole, Cox, 1981).

Table 2. **Secondary metabolites produced by *A. amstelodami* 724 strain grown on CYA medium**

Number of metabolite	Rf of produced metabolites	Fluorescence in UV	Identified mycotoxins
1	0.14	–	Not identified
2	0.24	–	Not identified
3	0.60*	–	Patulin
4	0.72*	+	OTA
5	0.82	–	Sterigmatocystin

* Found both in mycelium and medium.

The ability of *A. amstelodami* 724 to produce OTA was confirmed quantitatively growing the fungi on grains (Table 3). The amount of OTA increased during growth, and the greatest amount was estimated after 21 days (116.4 µg/kg). The synthesis of patulin started at day 21 of growth. The results showed that fungi functioning on the food products were able to contaminate them with different amounts of one or a few toxic compounds.

Table 3. **Amount of mycotoxins produced by *A. amstelodami* 724 strain grown on wheat grain**

Time of growth, days	Amount of mycotoxins, µg/kg		
	Aflatoxins	OTA	Patulin
7	0	0.3	0
14	0	9.1	0
21	0	116.4	5.0

Micromycetes belonging to the genera *Penicillium, Aspergillus, Fusarium* and some other predominated on grains in Lithuania. They are able to produce mycotoxins of various composition. The highest concentrations of zearalenone and OTA were found in grains (Baliukonienė et. al., 2003). Significant amounts of OTA produced by *Eurotium amstelodami* were found on barley grown in Saudi Arabia (Al-Julaifi, 2003).

The *A. amstelodami* 724 strain was isolated from wheat grains stored in a food processing company, so the stored grains were a potential source of these fungi and their mycotoxins. Therefore, we studied health risks associated with *A. amstelodami* and its mycotoxins to laboratory animals *in vivo* and to cells in the culture *in vitro*.

It is known that mycotoxins for a long time can exert very pervasive, even subclinical and unnoticed effects on health. By the time the mycotoxin poisoning effects accumulate and the clinical symptoms manifest, significant damage is done. Such a delitescent toxic effect was registered in our experiments in the test group when the toxicity of *A. amstelodami* 724 was tested

during a period of 30 days. Though the animals seemed to be healthy and curious, after two weeks they became slothful, slow, anorexic, the fur left sparse and dirty. Our results have proven that under a 12-day influence of the test micromycete strain the mice showed a lower weight gain (Fig. 2). At the end of the test period (30 days) the body weight of experimental mice in both groups was about 20% less than in the control group. Hematological data of the test animals were in the physiological range; nevertheless, they showed a slight increase in the number of all white blood cells in comparison to the control group (Table 4). The most noticeable effect was registered in the scale of the number of eosinophils. The results of blood test imply that the percentage of eosinophils have increased more than twice in the experimental groups versus the control animals.
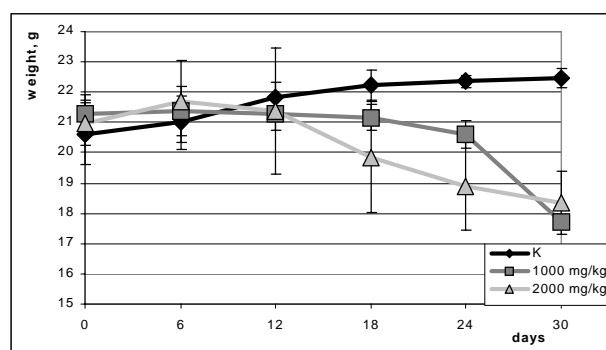

Fig. 2. Effects of *A. amstelodami* on Balb/c mice body weight during a 30-day toxicity test

OTA has been described as an immunosupressant and a potent nephrotoxic, hepatotoxic mycotoxin (Bennett, Klich, 2003). In most species of animals OTA affects such organs as liver, myocardium, gastrointestinal tract, lymphoid organs, skeletal system, haemopoietic tissues and reproductive organs. Tests on laboratory animals indicated the capability of this substance to trigger immunomodulation even at levels far below the toxicity threshold (Muller et al., 1999). The present study revealed the effects of OTA in the model cell cultures derived from the potent OTA-target organs – blood and liver. During the experiments there were made quantitative analyses of the effect of OTA treatment on the viability and proliferation of haemopoietic (Jurkat) and hepatic (MH-22A) cell lines.

According to our data, hepatic MH-22A cells proved to be more resistant to OTA than haemopoietic Jurkat cells (Fig. 3). Nevertheless, part of the cells in the culture of both lines proliferated after 48 h of exposure to OTA (Fig. 3, A). The ratio of cell number after the 48 h treatment to the control cell amount was similar in both cultures. After the treatment, only the study of cell viability revealed a higher sensitivity of Jurkat cells (Fig. 3, B). About 60% of the cells were obviously dead after exposure to OTA; almost all dead cells were apoptotic in this culture. On the contrary, OTA had a weak effect on apoptosis (8%) in hepatic MH-22A cells.

Table 4. **Haematological data after a 30-days of mice exposure to *A. amstelodami* 724 strain micromycete culture**

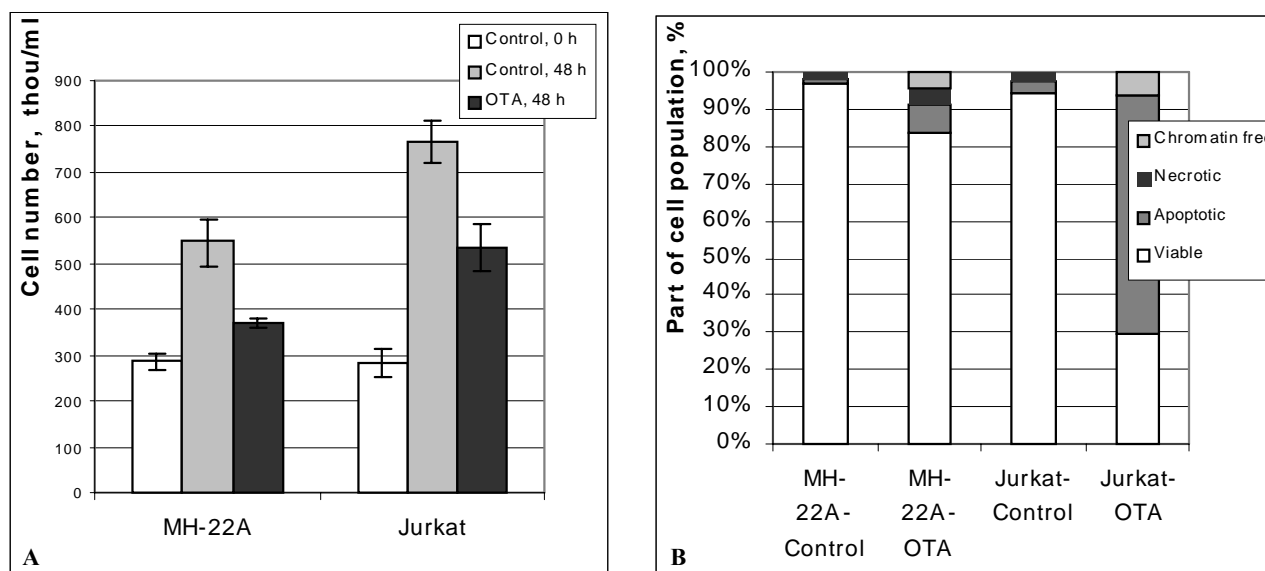|	WBC (K/µl)	Eosinophils		Monocytes	
		(K/µl)	%	(K/µl)	%
Normal range	*1.8 – 10.7*	*0.0 – 0.2*	*0.0 – 3.9*	*0.0 – 0.4*	*0.0 – 7.5*
Control	3.105 ± 0.602	0.136 ± 0.045	1.538 ± 1.473	0.23 ± 0.056	5.32 ± 1.94
1000 mg/ml	7.876 ± 2.236	**0.566 ± 0.321**	**6.776 ± 1.814**	0.505 ± 0.16	6.348 ± 1.011
2000 mg/ml	7.450 ± 0.930	**0.585 ± 0.066**	**7.953 ± 1.016**	0.445 ± 0.048	6.081 ± 0.925

Fig. 3. A – Evaluation of MH-22A and Jurkat cell proliferation after a 48-h treatment with OTA; **B** – Distribution in the percentage of viable, apoptotic, necrotic and chromatin-free MH-22A and Jurkat cells after 48 hours of exposure to OTA

The data have shown that OTA acts as a genotoxic agent in the haemapoietic cell line Jurkat, but it showed only a retardation of cell proliferation in the hepatic cell line MH-22A. The resistance mechanisms in detail are not yet fully understood. However, there are data that OTA-induced activation of ERK1/2 could trigger an important intracellular signaling pathway that mediates some of mycotoxin's effect in renal epithelia and liver-derived cells (Schramek et al., 1997; Baltriukiene, data unpublished).

Despite much evidence pointing toward an important role of *Aspergillus* species and their secondary metabolites in the health hazards, studies elucidating the possible mechanisms involved in this process have not yet been completed. Contradictory reports on DNA-damaging activity and on the carcinogenity of mycotoxins have appeared. It was shown that OTA may activate different cellular processes involved in the degradation of various kinds of cells (Seegers et al., 1994). One species of fungi produces a range of mycotoxins which have different targets and different mechanisms of action. Their effects may summarize or disperse.

The present investigation showed that the strain *A. amstelodami* 724 may be evaluated as a toxin producer. Filtrates of the fungi inhibited the growth of *B. stearothermophilus* and *B. megaterium*. According to Rf of thin-layer chromatography, such toxins as patulin, OTA and sterigmatocystin were identified. The ability of fungi to produce toxic secondary metabolites depended on growth duration. The highest amount of OTA synthesized by *A. amstelodami* 724 was noted after 21 days of growth on wheat grains. Health risks associated with *A. amstelodami* and its mycotoxin were proved using laboratory mice *in vivo* and cells in the culture *in vitro*.

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SVEIKATOS RIZIKOS VEIKSNIŲ, SUSIJUSIŲ SU *ASPERGILLUS AMSTELODAMI* IR JO PRODUKUOJAMŲ MIKOTOKSINŲ POVEIKIU, TYRIMAS

S a n t r a u k a

Darbo tikslas – įvertinti *Aspergillus amstelodami* gebėjimą gaminti mikotoksinus bei gyvų organizmų sveikatos riziką, susijusią su šio grybo užkratu. *A. amstelodami* 724 padermė buvo išskirta iš kviečių grūdų, saugomų grūdų sandėliuose. Bakterijų augimo zonos stabdymas (3–5 mm) parodė, kad tiriama mikromicetų kultūra yra toksiška. Iš šio mikromiceto micelio buvo išskirti penki mikotoksinai, o iš jų auginimo terpės – du. *A. amstelodami* 724 kultūroje aptiktas patulinas, ochratoksinas A (OTA), sterigmatocistinas. OTA – vienas iš šio mikromiceto gaminamų antrinių metabolitų. Nustatyta, kad po 21 grybo auginimo dienos OTA grūduose buvo 116,4 µg kg^{-1}.

Sveikatos rizikos tyrimas, panaudojant laboratorines peles, parodė, kad *A. amstelodami* 724 grybo biomasė po 12 poveikio dienų sukėlė toksines reakcijas – 20% krito gyvūnų svoris, jie prarado apetitą, tapo vangūs, pakito kailio išvaizda. Nors pagrindiniai pelių kraujo rodikliai kito fiziologinėse ribose, tačiau baltųjų kraujo kūnelių kiekis šiek tiek padidėjo. Ypač išaugo eozinofilų skaičius – jis buvo net 2 kartus didesnis už kontrolinių gyvūnų kraujyje registruotą eozinofilų kiekį.

Siekiant įvertinti tiriamos mikroskopinių grybų kultūros toksinio poveikio pasekmes, buvo analizuojamas OTA – vieno iš šio mikroskopinio grybo metabolitų – poveikis taikininių organų kilmės ląstelių kultūrose. Tuo tikslu naudotos kraujo (Jurkat) ir kepenų (MH-22A) kilmės ląstelių linijos. Nustatyta, kad OTA pasižymėjo genotoksiniu veikimu kraujo kilmės ląstelių kultūroje, bet MH-22A linijos ląstelių atžvilgiu registruotas tik proliferaciją stabdantis aktyvumas.

Gauti rezultatai patvirtino, kad tiriama mikromiceto *A. amstelodami* 724 padermė yra toksiška, o jos gaminamų toksinų kiekis priklauso nuo auginimo laiko. Tiek tyrimuose *in vivo*, tiek ir ląstelių kultūroje *in vitro* įvardytos tirtos mikromiceto padermės ir jos gaminamų metabolitų poveikio pasekmės.

Raktažodžiai: *Aspergillus amstelodami*, ochratoksinas A, toksiškumas, proliferacija, apoptozė