Mutagenic Effects of Selected Trichothecene Mycotoxins and their Combinations with Aflatoxin B_1

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Abstrakt

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The authors focused on the amplification of data on the mutagenicity of selected trichothecene mycotoxins (T-2 toxin, vomitoxin) and their combination with aflatoxin B_1, which is known to be a strong mutagen. Mutagenic activity was investigated using the Ames test in a prokaryote model at low doses (close to 0.1 LD_{50}). Whereas the individual trichothecene mycotoxins (T-2 toxin, vomitoxin) did not show any mutagenic activity in the test systems mentioned, in combination with AFB_1, or as a combination of all three mycotoxins, they showed a mutagenic effect significantly greater than AFB_1 alone in the Ames test (in strain TA98 at all concentrations) as well as in the micronucleus test (combination of T-2 toxin with AFB_1).

Keywords: mutagenic activity; Ames test; micronucleus test; mycotoxins; aflatoxin B_1; T-2 toxin; vomitoxin

In the spectrum of various toxic and genotoxic substances or their mixtures that contribute to environmental pollution in the Czech Republic, mycotoxins represent a potential risk even to the human population.

This fact has been repeatedly stated but the lack of information on the character of their genotoxic effects (WOOD 1992) does not allow to an objective assessment of the risk they pose. We have therefore focused on the amplification of basic knowledge of the mutagenicity of mycotoxins, secondary metabolites of certain moulds, which have several features in commen: relatively frequent occurrence in food chains, coincidence of several toxins in the same substrate, andnsufficient or contradictory information in the literature on the mutagenic effects of individual mycotoxins and their combinations (aflatoxin B_1, T-2 toxin, vomitoxin).

First of all, mycotoxins occur in low concentrations, namely in foodstuffs and feeds as well as in tissues and body fluids of man and domestic animals (RUPRICH 1995; TUREK 1996; LEWIS 1998). Such chronic influence of low doses of mycotoxins poses risks of genotoxicity (mutagenicity, carcinogenicity, embryotoxicity), immunosuppressive effects, and other nonspecific damage.

Most studies mainly follow up of the effects of individual toxins or deal with mycotoxin producers. Toxinogenic moulds often produce several toxins simultaneously (THIEL *et al.* 1991; MILLER & TRENHOLM 1994). Several species of toxinogenic moulds may appear in the same substrate, either simultaneously or in succession. A combined exposure to mycotoxins from various components of food or feed is also possible (SINHA 1998). For these reasons it is often necessary to also consider the *combined* biological effects of low levels of various mycotoxins.

MATERIAL AND METHODS

The Ames test: The principle of a Ames test in the prokaryote model (MARON & AMES 1983; ČERNÁ *et al.* 1989) consists in the follow-up of spontaneous and induced reverse mutations in special test strains of bacteria (auxotrophic strains of *his- Salmonella typhimurium*, strains TA98 and TA100). The S9 fraction of liver homogenate from the liver of laboratory rats induced by a mixture of polychlorinated biphenyls, Delor 103, was used for the modelling of metabolic transformation *in vitro*.

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The following substances with known mutagenic properties (reference mutagens) were used as a positive control: 2-aminofluorene at a concentration of 10 µg per dish as an indirect mutagen, in both, TA98 and TA100 4-nitro-ortho-phenylenediamine (NOFD) in TA98 at a concentration of 30 µg per dish, and sodium azide at a concentration of 10µg per dish in TA100, as direct mutagens.

Aminofluorene and NOFD were diluted with dimethyl sulphoxide (DMSO), sodium azide was dissolved in distilled water. The tested mycotoxins and the reference mutagens were dissolved in DMSO and the stock solution was further diluted with DMSO to concentrations desired. The concentrations were diluted by dissolving as that the corresponding amounts of the substances tested in 0.1 ml of solvent.

T-2 toxin was tested in the Ames test on strains TA98 and TA100 at the doses of 1.0, 0.25, and 0.1 µg/dish. For **vomitoxin** the doses were 8.0, 4.0, 2.0, and 0.8 µg/dish. **Aflatoxin B_1** was tested at the doses of 1.0, 0.5, 0.25, and 0.1 µg/dish. The combination of **T-2 toxin** and **aflatoxin B_1** was tested at the doses of 1.0 + 1.0, 0.5 + 0.5, 0.25 + 0.25, and 0.1 + 0.1 µg/dish. The combination of **vomitoxin** with **T-2 toxin** was tested at the doses of 8.0 + 1.0, 4.0 + 0.5, 2.0 + 0.25, and 0.8 + 0.1 µg/dish, respectively. The combination of **vomitoxin**, **T-toxin** and **aflatoxin B_1** was applied to the test strains at the doses of 8.0 + 1.0 + 1.0, 4.0 + 0.5 + 0.5, 2.0 + 0.25 + 0.25, and 0.8 + 0.1 + 0.1 µg/dish, respectively. After culturing for the required period of time the numbers of revertants were revealed with the aid of a counter from Laboratory Imaging, Prague. Each individual concentration of the substances studied and their combinations were tested in each experiment in three replicate dishes, and each experiment was repeated at least twice. In the first series of experiments aflatoxin B_1, T-2 toxin, vomitoxin, and the combinations of T-2 toxin + AFB_1, and vomitoxin + AFB_1 were tested. In the second experimental series aflatoxin B_1, the combination of vomitoxin with T-2 toxin, and the combination of all three mycotoxins were tested.

In each experiment the numbers of spontaneous revertants were counted in each strain in the presence of 0.1 ml of each corresponding solvent per dish (negative control – NC). The ratio of the mean number of induced revertants at the concentrations tested vs. the mean number of spontaneous revertants (coefficient C) was used as the basic criterion for assessing the mutagenic activity of a tested substance. The statistical evaluation of mutagenicity was carried out by determining the interval of reliability on the basis of Poisson distribution at the 5% level of significance. Statistically significant differences in the numbers of revertants found at each level between the substances tested and the controls (NC) and differences between both strains were evaluated by the *t*-test.

The micronucleus test: The test (SCHMID 1995; GREGOR et al. 1987; DECD 1983) assesses the mutagenic (clastogenic) activity of substances tested in a eukaryote mammalian model *in vivo* (ability to induce chromosomal breaks).

To obtain data on the mutagenic activity of *low doses* of the mycotoxins tested, we chose 0.1 LD_{50} as the starting point value for the concentrations to be tested in the micronucleus test. Experimental animals were given the tested mycotoxins and their combinations intraperitoneally in a single dose in respective concentrations. The concentrations were diluted so that the required amount of the tested mycotoxin calculated per 10 g of body weight of the mouse was dissolved in 0.1 ml of the solvent (7% DMSO). The control group was also given the same amount of the solvent.

White SPF laboratory mice, strain ICR, 20–25 g in body weight, supplied by Top-Velaz, were kept under standard conditions in the animal house at the 3rd Faculty of Medicine (A*ccredited as a facility for performing animal experiments according to § 14 of Public Notice No.311/ 1998 Sb. on the breeding and utilization of experimental animals.)*. The tested substances were applied to these at the following concentrations (in mg/kg): **T-2 toxin** 5.0 and 1.0, **vomitoxin** 10.0 and 5.0, **aflatoxin B_1** 5.0 and 1.0.

Concentrations of the mycotoxins were tested in single doses as presented in Table 2. The combination of AFB_1 after the pre-treatment with T-2 toxin was tested. The pre-treatment with T-2 toxin consisted in three oral doses in the course of two weeks. The dose of T-2 toxin applied was always the same (5.0 or 1.0 mg per kg of body weight), the single dose of AFB_1 was always 0.1 mg per kg body weight. Exposure to aflatoxin B_1 lasted 24 hours.

After histological processing of the bone marrow polychromatophilic erythrocytes and the frequency of micronuclei there were assessed under 1000 times magnification. In each experimental group of animals the mean number of micronuclei per 1000 cells was determined.

Mice treated i.p. with cyclophosphamide (reference mutagen) at doses of 20 and 40 mg/kg served as positive controls were used. Mice treated i.p. with solvent (7% DMSO) were used as negative controls. They reveated a significantly higher frequency of micronuclei in comparison to intact animals. The basic data on the mutagenicity of the sample tested were obtained by frequency comparison of micronuclei following the administration of the substance tested with those upon the administration of control substances.. Statistical evaluation of results of the micronucleus test was performed by the t-test at the 5% level of significance.

RESULTS AND DISCUSSION

The mean values of spontaneous revertants (negative control – NC) in the Ames test with *S .typhimurium* TA98 and TA100 (with metabolic activation) are presented in

Table 1a and b. The mean values of revertants induced with the reference mutagen in strain TA98 were 562.5 ± 25.3 without metabolic activation and 916.3 ± 41.9 upon metabolic activation. Results of the first series of experiments are presented in Table 1a, those of the second series in Table 2 (mean number of revertants and mean C value).

In the **micronucleus test** the mean number of micronuclei in polychromatophilic erythrocytes of the bone marrow in control animals was 2.9 ± 1.4 (7% DMSO). No statistically significant differences between males and females were found at the level of significance $\alpha = 0.01$. The differences found between the tested substances and control groups were assessed with help of the *t*-test at the level of significance $\alpha = 0.05$. The mean numbers of micronuclei in polychromatophilic erythrocytes of the bone marrow in all the substances tested, as well as the mutagenic activity of the individual doses are summarised in Fig. 1.

Aflatoxin B_1 significantly increased the number of induced revertants vs. spontaneous revertants (negative control). It had this effect in both strains, but only after metabolic activation by the S9 fraction of liver homogenate. A statistically insignificant difference was found only at a concentration of 0.1 μg per dish in strain TA98. The concentration of 0.1 μg/dish had the highest mutagenic activity in both strains. Mutagenic activity was directly dose dependent. Mean numbers of revertants, calculated

Table 1. Mutagenic activity of T-2 toxin, vomitoxin, and their combinations with aflatoxin B_1 in the Ames test in *S.typhimurium* test strains

Strain AFB_1 (μg/dish)	TA98 +S9		TA100+S9	
	number of revertants	C	number of revertants	C
1	169.3 ± 50.1	6.7*	1111.5 ± 383.4	7.3*
0.5	78.3 ± 16.0	3.1*	627.2 ± 144.8	4.1*
0.25	72.7 ± 26.8	2.9*	590.2 ± 317.5	3.9*
0.1	35.3 ± 13.1	1.4	291 ± 56.8	1.9*
T-2 toxin				
1	28.8 ± 4.5	1.1	181.6 ± 28.8	1.2
0.5	27.6 ± 5.3	1.1	178.2 ± 39.7	1.2
0.25	26.8 ± 1.8	1.1	173.7 ± 33.6	1.1
0.1	25.0 ± 3.0	1	187.5 ± 15.9	1.2
Vomitoxin				
8	28.3 ± 5.1	1.1	159.8 ± 10.5	1
4	24.8 ± 5.2	1	149.5 ± 17.4	1
2	26.5 ± 5.1	1	154.2 ± 22.4	1
0.8	23.8 ± 1.5	0.9	148.0 ± 19.0	1
T-2 toxin+AFB_1				
1.0 + 1.0	288 ± 95.5	11.4**	1682.9 ± 201.8	11.1**
0.5 + 0.5	201.2 ± 85.3	8.0**	1207.4 ± 382.3	7.9**
0.25 + 0.25	131.2 ± 36.2	5.2**	712.6 ± 239.4	4.7*
0.1 + 0.1	119.0 ± 64.4	4.7**	318.1 ± 99.2	2.1*
Vomitoxin+AFB_1				
8.0 + 1.0	348.3 ± 160.7	13.8**	740 ± 162.4	4.9*
4.0 + 0.5	186.6 ± 95.7	7.4**	748.7 ± 357.4	4.9*
2.0 + 0.25	178.8 ± 95.3	7.1**	617.8 ± 168.0	4.1*
0.8 + 0.1	111.3 ± 27.3	4.4**	361.5 ± 128.8	2.4*
NC	**25.3 ± 5.7**		**152.3 ± 21.3**	

+S9 – with metabolic activation with the S9 fraction of liver homogenate

C – ratio of the concentration studied to negative control

X^* – statistically significant frequences of induced revertants vs. negative controls

X^{**} – statistically ssignificant frequences of induced revertants vs. aflatoxin B_1

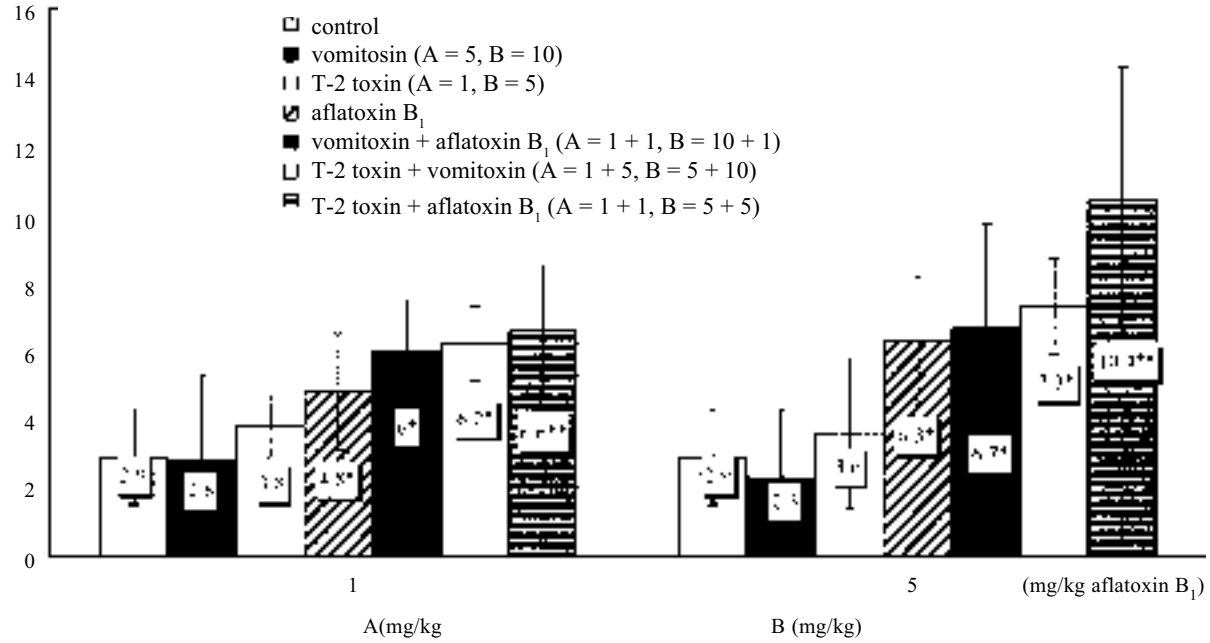

Fig. 1. The numbers of micronuclei in polychromatophilic erythrocyted of the bone marrow after individual i.p. aplication of T-2, vomitoxin, aflatoxin B_1 and combination T-2 and vomitoxin (mean of both sexual)

coefficient C values and standard deviations SD are presented in Tables 1 and 2.

Likewise, the micronucleus test rereated a significantly higher number of micronuclei in animals treated with aflatoxin B_1 at both concentrations, compared with the control group. Differences between the concentrations tested were also found. The number of micronuclei in polychromatophilic erythrocytes was significantly higher at a dose of 5.0 mg/kg than at 1.0 mg/kg. The difference between males and females was insignificant.

Aflatoxin B_1 is one of the best-studied and longest known mycotoxins. In our study it was used as a reference mycotoxin that shows mutagenic activity in all prokaryote and eukaryote test systems. The mutagenic activity of aflatoxin B_1 in the Ames test was examined by many authors. Similarly as in our experiments, the mycotoxin is know to be mutagenic only after metabolic activation (COLES *et al.* 1977; BUENING *et al.* 1978; UENO *et al.* 1978; etc.). This mutagenic activity of aflatoxin B_1 is ascribed to the metabolite 8,9-epoxide-aflatoxin B_1 which is formed from aflatoxin B_1 through oxidation by cytochrome-P450-monooxygenases and forms adducts with DNA (GUENGERICH *et al.* 1994). The adducts have a capacity of long-term persistence, namely in cells with low proliferative activity. They induce chromosomal aberrations, the formation of micronuclei and exchanges of sister chromatids detectable in bone marrow cells of various laboratory species, as well as dominant lethal mutations in laboratory mice and rats (NIX *et al.* 1981; BÁRTA *et al.* 1990, 1991; MCQUEEN & WAY 1991 etc.).

Data concerning the mutagenicity of trichothecene mycotoxins are ambiguous in studies *in vitro* as well as *in vivo*.

In trichothecene mycotoxins, T-2 toxin and vomito-xin, no increase in the number of revertants was found in the Ames test following metabolic activation or without it. Likewise, we have not found any statistically significant increase in the frequency of micronuclei in the micronucleus test. These expected results are in keeping with data in the literature (WEHNER *et al.* 1978; KUCZUK *et al.* 1978).

In the Ames test the combination of T-2 toxin and vomitoxin did not show any mutagenic activity after metabolic activation, or without it. This is valid for at most concentrations tested with the exception of the TA98 strain after metabolic activation at the dose of l.0 µg per dish. We have not found any published data on the combined mutagenic action of these two trichothecene mycotoxins in bacterial models. However, in the micronucleus test this combination induces a statistically significant increase in the frequency of micronuclei at both concentrations tested in comparison with the control group. Significant differences between the concentrations tested or between males and females were not detected.

The mutagenic activity of the combination of T-2 toxin and AFB_1 in the Ames test after metabolic activation differed significantly from the mutagenicity of AFB_1 alone in strain TA98. The number of revertants was higher than in AFB_1 alone at all the concentrations tested. Strain TA100 produced significantly more revertants at concentrations 1.0 + 1.0 and 0.5 + 0.5 µg/dish, compared with

Table 2. Mutagenic activity of mutual combinations of T-2 toxin, vomitoxin, and aflatoxin B_1 in the Ames test in *S. typhimurium* test strains

Strain AFB_1 (μg/dish)	TA98 +S9		TA100+S9	
	number of revertants	C	number of revertants	C
1	435.7 ± 82.7	11.2*	1854.2 ± 149.0	11.7*
0.5	371.0 ± 94.5	9.6*	1423.6 ± 160.4	9.2*
0.25	195.3 ± 98.2	5.1*	559.5 ± 173.6	3.5*
0.1	243.6 ± 41.2	6.3*	351.1 ± 129.5	2.2*
Vomitoxin + T-2 toxin				
8.0 + 1.0	82.5 ± 11.5	2.1*	218.0 ± 35.6	1.4
4.0 + 0.5	30.3 ± 4.5	0.8	158.2 ± 16.9	1
2.0 + 0.25	64.3 ± 11.1	1.7	168.2 ± 37.2	1.1
0.8 + 0.1	25.8 ± 7.2	0.7	211.5 ± 22.9	1.3
Vomitoxin+T-2 toxin+AFB_1				
8.0 + 1.0 +1.0	677.3 ± 169.0	17.5**	899.2 ± 130.8	5.7**
4.0 + 0.5 + 0.5	668.7 ± 161.7	17.3**	808.3 ± 88.7	5.1**
2.0+0.25+0.25	468.4 ± 208.0	12.1**	507.0 ± 219.8	3.2*
0.8 + 0.1 + 0.1	214.1 ± 101.7	5.5*	372.3 ± 194.0	2.3*
NC	**38.6 ± 8.5**		**158.6 ± 27.0**	

+S9 – with metabolic activation with the S9 fraction of liver homogenate

C – ratio of the concentration studied to negative control

X^* – statistically significant frequences of induced revertants vs. negative controls

X^{**} – statistically significant frequences of induced revertants vs. aflatoxin B_1

AFB_1 alone. In two lower concentrations the differences were insignificant. In the study of the combination of T-2 toxin and aflatoxin B_1 in the animal model there two modes of applying the toxins were compared. The aim of that was to assess the influence of the immunosuppressive effect of T-2 toxin on the modulation of the mutagenic activity of aflatoxin B_1.

There were two patterns of applying T-2 toxin in combination with AFB_1 to experimental animals. One was based on applying T-2 toxin in three doses in the course of two weeks before the application of AFB_1 to verify the mutagenic effect of AFB_1 in an organism with immunity impaired by T-2 toxin. In the other alternative both toxins were applied simultaneously. In the experimental groups that were applied a dose of 5.0 mg/kg animals died just as in all other repeated experiments. That confirms the synergic toxic effect of higher doses, as was described by COFFEY et al. (1990). Therefore all groups were given a dose of only 1.0 mg/kg of AFB_1.

Similarly, deaths repeatedly occurred in animals upon application of the combination of AFB_1 + T-2 toxin + vomitoxin at both concentrations, therefore the results were not evaluated.

The mutagenic effect of both application patterns of these mycotoxins was not significantly different at the level of significance $\alpha = 0.45$. However, we found a significantly higher frequency of micronuclei upon application of the combination T-2 toxin + aflatoxin B_1 in comparison with the effect of aflatoxin B_1 alone in both modes of application.

In the combination vomitoxin + AFB_1 with metabolic activation the numbers of induced revertants were significantly greater than the numbers of spontaneous revertants at all concentrations and in both test strains. In strain TA98 all concentrations of this combination induced signifidantly more revertants than AFB_1 alone, the mutagenic activity of the combination being higher.

In strain TA100 no mutagen combinations significantly differ from the same concentration of AFB_1 alone. The concentration 8.0 + 1.0 μg/dish even induced a lower number of revertants than the corresponding concentration of AFB_1 alone, but the difference was not significant statistically at the level of $\alpha = 0.05$ (the probability of a difference between the groups was 91%).

Under simultaneous i.p. application of the combination vomitoxin + aflatoxin B_1 deaths repeatedly occurred in the groups that were applied 10.0 mg/kg of vomitoxin and 1.0 mg/kg of aflatoxin B_1. The resulting numbers of animals were too small for statistical evaluation and no further conclusions were drawn. In the group where the combination of both toxins at a dose of 1.0 + 1.0 mg/kg was applied the numbers of micronuclei in polychromato-

philic erythrocytes differed significant by from the control group. No statistically significant difference between these mice and the groups of laboratory mice that were treated with AFB_1 alone were found, although the value approximated a statistically significant difference at the level of significance $\alpha = 0.05$ (the probability of a difference between both groups was 92%).

The combination of mycotoxins, AFB_1, T-2 toxin and vomitoxin induced a statistically significant increase in the numbers of revertants at all the concentrations after metabolic activation only.

In strain TA98 there has been found a higher frequency of revertants induced by the mycotoxin mixture was found at all concentrations, compared with the effect of AFB_1 alone after metabolic activation, except for the concentration 0.8 + 0.1 + 0.1 μg/dish, which did not differ from AFB_1 alone to any statistically significant degree.

In strain TA100 the numbers of revertants were even lower in comparison with the effect of AFB_1 The difference was statistically significant at the two highest concentrations, the two lowest concentration (2.0 + 0.25 + 0.25 and 0.8 + 0.1 + 0,1) caused only insignificant changes. Mutagenic activity expressed by the coefficient C (P/NC) at all concentrations, after metabolic activation was significantly lower in strain TA100 than in strain TA98.

The two *Salmonella typhimurium* strains employed in the Ames test appear to react differently. Statistically, strain TA98 produced significantly greater numbers of induced revertants at all concentrations of aflatoxin B_1 with vomitoxin, and at all concentrations of aflatoxin B_1 with T-2 toxin as compared with aflatoxin B_1 alone. The combination of aflatoxin B_1 with T-2 toxin and vomitoxin in TA98 induced the highest mutagenic activity of all the combinations tested. In strain TA100 the numbers of revertants at the two highest concentrations of aflatoxin B_1 + T-2 toxin + vomitoxin were always significantly lower than in aflatoxin B_1 alone. Mutagenic and cytotoxic effects of these mycotoxins apparently combine. A different type of mutation in the histidine operon, with which the two strains are equipped certainly also plays its role (MARON & AMES 1983; BONEAU *et al.* 1991).

Results of the micronucleus test and of the Ames test were compared using the correlation coefficient. The values of the correlation coefficient between the micronucleus test and the Ames test were 0.94 in strain TA98 and 0.92 in strain TA100. Comparing both *S. typhimurium* strains, TA98 and TA100, the correlation coefficient equalled 1.0. Both tests seem to be suitable for the follow-up of the combined effects of even small doses of mycotoxins.

Data in the literature on the combined effects of trichothecene mycotoxins and aflatoxin B_1 in animals are not cosistent. In certain cases the effects of combinations were greater than the effect of individual mycotoxins alone (KUBENA *et al.* 1989; HUFF *et al.* 1986, 1988a, b etc.), sometimes the effect seemed to be additive (HUFF *et al.* 1986), but in other cases the effects of combinations did not differ from those of aflatoxin B_1 alone (HARVEY *et al.* 1989). The combinations of mycotoxins tested by us mostly showed a potentiation effect *in vivo* although in some combinations of higher concentrations the toxic effect of mycotoxins predominated. However, data on the mutagenic activity of mycotoxin combinations obtained by us, are not available in the literature and should therefore be subjected to further research because combined mycotoxins represent a real health risk to the human population.

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Souhrn

ŠMERÁK P., BÁRTAII., POLÍVKOVÁ Z., BÁRTOVÁ, J., SEDMÍKOVÁ M. (2001): **Mutagenní účinky vybraných mykotoxinů a jejich kombinací s aflatoxinem B_1**. Czech J. Food Sci., 1**9**: 90–96.

Práce byla zaměřena na rozšíření údajů o mutagenitě vybraných trichothecenových mykotoxinů (T-2 toxin, vomitoxin) a jejich kombinací s aflatoxinem B_1, který je znám jako silný mutagen. Mutagenní aktivita byla testována Amesovým testem na prokaryotním modelu a mikronukleus testem na eukaryotním modelu v nízkých dávkách (blízkých 0,1 hodnoty LD_{50}). Zatímco jednotlivé trichotecenové mykotoxiny (T-2 toxin**,** vomitoxin) nevykazovaly v uvedených testovacích systémech mutagenní aktivitu, v kombinaci s AFB_1, resp. v kombinaci všech tří mykotoxinů, jevily jak v Amesově testu (u kmene TA98 ve všech koncentracích), tak i v mikronukleus testu (kombinace T2 toxinu s AFB_1), mutagenní efekt významně vyšší než samotný AFB_1. Některé koncentrace kombinace vomitoxinu s T-2 toxinem a s AFB_1 však v Amesově testu (kmen TA100) indukovaly statisticky významně nižší četnosti revertant ve srovnání s účinkem samotného AFB_1.

Klíčová slova: mutagenní aktivita; Amesův test; mikronukleus test; mykotoxiny; aflatoxin B_1; T-2 toxin; vomitoxin

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