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Toxic effects of *Ustilago maydis* and fumonisin B_1 in rats

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The toxicity of *Ustilago maydis* and the possible synergism with fumonisin B_1 (FB_1) were studied in Fischer rats by evaluating pathological changes and biochemical parameters in blood serum (LDH, ALT, GGT, ChE) and tissue homogenate of brain and liver (AChE, ChE, GGT, ALP). One experimental group (US) consumed diet with 70% of *U. maydis* galls and the other group (US+FB_1) was fed pellets containing 70% of *U. maydis* galls and 1 mg of FB_1 per kg of diet for 17 days. Control group (C) consumed standard pellets. During the trial, experimental animals were more excited, showing hyperactivity. Body mass gains slightly increased in both groups compared to the control. Gross pathological changes in liver, lungs, uterus and ovaries were more pronounced in the US+FB_1 than in the US group. Specific catalytic activities of AChE decreased by 61% and by 63% in the liver and brain homogenate of the US group ($p < 0.05$) compared to the control, indicating neurotoxic activity of *U. maydis*. Also, specific catalytic concentration of AChE and ALP was significantly decreased in the liver of the US+FB_1 group ($p < 0.05$). Activity of LDH in the blood serum was increased up to 166% and 165% in the US+FB_1 group ($p < 0.05$) compared to the control and US group values, respectively, which indicates that FB_1 was responsible for the disruption of cell membrane integrity. These findings suggest that *Ustilago maydis* and FB_1 showed neurotoxicity in Fischer rats, which could be related to the alkaloids of *U. maydis* and disruption of sphingolipid metabolism by FB_1 activity.

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Ustilago maydis is a facultative biotrophic basidiomycete, causing smut disease in maize. A hallmark of the disease is the induction of plant tumours (galls) filled with masses of black teliospores. *Ustilago maydis* infection can inhibit plant development,

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leading to necrosis, hyperplasia and hypertrophy of infected organs (1). Earlier studies observed inflammation of the respiratory system, lung emphysema, neurotoxicity, liver and kidney necrosis, hyperplasia of small intestine and reproductive toxicity in various animals (rats, horses, cows, pigs, poultry) fed with *Ustilago* species (2–5). The corn smut fungus produces several secondary metabolites including ustizein, guanacine, itaconic and ustilagic acid, alkaloids ustilagin and trimethylamine (6–8), which could be related to the described pathology in animals. The P4 strain of *U. maydis* secrets a toxin KP4, encoded by a fungal virus. This toxin was shown to inhibit the calcium channel activity in mammalian cells (9). Nowadays, there is some disagreement on *U. maydis* toxicity. In Mexico and other Latin American countries, *U. maydis* has been traditionally used as human food named huitlacoche (10). On the one side, there is the nutrient potential of *U. maydis* galls that includes carbohydrates, proteins, fats, vitamins and minerals, and on the other side, its possible toxic effect on animals and humans.

Fumonisin B_1 (FB_1) is a mycotoxin produced mainly by *Fusarium verticillioides*, a common contaminant of maize and other grains (11). This mycotoxin has been associated with various diseases in animals and humans, including equine leukoencephalomalacia, porcine pulmonary edema, immunosuppression, liver and kidney toxicity, liver cancer, and has been connected with human oesophageal carcinoma in Southern Africa and China (12–16). A proposed mechanism of FB_1 toxicity based on its ability to inhibit the biosynthesis of ceramide and complex sphingolipids through inhibition of the enzyme ceramide synthase (17). Several studies in animals (rats, mice, rabbits, swine, horses, fish) fed high and low doses of FB_1 observed certain changes in haematological and biochemical parameters, such as lower haematocrits, reduction of red and white blood cell counts, abnormal erythrocyte formation, increase of liver enzyme activities, increase of biochemical parameters of kidney injury and increase in serum cholesterol (18–22). The importance of FB_1 as a toxic agent has been well studied in various animal models, especially rats, but its possible synergism with other co-occurring mycotoxins and/or frequent fungal contaminants of maize, such as *U. maydis*, remains a poorly investigated area.

The work reported here was undertaken to investigate the toxicological profile of *U. maydis* in Fischer rats and its possible synergism with FB_1 by evaluating pathological changes and some biochemical parameters.

EXPERIMENTAL

Materials

Ustilago maydis galls were collected in maize-growing fields near Zagreb (Granešina) in February, 2003, one month before the experiment.

Fumonisin B_1 was isolated and detected according to the established method by Pepeljnjak *et al.* (22). Briefly, *F. vertocillioides* (syn. *moniliforme*) cultures growing on YES (yeast exstract 20 g, suchrose 40 g, sterile water 1000 mL) were homogenised and portions of 50 mL were extracted with 50 mL acetonitrile/water (9:1) for 10 min and then filtered. Filtrate (50 mL) was extracted with *n*-hexane (2 x 25 mL). Upper hexane phase was discarded and the water-soluble phase, adjusted to pH 8–9 with 25 mL of saturated

$NaHCO_3$, was then shaken with 25 mL of chloroform for subsequent purification. Upper water-soluble phase was partially evaporated at 80 °C and then concentrated in vacuum by lyophilisation. Lyophilisate was dissolved in acetonitrile/water (1:1) and analysed with an FB_1 commercial sample on silica gel GF_{254}. Plates were developed in acetonitrile/toluene/water (93:5:2). Visualisation of FB_1 was performed under UV light (366 nm). The white crystalline material was tested for purity by thin-layer chromatography on silica gel GF_{254}.

Animals and treatment

Fifteen adult female Fischer rats weighing approximately 200 g were obtained from the Animal Unit of the Faculty of Pharmacy and Biochemistry, Zagreb, Croatia, and housed five per cage. The rats were given food and water *ad libitum* (standard laboratory pellets, declared not to contain mycotoxins, PLIVA d.d., Croatia). Animals were divided in two experimental (n = 2 x 5) and one control (n = 5) group. First experimental group (US) consumed diet containing 70% *U. maydis* galls homogenised with pellets; the second experimental group (US+FB_1) consumed diet containing 70% *U. maydis* galls and 1 mg of FB_1 per kilogram of diet (total FB_1 amount = 2 mg kg^{-1} of b.m.); control group (C) consumed standard pellets. Body weight was recorded on the first and the last day of the experiment, which was terminated after 17 days.

Animals were sacrificed by cervical dislocation after light ether anaesthesia. Pathological changes in the liver, kidneys, brain, lungs, spleen, intestine, heart, uterus and ovaria in experimental groups were visually examined and compared to the control.

Experiment was ethically approved by the Ethics Committee of Faculty of Pharmacy and Biochemistry, University of Zagreb.

Preparation of liver and brain tissue homogenates

Liver and brain tissues were used to make homogenates in 10 g L^{-1} concentrations. Tissues were quickly excised and homogenised at 4 °C in an Ultra-Turrax homogenizer in cold 0.32 mol L^{-1} sucrose buffered with 0.05 mol L^{-1} Tris-HCl medium supplemented with phenylmethyl-sulphonyl-fluoride as a proteinase inhibitor, pH 7.4, two times at 12000 rpm for 30 s.

Analytical methods

Acetylcholinesterase (acetylcholine acetyl-hydrolase, AChE, EC 3.1.1.7) activity was determined by a colorimetric assay. Enzyme activity was calculated via acetylcholine (ACh) concentrations at the start (ACh 0.5 mmol L^{-1}) and after substrate incubation in phosphate buffered solution (pH 7.2) at 37 °C for 30 min. ACh concentration was estimated by the method of Augustinsson (23). Cholinesterase (acylcholine acyl-hydrolase, ChE, EC 3.1.1.8) activity was determined according to Ellman *et al.* (24). Gamma glutamyl transferase (GGT, EC 2.3.2.2) activity was determined according to Szasz (25). The activity of alkaline phosphatase (ALP, EC 3.1.3.1) was determined by the kinetic method of McComb and Bowers (1972) on a Pye Unicam SP 8-100 UV/Vis spectrophotometer. Lactate dehydrogenase (LDH, EC 1.1.1.27) activity was determined using 50 μL of blood serum and 2 mL of working reagent (61.43 mmol L^{-1} Tris buffer, 0.20 mmol L^{-1} NADH,

pH 7.4) and 10 μL of substrate (21.5 mmol L^{-1} pyruvate). Absorbances were measured at 340 nm every 30 s over a 5-min time period on the spectrophotometer. The activity of alanine aminotransferase (ALT, EC 2.6.1.2) was determined by the UV kinetic method according to IFCC recommendation, using a commercial test manufactured by Herbos Dijagnostika. Protein concentrations in liver and brain homogenates were determined by the method of Lowry *et al.* (26).

The data presented as mean values and standard deviation were statistically analysed by the Kruskal-Wallis test followed by a multiple comparison procedure (Dunns test). The level of $p < 0.05$ was considered statistically significant for all tests performed.

RESULTS AND DISCUSSION

All animals survived to the end of the study. From the first to the seventh day, the behaviour of animals was similar in each group while from the seventh day to the end of experiment feed consumption was increased in experimental groups and animals were more excited, showing hyperactivity. At the end of the study, body mass gains unsignificantly increased in both experimental groups (Fig. 1). These findings could be attributed to the nutrient potential of *U. maydis* (carbohydrates, proteins, fats), stimulation of appetite and metabolism. Previous studies on Leghorn chickens also revealed an increase in body mass after feeding 2.5–10% of *U. maydis* (4).

Gross examination of dissected organs (Table I) revealed significant pathological changes in the liver, kidneys, brain, lungs, stomach, intestine, uterus and ovaries. Pathological changes in the liver, lungs, uterus and ovaries were more pronounced in the group treated with both *U. maydis* and FB_1. In the chickens fed *U. maydis* only, a slight chronic catarrh with hyperplasia of glandular stomach and small intestine was found (4). Outbreaks of pneumonia in cattle in Bulgaria were described as a consequence of *U. maydis* spores inhalation. The animals in these outbreaks had lung emphysema and a mass of teliospores was found in their lungs. Convulsions and cerebral meningitis with spores present in the brain were found in rats and dogs treated with *U. maydis* (2). In our previous unpublished experiment done in 2001 with three Fischer rats fed the same amount of *U. maydis* for 12 days, some severe neurotoxic symptoms were manifested such as body stiffness, and the left-side twisted head of one animal. In the present study,

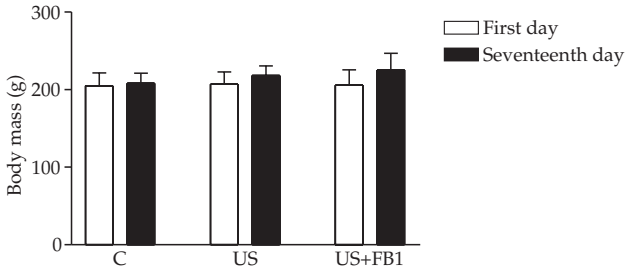

Fig. 1. Effect of *U. maydis* and FB_1 on body mass gain in Fischer rats (C – control group, US – group treated with *Ustilago maydis*, US+FB_1 – group treated with *Ustilago maydis* and fumonisin B_1).

Table I. Gross pathological changes in Fischer rats fed U. maydis *and* FB_1

	Control	US	US+FB_1
Liver	compact, brown, normal	dark brown, hyperaemic	yellow discoloration, lipid infiltration, hyperaemic
Kidney	solid capsule, dark red, normal	shrieked, hyperaemic	shrieked, hyperaemic
Brain	grey, visible capillaries, no bleeding	hyperaemic	hyperaemic
Heart	normal	normal	normal
Lungs	light red, no bleeding	hyperaemic	swollen, hyperaemic
Stomach and intestine	stomach mucous smooth, pale red, intestine normal pale yellow	stomach and intestine hyperaemic, fragile to touch	stomach and intestine hyperaemic, fragile to touch
Spleen	normal	normal	normal
Ovaries and uterus	few Graff follicles, uterus pale red	swollen ovaries, uterus hyperaemic	swollen, hydropic, hyperaemic ovaries, many Graff follicles, uterus hyperaemic

such symptoms were not observed in any animal probably due to lower toxic potential of *U. maydis*. More severe symptoms were found in the liver, lungs, uterus and ovaries of the US+FB_1 group, which could be attributed to the synergistic effect of *U. maydis* and FB_1, which is known to induce hepatotoxicity, pulmonary oedema and reproductive toxicity in animals (20, 27, 28).

In order to examine the effects of *U. maydis* and FB_1, we determined the catalytic activities of AChE, ChE, ALT, ALP, GGT and LDH in liver and brain tissue homogenates as well as in the blood serum of the experimental animals. In the liver tissue homogenate, significant decreases in catalytic activity of AChE and ALP were found in both experimental groups and the US+FB_1 group, respectively ($p < 0.05$) (Fig. 2). ChE and GGT activities were not significantly different from the control values. In the brain tissue, significant decreases of AChE and GGT activities were recorded only in the US group ($p < 0.05$) (Fig. 3). LDH significantly increased and ALT was significantly decreased in the blood serum samples of the US+FB_1 group (Table II). Our results showed that the specific catalytic activities of AChE in the liver and brain homogenate of the US group decreased by 61% and 63% compared to the control, respectively. Specific catalytic concentration of this enzyme also decreased in the US+FB_1 group liver homogenates. AChE is a specific cholinesterase located in the synapse, especially on postsynaptic membrane, but it can be also found in lungs, spleen and erythrocytes. Alkaloids and organophosphoric compounds are well known inhibitors of AChE activity, but numerous molecules are moderators of this enzyme as well. Inhibited activity of AChE results in increased concentration of acetylcholine, which reflects on the experimental animal behaviour. The treated animals seem hyperactive. Chronic inhibited activity of AChE can lead to neurological disorders. Secondary metabolites of *U. maydis* include alkaloids, some of which could be considered potentially toxic and they can influence, directly or indirectly, the activity of AChE.

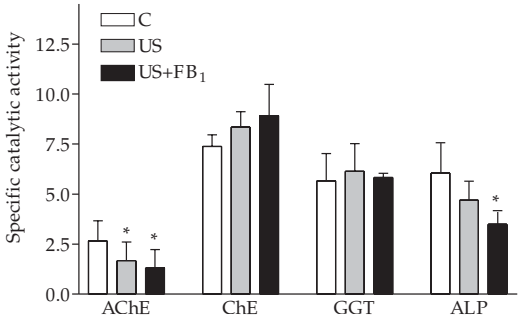

Fig. 2. Specific catalytic activities of acetylcholinesterase (AChE), cholinesterase (ChE), gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) in liver tissue of control and treated rats. Values are mean ± SD. Enzyme activity for AChE and ChE are given in μmol min^{-1} per miligram of protein × 10^{-3}. Enzyme activity for GGT and ALP are given in μmol min^{-1} per miligram of protein.
* Statistical significance of the difference between treated and control rats at the confidence level $p < 0.05$ (C – control group, US – group treated with *Ustilago maydis*, US+FB_1 – group treated with *Ustilago maydis* and fumonisin B_1).

LDH is the enzyme of the cytoplasm and its increased activity in blood serum is present in damaged plasmatic membranes of cells in different tissues. In this study, significantly increased catalytic activities of LDH were found in the US+FB_1 group (166% and 165%), when calculated according to the control values and the US group values, respectively. The activity of LDH is increased only in the US+FB_1 group while the LDH activity in the US group is almost equal to the control. This leads to the conclusion that FB_1 is responsible for the disruption of cell membrane integrity. At the same time, *U. maydis* shows no cytotoxic effects.

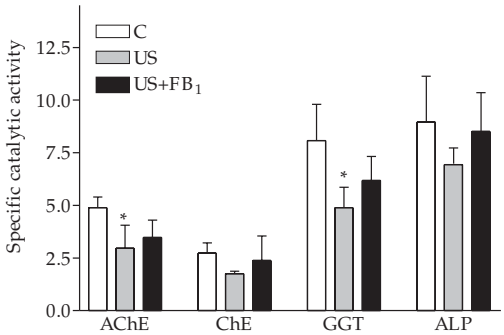

Fig. 3. Specific catalytic activities of acetylcholinesterase (AChE), cholinesterase (ChE), gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) in brain tissue of control and treated rats. Values are mean ± SD. Enzyme activity for AChE and ChE are given in μmol min^{-1} per miligram of protein × 10^{-3}. Enzyme activity for GGT and ALP are given in μmol min^{-1} per miligram of protein.
* Statistical significance of the difference between treated and control rats at the confidence level $p < 0.05$ (C – control group, US – group treated with *Ustilago maydis*, US+FB_1 – group treated with *Ustilago maydis* and fumonisin B_1).

Table II. Catalytic activities of lactate dehydrogenase (LDH), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), and cholinesterase (ChE) in blood serum of Fischer rats[a]

Group	*n*	LDH (U/L)	ALT (U/L)	GGT (U/L)	ChE (U/L)
C	5	1576.9 ± 444.7	12.2 ± 2.7	5.5 ± 1.2	0.038 ± 0.005
US	5	1586.6 ± 350.4	10.2 ± 2.2	6.3 ± 1.1	0.037 ± 0.011
US+FB_1	5	2621.1 ± 410.9[b]	6.9 ± 1.6[b]	9.1 ± 3.6	0.029 ± 0.006

[a] Data are expressed as mean ± SD.
[b] $p < 0.05$, significantly different from control values.
C – control group, US – group treated with *Ustilago maydis*, US+FB_1 – group treated with *Ustilago maydis* and fumonisin B_1.

This study was not foremost focused on the possibility that *U. maydis* could be infected by mycoviruses but this fact ought to be taken into account. Mycoviruses generally have double-stranded RNA genomes. Effects of these viruses on the fungal hosts are usually uncertain but some are associated with debilitation of the host fungus, hypovirulence of the pathogen, or antifungal toxin production (29). Best characterised are the killer-toxin-producing viruses of *U. maydis*. It was noted that the cells infected with each of the three virus types produced diffusible proteinaceous toxins called KP1, KP4, and KP6, respectively. The infected *U. maydis* cells produce inhibitory factors called killer toxins, which are pathogens for the other type of fungi or plant cells. However, there are some killer toxins that are potentially pathogenic to mammalian cells. KP6α, for example, suggests a multimeric assembly with a central pore, which may be involved in disruption of potassium ion balance (30), while KP4 suggests a possible function as an ion channel inhibitor (9).

Our results are in agreement with the research carried out by Rumora *et al.* (31) regarding cytotoxic and genotoxic effects of FB_1 in the RK13 cell line. They have observed increased activities of LDH and GLDH in the medium of FB_1 treated cells. A very sensitive parameter of liver tissue damage or hepatocellular injury is the increase of ALT activity. However, in our study, the enzyme activity was not increased compared to the control. This leads to the conclusion that the increase of LDH activities could be a result of muscle tissue injury or of other organs and not the liver. Themur *et al.* (32) reported unsignificant changes in ALT and GGT activity in serum observed in the rats treated with a total of 319, 544 and 810 mg of FB_1 per kilogram of b.m. for 30, 60 and 90 days (32). In contrast to our results, the ALT activity was significantly increased in the serum of rats and carp treated with FB_1 (15 mg kg^{-1}, 0.5 and 5 mg kg^{-1} of b.m., respectively) (22, 33).

CONCLUSIONS

Ustilago maydis and FB_1 showed neurotoxicity in Fischer rats, which was indicated by hyperactivity, brain hyperaemia and especially by decreased AChE activity. These pathological changes could be related to the alkaloids of *U. maydis* and disruption of sphingolipid metabolism by FB_1 activity. Pathological changes in liver, lungs, uterus and ovaries were more pronounced in the US+FB_1 than in the US group, indicating possible

synergism. Increased activity of LDH in the serum was observed only in the US+FB_1 group, which led to the conclusion that FB_1 is responsible for cytotoxic effects. Such activity of FB_1 could increase the toxicity of *U. maydis* and other co-contaminants of fungal origin that can be found in foods and feeds.

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S A Ž E T A K

Toksični učinci *Ustilago maydis* i fumonizina B_1 u štakora

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Toksično djelovanje kukuruzne snijeti *Ustilago maydis* i mogući sinergizam s fumonizinom B_1 (FB_1) ispitivani su na štakorima soja Fischer. Toksični učinci su procijenjeni na temelju organoleptičkog pregleda organa žrtvovanih životinja i mjerenja biokemijskih parametara u serumu (LDH, ALT, GGT, ChE), te homogenatu tkiva jetre i mozga (AChE, ChE, GGT, ALP). U jednoj pokusnoj skupini (US) životinje su hranjene smjesom biomase i snijeti masenog udjela 70%, dok je druga pokusna skupina (US+FB_1) primala smjesu biomase i snijeti masenog udjela 70%, te fumonizina B_1 (1 mg po kilogramu hrane) tijekom 17 dana. Kontrolna skupina je hranjena standardnom laboratorijskom hranom. Tijekom pokusa životinje su bile ekscitirane. Masa pokusnih životinja bila je na kraju pokusa nešto veća u odnosu na kontrolnu skupinu. Uočene patološke promjene na jetri, plućima, uterusu i ovarijima bile su više izražene u skupini US+FB_1. Specifična katalitička aktivnost AChE u homogenatu jetre i mozga bila je značajno smanjena u US skupini (61, odnosno 63%) u odnosu na kontrolu ($p < 0,05$), što ukazuje na neurotoksično djelovanje snijeti. Osim toga, zabilježena je značajno smanjena aktivnost ovog enzima i ALP u homogenatu jetre US+FB_1 skupine ($p < 0,05$). Specifična katalitička aktivnost LDH u serumu bila je značajno povećana u US+FB_1 skupini (165%, odnosno 166%) u odnosu na US i kontrolnu skupinu, što ukazuje da su oštećenja stanične membrane rezultat djelovanja FB_1. Neurotoksični učinci u štakora soja Fischer vjerojatno su posljedica djelovanja alkaloida *U. maydis* i poremećaja metabolizma sfingolipida uslijed djelovanja FB_1.

Ključne riječi: Ustilago maydis, fumonizin B_1, neurotoksičnost, citotoksičnost

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