Combined effects of repeated low doses of aflatoxin B_1 and T-2 toxin on the Chinese hamster

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ABSTRACT: The aim of this work was to find out how repeated low doses of aflatoxin B_1 (AFB_1) and T-2 toxin would influence the Chinese hamster and if the amplifying of these effects would occur with the application of both toxins together. The animals were treated with 10 ml/kg of 7% dimethylsulfoxid (DMSO) in the control group (C), 1.0 mg/kg of AFB_1 in group A, 1.0 mg/kg of T-2 toxin in group T2, and 1.0 mg/kg of AFB_1 + 1.0 mg/kg of T-2 toxin in group T2/A. All mycotoxins were dissolved in 10 ml/kg of 7% DMSO. These doses were administered intragastrically twice a week for a period of three weeks. General health condition, histological picture of some internal organs, some biochemical blood serum indicators of liver and kidney functions, and leucogram were monitored. No differences in prosperity or weight gains appeared during the course of the experiment. The histological examination did not show any changes in the investigated organs in any experimental group. On the contrary, differences were found in the biochemical blood serum profile. ALT and AST activities decreased significantly in T2/A group animals compared with the other medicated groups (T2 – 24.46 μkat/l; 45.18 μkat/l; A – 18.17; 41.84; T2/A – 4.74; 14.21). A similar decrease appeared in GMT activity as well, but it was significant only in comparison with the T2 group (T2 – 0.6 μkat/l; T2/A – 0.25). ALP activity was increased in the experimental groups compared with the control, significantly in the T2 group (C – 5.0 μkat/l; T2 – 6.92). LDH activity was lower in the T2 and T2/A groups, significantly when the T2/A group was compared with the A group (A – 94.05 μkat/l;; T2/A – 37.48). The cholesterol level was significantly increased in group A compared with the C and T2 groups. A smaller increase in the T2/A group was significant when compared with the T2 group as well (C – 3.05 mmol/l; T2 – 2.85; A – 3.59; T2/A – 3.27). Total and conjugated bilirubin concentrations decreased in group order A – C – T2 – T2/A, when differences among the A, T2 and T2/A groups were significant (T2 – 1.0 mmol/l; 0.36 mmol/l; A – 2.36; 0.85; T2/A – 0.69; 0.21). A glycemia decrease in medicated groups was significant in the T2/A group, while it approached a significant level in the T2 group (C – 10.46 mmol/l; T2 – 9.01; T2/A – 8.91). The main liver condition indicators seemed to be influenced by the T-2 toxin and AFB_1 combination more than by individually applied toxins. We assume the amplification of the mycotoxin effects on proteosynthesis. The ALT activity especially was probably influenced more than in the additive manner. All the medicated groups showed a significant increase in the monocyte percent count (T2 – 9.8%; A – 9.62; T2/A – 8.85; C – 6.65). The differences observed in other leucocyte types were not significant. There were no differences in the effects of individual mycotoxins and their combination on the leucogram level.

Keywords: mycotoxin; trichothecens; interaction; genotoxicity; biochemistry; differential count; histology

INTRODUCTION

Since the time when the risks associated with mould contamination of foods and feeds wetre recognized, a series of measures to reduce the risk of acute mycotoxicoses to minimal levels has been introduced. Nevertheless, relatively frequent contamination of materials with low doses of mycotoxins remains (Moss, 1996; Clear *et al*., 2000; Fazekas *et al*., 2000). The presence of two or more mycotoxins simultaneously in one substrate is also common (Gonzalez *et al*., 1999; Desjardins *et al*., 2000) regardless of the multicomponental composition of human and animal nutrients. This leads to the intake of various mycotoxin combinations, the common effects of which on the animal organism may differ from the effects of the same amount of these mycotoxins received individually (Prelusky *et al*., 1994).

Aflatoxin B_1 (AFB_1) is a strong hepatotoxin affecting a number of other organs and body systems, as well as a potent mutagen and carcinogen (Eaton and Groopman,

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1994). On the contrary, data about mutagenity of trichothecene mycotoxins are ambiguous in studies both *in vitro* and *in vivo*. The mutagenic effect of these mycotoxins has not been proved in most of these studies (for example – Zhu *et al*., 1987). However, cytotoxic and immunotoxic effects or/and organ toxicity of trichothecens are well known (Prelusky *et al*., 1994; Pestka and Bondy, 1994; Iverson *et al*., 1995; Huszenicza *et al*., 2000). T-2 toxin is considered to be one of the most toxic representatives of this group (IARC, 1993).

In studying the effects of mycotoxins we focused on the effect of low toxin doses because in higher doses the genotoxic effects are often hidden by the toxic effect of the toxin (Haschek, 1989).

The aim of the presented work was to evaluate single and common effects of repeated low doses of AFB_1 and T-2 toxin on general health conditions, a histological picture of selected organs, biochemical markers in the blood serum, and leucogram in the Chinese hamster.

MATERIAL AND METHODS

Model animals

48 Chinese hamster males 6–8 weeks old were used in the experiment. The animals came from the race HsdHan : CHIN, a line imported from England in 1995 and further bred at the 3rd Faculty of Medicine, Charles University. The animals were housed in a battery breeding system bedded with shavings, with a controlled light regime of 12/12 hours, temperature of 20°C ± 2°C, relative humidity of 60% ± 10% and complete air recirculation 10–14 times per hour. The animals were supplied with water *ad libitum* and were fed with a commercial granulated mixture for laboratory rodents with the addition of animal proteins.

Conducting of the experiment

The animals were divided into four groups and treated with 10 ml/kg of solution by peroral intubation twice a week for three weeks as follows:

– group A: 13 animals – 1.0 mg AFB_1 per kg dissolved in 7% DMSO (dimethylsulfoxide),
– group T2: 13 animals – 1.0 mg T-2 toxin per kg dissolved in 7% DMSO,
– group T2/A: 14 animals – 1.0 mg AFB_1 per kg and 1.0 mg T-2 toxin per kg dissolved in 7% DMSO,
– group C (control): 8 animals – 7% DMSO alone.

All mycotoxins and chemicals used were supplied by Sigma – Aldrich, Ltd.

All animals were treated intraperitoneally with 0.1 ml of 0.04% colchicine per kg two hours before they were destroyed. The animals were destroyed in a halotan anesthesia by heart-puncture exsanguination 24 hours after the last application of mycotoxins.

The animals were weighed at the time of each treatment, and their general health status was monitored through the whole experiment. After exsanguination a necropsy was performed. The material for the pathohistological examination was fixed in 4% formaldehyde for 2 hours and further processed by standard histological procedures using hematoxilin-eosin staining, or, in indicated cases, PAS reaction as well. Samples of the liver, lung, kidney, spleen, pancreas, jejunum, thymus and testicles were taken for the examination. The biochemical examination of blood serum was performed with the automatic analyser RAXT Technicon by Bio-La-Tests. In control group 2 samples were not analysed due to strong haemolysis. The following parametres were monitored: urea nitrogen (urea), creatinine (crea), total and conjugated bilirubin (bilir and bilir-k), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyltransferase (GMT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), cholesterol (cholest) and glucose (gluc). The leukogram was read from blood smears prepared during the animal exsanguination and stained with a Pappenheim-type stain.

Statistical processing of the data

Statistical processing of the data was performed with Excel 97 and Statgraphic softwares. The differences between the individual experimental groups were measured by corresponding *t*-test after previous evaluation of the variance consonance on the bases of the *F*-test. The values tested as extreme deviations and impairing the normal distribution frequency of the data were excluded from the statistical processing in isolated cases. The differences with $P(H_0) < 0.05$ were evaluated as statistically significant, the differences with $0.1 < P(H_0) > 0.05$ as almost statistically significant.

RESULTS AND DISCUSSION

No differences in prosperity or weight gains appeared during the course of the experiment. No deviations from the norm were found either by necropsy or by histological examination of selected organs.

The obtained values of the biochemical parametres are presented in Table 1.

Exposure to aflatoxin B_1 induced a significant elevation of the cholesterol concentration compared to the control. The value deviations of the other observed parametres were insignificant. The elevation of the cholesterol level after AFB_1 administration in our experiments does not correspond fully to the written data indicating rather its decline (Miller and Wilson, 1994; Harvey *et al*., 1995a;

Table 1. Serum biochemical values of Chinese hamsters treated with aflatoxin and/or T-2 toxin. The values of each serum constituent designated with the same index do not differ at the significance level $P = 0.05$

	Group	No.	Average	Variance		Group	No.	Average	Variance
Urea (mmol/l)	C	6	13.39[a]	1.895	LDH (μkat/l)	C	6	63.78[ab]	1 181
	T2	13	12.90[a]	9.774		T2	11	43.83[ab]	709
	T2/A	14	12.06[a]	10.827		T2/A	12	37.48[a]	979
	A	13	13.07[a]	9.132		A	13	94.05 [b]	6 852
Crea (μmol/l)	C	6	98.65[a]	2 382	Bilir (μmol/l)	C	6	1.35[abc]	0.932
	T2	13	130.45[a]	2 534		T2	11	1.00[a]	0.060
	T2/A	14	121.17[a]	3 168		T2/A	12	0.69 [b]	0.049
	A	13	119.49[a]	2 225		A	13	2.36 [c]	1.730
ALT (μkat/l)	C	6	16.83[ab]	160.7	Gluc (mmol/l)	C	6	10.46[a]	2.807
	T2	12	24.46[a]	513.8		T2	13	9.01[ab]	1.729
	T2/A	11	4.74 [b]	14.1		T2/A	14	8.91[b]	1.335
	A	12	18.17[a]	327.3		A	13	9.26[ab]	3.310
AST (μkat/l)	C	6	39.58[ab]	625	Cholest (mmol/l)	C	6	3.05[ab]	0.026
	T2	12	45.18[a]	1 486		T2	13	2.85[a]	0.314
	T2/A	11	14.21 [b]	113		T2/A	13	3.27 [bc]	0.106
	A	12	41.84[a]	1 781		A	13	3.59 [c]	0.255
GMT (μkat/l)	C	6	0.82[ab]	0.398	Bilir-k (μmol/l)	C	6	0.54[abc]	0.310
	T2	13	0.60[a]	0.276		T2	11	0.36[a]	0.006
	T2/A	14	0.25[b]	0.076		T2/A	12	0.21 [b]	0.006
	A	13	0.55[ab]	0.327		A	13	0.85 [c]	0.299
ALP (μkat/l)	C	6	5.00[a]	1.067					
	T2	13	6.92[b]	3.434					
	T2/A	14	6.39[ab]	6.268					
	A	13	5.96[ab]	3.636					

Edrington *et al*., 1997; Verma *et al*., 1998). Nevertheless, the dose of 1 mg/kg of AFB_1 administered twice a week only, as performed in our experiments, was relatively low in comparison with the doses used in the cited papers. Moreover, the Chinese hamster is relatively resistent to AFB_1 effects in comparison with other laboratory animal species. Consequently, the beginning of liver metabolism destabilisation, or a phase of increased lipid mobilisation only, might be involved. The conclusion that the scheme of medication used in our experiments did not elicit any serious defects of the liver or the kidneys is also supported by negative findings of the other monitored parametres, whereas the previously cited authors as well as a number of others (Harvey *et al*., 1995b, c; Zaky *et al*., 1998) reported significant alterations of the blood serum biochemical profile (especially in transaminase activity).

T-2 toxin application evoked in comparison with the control a significant increase in ALP activity, and glucose serum concentration dropped to nearly the significance level ($P = 0.0567$). The other biochemical indicators were not significantly influenced. Regarding opposite trends of influencing the LDH activity and cholesterol concentration values by the application of T-2 toxin (decrease) and AFB_1 (increase), groups T2 and A differed almost significantly ($P = 0.0566$) or significantly in these parametres. According to Smith (1992), the site of the main T-2 toxin toxic effect is the liver, since the enzymatic activities of the mitochondrial electron transport system and cytochrom P-450 decrease. The author makes a connection between at least some of these changes and the affected proteosynthesis. Edrington *et al*. (1997) observed a decrease of cholesterol and urea levels, mean corpuscular volume, and weight gains in the 2nd and 3rd experimental weeks, and an increased incidence of oral lesions in chickens, fed with a T-2 toxin-contaminated mixture. However, the same authors found no significant deviations from the control except a lowered weight gain and the occurrence of oral lesions in further experiments with the same T-2 toxin doses – 5, 6, and/or 8 ppm (Kubena *et al*., 1997, 1998). Hoehler and Marquardt (1996) observed besides a lower feed intake and weight gain in chickens fed with 4 and/or 5 ppm of T-2 toxin in the diet a simultaneous feed conversion

rate increase and a decrease of vitamin E content in liver. The monitored biochemical parametres of blood serum were not influenced. The authors conclude from the tocopherol level change that peroxidation processes are involved in T-2 toxin effects. Mezes *et al*. (1999) arrived at a similar conclusion. Niyo *et al*. (1988) after a three-week medication of rabbits and Pang *et al*. (1987) after a single-toxin application to pigs observed a lower ALP activity. A decrease of the glucose and albumine concentration levels further appeared in pigs. Our findings do not correspond fully to all the cited results, but they indicate mild hepatic affection.

The administration of T-2 toxin/AFB_1 combination significantly decreased the glucose concentration compared to the control. The decline in ALT, AST and GMT activity along with the increase in ALP activity approached the level of significance (P = 0.0712; 0.0554; 0.0772; 0.0953). The influence on ALT, AST, total and conjugated bilirubin and essentially also on GMT was greater after the administration of the toxins combination than after individual applications. This might be connected at least in part with both toxins' effects on proteosynthesis. LDH activity was comparable with findings caused by the administration of T-2 toxin alone, but it was significantly different from the A group. Glycemia was influenced by application of single toxins and their mutual combination to the same extent, although compared to the control, its decrease was statistically significant only in the T2/A group. ALP activity and cholesterol concentration after influencing with the T-2 toxin/AFB_1 combination were found between values induced by application of the single T-2 toxin or AFB_1, when the cholesterol level differed significantly or almost significantly (P = 0.0717) from these groups. The monocyte percentage value increase was lower than after the administration of individual toxins. A combination of the same mycotoxins was tested by Tamimi *et al*. (1997) in rats. They observed a synergistic effect on most of the tested parametres. Similarly, Harvey *et al*. (1990) found an increased effect of this combination in pigs, but it was evaluated as less than additive. On the contrary, Huff *et al*. (1988a) assessed the effect of both toxins in chickens as synergistic at least in some parameters. In another work the same authors (Huff *et al*., 1988b) evaluated this mycotoxin combination as one of the most effective among mycotoxin combinations tested in pigs and poultry. Kubena *et al*. (1997) found an increased effect of fumonisin B_1 and T-2 toxin combination on the basis of some indicators (feed intake, mortality, stomach and pancreas relative weight), although these toxins affected antagonistically, for example, oral lesions. The results of our observations show certain differences in the method of influencing individual monitored parametres. However, it seems that the crucial indicators of the hepatic state were influenced by the combination of the T-2 toxin and AFB_1 more than by individual toxins. Especially ALT and AST activities seem to be influenced to a more than additive extent.

All the medicated groups showed a significant increase in the monocyte percentual count. The differences observed in other leucocyte types were not significant. The obtained leukogram values are summarized in Table 2.

Table 2. Leucogram of the experimental animals. The values of each blood cell type designated with the same index do not differ at the significance level P = 0.05

	Group	No.	Average	Variance
Neutrophils	C	8	30.94[a]	41.346
(%)	T2	13	31.11[a]	28.852
	T2/A	14	33.00[a]	57.674
	A	13	32.70[a]	23.738
Eosinophils	C	8	3.95[a]	0.403
(%)	T2	13	3.66[a]	0.436
	T2/A	14	3.36[a]	0.579
	A	13	3.43[a]	0.586
Basophils	C	8	0.33[a]	0.202
(%)	T2	13	0.47[a]	0.207
	T2/A	14	0.49[a]	0.199
	A	13	0.21[a]	0.156
Lymphocytes	C	8	58.14[a]	43.620
(%)	T2	13	54.96[a]	52.521
	T2/A	14	54.29[a]	68.544
	A	13	54.04[a]	30.304
Monocytes	C	8	6.65[b]	0.831
(%)	T2	13	9.80[a]	4.872
	T2/A	14	8.85[a]	3.543
	A	13	9.62[a]	3.335

According to other authors (Ray *et al*., 1986; Miller and Wilson, 1994; Fernandez *et al*., 1996) AFB_1 effects on hematological indicators were reflected rather in changes of the red blood picture and packed cell volume, but changes of the white blood picture were repeatedly observed as well – an increase in the leucocyte and neutrophile granulocyte numbers and a decline in the lymphocyte and monocyte numbers and/or a decrease of the lymphocytes/neutrophiles ratio (Harvey *et al*., 1995a; Kececi *et al*., 1998). Nor did we observe such an effect in the form of an insignificant trend, but rather, on the contrary, a significant increase of monocytes. In view of the absence of data concerning the general leucocyte count, it is impossible to define what process is involved in this finding.

As to the T-2 toxin effects, Corrier and Ziprin (1987) state that T-2 toxin induces lymphopenia and lowers neutrophil chemotactic migration. Niyo *et al*. (1988) observed

decreased mean corpuscular volume, leucocyte count, and – just below the significance level – decreased neutrophile count in rabbits after a 3-week administration of 0.75 mg/kg/day of T-2 toxin. Similarly, Gentry *et al*. (1984) found a distinct decrease in leucocyte count corresponding to a neutrophile decrease after a single application of T-2 toxin to calves. On the contrary, Pang *et al*. (1987) found marked neutrophilia after a single T-2 toxin administration. Hayes *et al*. (1980) observed anemia, lymphopenia and eosinopenia in mice after 3 weeks. However, granulocytopoesis and erythropoesis regeneration occurred later. It is probable that due to the lack of data on leucocyte number, the changes observed in our experiments were based on the neutrophile and/or lymphocyte decrease rather than on the real increased number of monocytes.

In an other experiment with the same scheme of medication, we observed that the percentage of aberrant medular cells in all medicated groups was significantly higher than in the control group, but the differences between the individual types of toxic stress were not significant (unpublished data).

Many of the tests performed *in vitro* and/or *in vivo* confirmed the mutagenic activity of aflatoxin B_1. It induced sex-fixed recessive lethal mutations and somatic mutations in *Drosophila melanogaster* (Nix *et al*., 1981). In addition, DNA breaks in cell cultures *in vitro*, unscheduled DNA synthesis in different animal and human cell types *in vitro*, gene mutations, chromosomal aberrations, and sister chromatides exchanges in tissue cultures of the Chinese hamster, human lymphocytes and fibroblasts *in vitro* were observed after aflatoxin B_1 influence (McQueen and Way, 1991). In *in vivo* systems it induced chromosomal aberrations, micronuclei formation, and sister chromatides exchanges in bone medullar cells of various laboratory animal species and dominant lethal mutations in laboratory mice and rats (Bárta *et al*., 1990).

We found a significant increase in the frequency of chromosomal aberrations in the T-2 toxin treated group compared to the control (unpublished data). Interestingly, in another study performed on mice, a single T-2 toxin application did not induce any significant increase in the micronucleus count in the bone marrow polychromatophile erythrocytes compared to the control group (Bárta *et al*., 1997). Also Norppa *et al*. (1980) did not observe any increased micronucleus occurrence in the Chinese hamster bone marrow after a single *i.p.* T-2 toxin application of a similar dose. On the contrary, Haschek (1989) states that a moderate increase in the chromosomal aberration frequency in the bone marrow cells was observed in Chinese hamsters after a single T-2 toxin *i.p*. administration of similar doses. The effect did not depend on the applied dose. Zhu *et al*. (1987) observed a higher incidence of micronuclei in Chinese hamster fibroblast culture after exposure to T-2 toxin as well. In cultivated Chinese hamster hepatocytes T-2 toxin induced gene mutations, sister chromatide exchanges, and chromosomal aberrations (Oldham *et al*., 1980). Also, other authors observed the mutagenic effect of T-2 toxin in some cases only. Lafarge-Frayssinet *et al*. (1981) state that no DNA damage occurred in hepatocytes after T-2 toxin administration to mice. Single DNA strand breaks were observed in the spleen and thymus lymphocytes. In *in vitro* studies, no nuclear DNA changes were found in the rat hepatocytes. However, DNA damage was observed in the spleen and thymus lymphocyte cell cultures after T-2 toxin treatment.

The combined medication of hamsters led to an increase in the frequency of chromosomal aberrations as compared to the control, but there were no differences in comparison with the effects of individual toxins (unpublished data). However, in a study on mice (in different application schemes) significantly higher micronucleus frequency was found in polychromatofile bone marrow erythrocytes after treatment with a T-2 toxin and AFB_1 combination compared to the effect of AFB_1 alone (Bárta *et al*., 1997).

Neither T-2 toxin nor AFB_1 induced under the conditions of the medication scheme used any changes detectable clinically, by dissection or by basic histological examination. There were no major alterations in the monitored biochemical parametres, only signs of mild hepatic alteration appeared. It would be necessary to complete at least some hematological parametres to evaluate the increased monocyte percentage in all medicated groups. Neverthless, a mutagenic effect appeared in all medication variants. Along with this, the results of biochemical screening point to an evident interaction of both mycotoxins under study in the case of their simultaneous administration, although the amplification of their genotoxic effect was not demonstrated. This emphasizes the importance of risks connected with food and feed contamination by low mycotoxin doses.

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