Potential hazard of simultaneous occurrence of aflatoxin B_1 and ochratoxin A

M. SEDMÍKOVÁ[1], H. REISNEROVÁ[1], Z. DUFKOVÁ[1], I. BÁRTA[2], F. JÍLEK[1]

[1]*Department of Veterinary Science, Czech University of Agriculture, Prague, Czech Republic*
[2]*Division of General Biology and Genetics, Charles University, 3rd Faculty of Medicine, Prague, Czech Republic*

ABSTRACT: Contents of aflatoxin B_1 (AFB_1) and ochratoxin A (OA) in the samples of wheat and barley were detected by RIA method. Average contents of AFB_1 in the samples of wheat and barley were 2.4 μg/kg and 2.2 μg/kg, respectively. Average contents of OA in the samples of wheat and barley were 3.1 μg/kg and 2.4 μg/kg, respectively. The contents of mycotoxins corresponded to the allowed limits in food. During the studies of mutagenic activity of mycotoxins and their combinations by means of Ames test it was found that OA could increase the mutagenicity of AFB_1 in the case of their simultaneous occurrence in the same substrate. ***For free full paper in pdf format see http://www.vri.cz/vetmed.asp***

Keywords: mycotoxins; residua; cereals; Ames test; mutagenicity

INTRODUCTION

Mycotoxins as secondary metabolites of toxigenic moulds can represent a great risk for human and animal health. In addition to general toxicity, their biological effects include also immunosuppressive, estrogenic and genotoxic effects. Aflatoxin B_1 (AFB_1) and ochratoxin A (OA) belong to the most frequently occurring mycotoxins. This has repeatedly been demonstrated but the risk resulting from their simultaneous occurrence could not be assessed due to the lack of information about their combined biological effects.

AFB_1 is one of the most deeply studied mycotoxins known for a long time that belongs to the group of toxins produced by the genus *Aspergillus (A. flavus, A. parasiticus, A. nominus)* (Sargeant *et al*., 1961). Many authors described its hepatotoxic and hepatocarcinogenic effects in laboratory rats, mice, pigs, monkeys, ducks, trout and other animals (Wogan *et al*., 1971; Ball *et al*., 1995 and others). The metabolic transformation of AFB_1 plays an important role in its biological activity. The most important of the metabolites, 8,9-epoxide aflatoxin B_1, is formed in the organism by oxidation of cytochrome P450 monooxidases. It forms adducts with DNA and has a marked mutagenic activity (Guengerich *et al*., 1994; Bailey *et al*., 1996). The mutagenic effect of AFB_1 was confirmed by many tests carried out both *in vitro* and *in vivo*. In Ames test, this mycotoxin caused mutations after metabolic activation only (Ueno *et al*., 1978 and others). In different species of laboratory animals, the exposure to AFB_1 led to gene mutations, chromosomal aberrations, formation of micronuclei, sister chromatid exchanges in bone marrow cells and dominant lethal mutations (Bárta *et al*., 1990; Eaton and Groopman, 1994; McQueen and Way, 1991 and others).

Ochratoxin A is the metabolite of some mould species of the genera *Aspergillus* (*A. ochraceus*, *A. sulphureus* and others) and *Penicillium*, especially *P. viridicatum* (Lai *et al*., 1970). It is a strong nephrotoxin causing nephropathies in different species of monogastric animals, but many authors also described its hepatotoxic, teratogenic, carcinogenic and immunosuppressive effects (Bendele *et al*., 1985; Castegnaro *et al*., 1991). OA inhibits proteosynthesis, mitochondrial respiration and ATP formation and increases lipid peroxidation and formation of free radicals (Höhler, 1998; Fink-Gremmels *et al*., 1995; Marquardt and Frohlich, 1992). As a possible carcinogen for humans, the toxin was classified as 2B cancer compound (Petzinger and Ziegler, 2000).

The cereals belong to the substrates most frequently contaminated by moulds and mycotoxins. However, a high contamination of human food and animal feed by AFB_1 and OA occurs only exceptionally in climatic conditions of this country (Ruprich *et al*., 1991; Láníková *et al*., 1992). The cereals can be contaminated by AFB_1 and OA and their metabolites before the harvest, or dur-

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ing the harvest, or gradually during the storage and processing (Kurtzman *et al*., 1987). The genus *Aspergillus* requires a higher minimum temperature for the production of its metabolites and it is rather a storage mould in this country. The genus *Penicillium* is a mould occurring both in fields and stores and requires a higher content of water in the substrate at lower environmental temperatures (Jesenská, 1987).

Many papers have dealt with the effect of individual toxins, but the interactions between mould metabolites in food and feed have been reported very rarely. Huff *et al*. (1988) studied the combined effect of aflatoxin and ochratoxin in broiler chickens. Their effect was synergistic. The antagonistic effect of AFB_1 and OA was manifested by the fact that the number of liver lipids was not increased in the presence of OA, although in other cases this is a diagnostic symptom of aflatoxicosis (Huff and Doerr, 1981; Huff *et al*., 1983). The toxicity of some mycotoxin combinations was also tested in poultry and pigs, where AFB_1 together with OA were found to be most toxic (Huff *et al*., 1988).

The aim of our study was to assess whether AFB_1 together with OA can be present in the same malt barley and food wheat substrates and what is the possible risk of their simultaneous occurrence.

MATERIAL AND METHODS

Radioimmunochemical method (RIA)

The samples of wheat and barley used in our study were obtained from the experimental station of Czech University of Agriculture in Prague-Uhříněves. A total of randomly collected 30 samples of wheat and 30 samples of barley were tested. Mycotoxin residues in the samples were demonstrated by RIA method. The method according to Píchová *et al*. (1985) was used for AFB_1 and that according to Chu (1976) for ochratoxin. The studies were carried out in the radioimmunochemical laboratory of the Czech University of Agriculture in Prague. Rabbit antiserum against the conjugate of bovine serum albumin and I^{125} labeled radioligand were used for the analysis. After incubation with antiserum and AFB_1 the linked and free radioligands were separated using the suspension of activated carbon coated with dextran. Polyethylene glycol was used for separation in the case of OA. The chemicals were supplied by SIGMA-ALDRICH. The number of impulses of the linked fraction in individual samples and standards was determined by NA-3601 gamma automatic machine (Tesla Liberec, Czech Republic).

Ames test

Ames test (Maron and Ames, 1983) was applied for the determination of mutagenic activity of mycotoxins under study. Auxotrophic indicator his^- strains of *Salmonella typhimurium* TA98 and TA100 were used in our experiments. S9 fraction of liver homogenate from laboratory rat liver induced by Aroclor 1254 (mixture of polychlorinated biphenyls) was used for metabolic activation. This fraction was obtained from the Reference Laboratory for Ames test of the State Institute of Health, Prague. 2-aminofluorene as indirect mutagen (10.0 µg per plate) and 4-nitro-ortho-phenylenediamine (30.0 µg per plate, strain TA98) and sodium azide (10.0 µg per plate, strain TA100) as direct mutagens were used as reference mutagens (positive control). The tested mycotoxins (AFB_1 and OA) were dissolved in concentrated DMSO (dimethylsulphoxide). Mycotoxins and reference mutagens were supplied by SIGMA-ALDRICH. The numbers of revertants were counted by the colony counting of computer image analysis Lucia (Laboratory Imagin). The concentrations of mycotoxins and their combinations were tested on six plates at least.

Statistical evaluation of significant differences between the separate groups was carried out by *t*-test, the significance level being $\alpha = 0.05$.

Table 1. Residua of mycotoxins in samples of barley and wheat detected by RIA method

		n	Mean content (µg/kg) $\bar{x}$ ± SD	Maximum content (µg/kg)	Percentage of samples below detection limit	Acceptable contents (µg/kg)
AFB_1	barley	30	2.1 ± 1.4	4.0	13.3	NPM
	wheat	30	2.4 ± 1.4	4.6	16.7	20.0
OA	barley	30	2.4 ± 1.4	4.3	23.3	PM
	wheat	30	3.1 ± 0.8	4.5	0.0	10.0

Detection limits: AFB_1 0.3 µg/kg, OA 2.0 µg/kg
NPM: maximal acceptable contents in food
PM: acceptable contents in food (Decree of the Ministry of Health, No. 298/1997)

RESULTS AND DISCUSSION

The mean AFB_1 content in wheat samples demonstrated by RIA method was 2.4 µg/kg, the maximum value was 4.6 µg/kg. In 16.7% of samples the AFB_1 content was lower than the detection limit of RIA method (0.3 µg/kg). The mean AFB_1 in barley samples was 2.1 µg/kg, the maximum value was 4.0 µg/kg. In 13.3% of samples the AFB_1 content was lower than the detection limit of the used method.

The mean OA content in wheat samples was 3.1 µg/kg, the maximum value was 4.5 µg/kg. In none of the samples the OA values were lower than the detection limit of the method (2.0 µg/kg). The mean OA content in barley samples was 2.4 µg/kg, the maximum value was 4.3 µg/kg. In 23.3% of samples the OA content was lower than the detection limit of the method.

The data on the mean toxin content detected by RIA method, standard deviation, maximum value, and percentage of samples below detection limit are summarized in Table 1.

The mean AFB_1 and OA contents in wheat and barley samples demonstrated by RIA method met the requirements of the Decree of the Ministry of Health of Czech Republic (1997) as a part of the law on human food. Some of the samples contained the highest possible allowed amount of toxins (with the exception of baby food). However, it should be considered that both toxins were present in the same substrate, which might have changed their biological effects (Sokol *et al*., 1988).

In another part of our experiments, the mutagenic activities of AFB_1 and OA, and particularly their combinations were studied by means of Ames test using the detection system of auxotrophic strains of *Salmonella typhimurium* (Tables 2 and 3).

The increase in numbers of revertants induced by AFB_1 was statistically significant in comparison with the negative control at all concentrations of the strains TA98 and TA100 after metabolic activation with S9 fraction of liver homogenate.

Many authors also recorded the mutagenic activity of AFB_1 after metabolic activation only. AFB_1 is an indirect mutagen and the mutagenic activity is produced by the metabolite 8,9-epoxide aflatoxin B_1, which is formed from aflatoxin B_1 by oxidation with cytochrome P450 monooxidases and forms adducts with DNA (Guengerich *et al*., 1994; Ueno *et al*., 1978 and others). S9 fraction of liver

Table 2. Mean numbers of revertants in TA98 strain of *S. typhimurium* after treatment with AFB_1, OA and their combinations

Dose (µg/plate)	–S9			+S9		
	n	$\bar{x}$ ± SD	K	*n*	$\bar{x}$ ± SD	K
Aflatoxin B_1						
2.0	6	30.5 ± 4.2[a]	1.3	6	804.8 ± 207.7[b]*	26.1
1.0	6	31.7 ± 5.0[a]	1.3	6	1 091.2 ± 726.7[b]*	35.4
0.5	6	27.7 ± 8.9[a]	1.1	6	928.0 ± 286.0[b]*	30.1
0.25	6	28.0 ± 5.4[a]	1.2	6	666.3 ± 116.3[b]*	21.6
Ochratoxin A						
4.0	6	29.7 ± 4.2[a]	1.2	6	37.8 ± 3.3[a]	1.2
2.0	6	23.2 ± 2.5[a]	1.0	6	39.3 ± 6.1[a]	1.3
1.0	6	27.0 ± 4.0[a]	1.1	6	40.0 ± 4.6[a]	1.3
40.5	6	30.2 ± 4.5[a]	1.2	6	37.5 ± 5.3[a]	1.2
Aflatoxin B_1 + Ochratoxin A						
2.0 + 4.0	–	–	–	6	1 475.0 ± 213.0[b]*	47.8
1.0 + 2.0	–	–	–	6	1 971.0 ± 72.4[b]*	63.9
0.5 + 1.0	–	–	–	6	1 552.3 ± 176.1[b]*	50.3
0.25 + 0.5	–	–-	–-	6	991.5 ± 125.3[b]*	32.2
NK – concentrated DMSO						
	6	24.2 ± 5.9[a]	1.0	6	30.8 ± 6.9[a]	1.0

–S9 = without metabolic activation with S9 fraction of liver homogenate
+S9 = with metabolic activation
K = ratio of induced to spontaneous revertants (NK)
[a,b] statistically significant differences in the same column are indicated by different superscripts ($\alpha = 0.05$)
* statistically significant differences between the same concentrations of AFB_1 alone and AFB_1 and OA ($\alpha = 0.05$).

Table 3. Mean numbers of revertants in TA100 strain of *S. typhimurium* after treatment with AFB_1, OA and their combinations

Dose (μg/plate)	–S9			+S9		
	n	$\bar{x}$ ± SD	K	*n*	$\bar{x}$ ± SD	K
Aflatoxin B_1						
2.0	6	239.5 ± 21.3[a]	1.3	6	1 723.5 ± 97.0[b]	8.9
1.0	6	220.2 ± 19.0[a]	1.1	6	1 243.5 ± 434.4[b]	6.4
0.5	6	212.0 ± 25.2[a]	1.1	6	1 211.8 ± 201.6[b]*	6.3
0.25	6	195.7 ± 19.5[a]	1.0	6	1 447.8 ± 72.9[b]	7.5
Ochratoxin A						
4.0	6	181.7 ± 8.4[a]	0.9	6	180.8 ± 13.0[a]	0.9
2.0	6	186.3 ± 13.6[a]	1.0	6	202.7 ± 23.8[a]	1.0
1.0	6	181.8 ± 14.0[a]	0.9	6	178.8 ± 12.6[a]	0.9
0.5	6	185.7 ± 8.2[a]	1.0	6	189.2 ± 7.8[a]	1.0
Aflatoxin B_1 + Ochratoxin A						
2.0 + 4.0	–	–	–	6	1 213.7 ± 114.6[b]	6.3
1.0 + 2.0	–	–	–	6	1 059.2 ± 72.8[b]	5.5
0.5 + 1.0	–	–	–	6	1 499.2 ± 119.6[b]*	7.8
0.25 + 0.5	–	–	–	6	1 500.5 ± 156.3[b]	7.8
NK – concentrated DMSO						
	6	191.5 ± 12.0[a]	1.0	6	193.4 ± 14.0[a]	1.0

–S9 = without metabolic activation with S9 fraction of liver homogenate
+S9 = with metabolic activation
K = ratio of induced to spontaneous revertants (NK)
[a,b,] statistically significant differences in the same column are indicated by different superscripts ($\alpha = 0.05$)
* statistically significant differences between the same concentrations of AFB_1 alone and AFB_1 and OA ($\alpha = 0.05$).

homogenate has monooxygenase activity (Maron and Ames, 1983).

OA did not induce a statistically significant increase in the number of revertants in any of the two strains of *S. typhimurium* at any of the tested concentrations, not even with metabolic activation or without it. In contrast to AFB_1, the metabolic activation is not significant for the mutagenic effect of OA. It does not require enzymatic conversion of its structure (Moulé, 1984). Neither in the previous studies using Ames test was a statistically significant increase in the number of revertants found in strains TA98 and TA100, with metabolic activation or without it (Bárta *et al*., 1997). In *in vitro* systems, the mutagenic effect was observed in eucaryotic cells only (Castegnaro *et al*., 1991).

AFB_1 in combination with OA induced a statistically significant increase in the number of revertants at all concentrations in TA98 strain with metabolic activation, compared to negative control and OA alone. In comparison with AFB_1 alone, the combination of the two mycotoxins produced a statistically significant increase in the mutagenic effect too. In TA100 strain with metabolic activation, the combination of AFB_1 and OA induced a statistically significant increase in the number of revertants at all concentrations in comparison with the negative control and OA alone. However, in comparison with AFB_1 alone, the combination of both toxins induced a statistically significant increase in the number of revertants in the same strain at the concentration of 0.5 + 0.1 μg/plate only. At the two highest concentrations, the number of revertants induced by the two toxins together was even lower than that induced by AFB_1 alone. It is possible that this was caused by the toxic effect of higher concentrations of toxin combination in TA100 strain, which suppressed the possible mutagenicity.

The combined effect of AFB_1 and OA was also studied by Huff *et al*. (1988) in broiler chickens. The effect of toxins was synergistic. However, Huff and Doerr (1981) and Huff *et al*. (1983) observed an antagonistic effect of AFB_1 and OA, which was evident from the fact that the number of liver lipids did not increase in the presence of OA. In other cases, the increased number of lipids is a diagnostic symptom of aflatoxicosis. Sokol *et al*. (1988) reported that OA supports the carcinogenic effect of AFB_1.

The ability of ochratoxin A to increase the mutagenic effect of aflatoxin B_1 may be due to the relation of these two toxins to proteosynthesis. Inside the cell, OA induces the formation of genotoxic free radicals which in-

teract with the molecules of DNA, RNA and proteins and this leads to the inhibition of their synthesis. The effect of AFB_1 is involved in the transcription and that of OA in proteosynthesis during translation (Creppy *et al*., 1995).

A comparison of the two *Salmonella typhimurium* strains used for the Ames test shows that their reaction to the presence of the tested agents is not the same. In the opinion of Jurado *et al*. (1993) the TA100 strain is more sensitive. In our experiments, the mutagenic effect of the tested mycotoxins was more pronounced in TA98 strain whereas TA100 strain was found to be more sensitive to their toxic effect. Besides the difference in the type of mutation in histidine operon, the two strains also differ in other incorporated mutations which increase their sensitivity. The different reaction to the presence of a mutagenic agent may be due to the genetic difference between the non-isogenic standard strains (Maron and Ames, 1983).

Our results suggest that OA can significantly increase the mutagenic effect of AFB_1. The Decree giving the maximum allowed limits of mycotoxins in human food and animal feed only gives the maximum values for separate toxins and does not take into consideration their stronger combined effects. It is therefore necessary to revise it.

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Corresponding Author:

Ing. Mgr. Markéta Sedmíková, PhD., Czech University of Agriculture, 165 21 Prague-Suchdol, Czech Republic
Tel. +420 2 24 38 29 33, fax +420 2 20 92 03 15, e-mail: sedmikova@af.czu.cz