Research Article

The involvement of oxidative stress in ochratoxin A and fumonisin B_1 toxicity in rats

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The aim of this study was to find out whether very low doses of nephrotoxic and hepatotoxic mycotoxins ochratoxin A (OTA) and fumonisin B_1 (FB_1) induce oxidative stress in rat kidney and liver and whether their effect is synergistic. Rats were treated orally with OTA (5 ng/kg b.w. and 50 μg/kg b.w.) and FB_1 (200 ng/kg b.w. and 50 μg/kg b.w.), or their combinations. Malondialdehyde (MDA) and protein carbonyls (PCs) concentration in kidney was affected with lower dose of OTA than in liver ($p < 0.05$). FB_1 did not affect MDA and PCs concentrations in the liver, while in the kidney both FB_1 doses increased MDA concentration ($p < 0.05$). The combination of the lower doses of OTA + FB_1 increased the MDA and PCs concentration both in the liver and the kidney, compared to controls and animals treated with respective doses of mycotoxins ($p < 0.05$). The combinations of mycotoxins reduced the catalase activity only in the kidney when compared to controls ($p < 0.05$). In contrast to the increased kidney concentrations of MDA and PCs even with very low doses of OTA and FB_1, the activity of catalase and SOD does not change. Combinations of OTA + FB_1 affected almost all parameters, which indicates their potential to produce oxidative damage.

Keywords: Catalase / Malondialdehyde / Mycotoxins / Protein carbonyls / Superoxide dismutase

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1 Introduction

Ochratoxin A (OTA) and fumonisin B_1 (FB_1) are the most toxic and the most common derivatives of the respective families of mycotoxins. They are both nephrotoxic and hepatotoxic, but the mechanism of their toxicity is not fully understood.

However, it is known that OTA inhibits protein synthesis [1], disturbs mitochondrial respiration [2] and causes the increase of lipid peroxidation [3]. OTA is nephrotoxic in all animal species tested and is believed to be involved in the etiology of endemic nephropathy (EN) [4, 5]. OTA is also hepatotoxic, immunosuppressive, genotoxic, and carcinogenic [6]. Although OTA may cause urothelial tumors, which are very frequent in region with EN, the International Agency for Research on Cancer (IARC) has classified it in Group 2B (potentially carcinogen for humans) due to a lack of epidemiological evidence [7].

The main mechanism of FB_1 toxicity is the perturbation of sphingolipid metabolism due to the structural similarity of FB_1 and sphingoid bases [8]. It is also shown that FB_1 increases lipid peroxidation in rat liver and primary rat hepatocytes [9]. The target organs and toxicity of FB_1 are species- and sex-specific. Thus FB_1 causes leukoencephalomalacia in horses, pulmonary edema in pigs, and it is nephrotoxic and hepatotoxic in laboratory rodents. The main target organ of FB_1 toxicity in rats is the kidney, while in mice it is the liver [4]. Renal carcinogenicity of FB_1 is more pronounced in male rats and liver carcinogenicity in female mice [10]. IARC classified FB_1 in the same group as OTA (Group 2B) [11].

Humans are exposed to OTA and FB_1 all over the world, mostly through ingestion of contaminated food. Respective OTA and FB_1 were found in 37 and 100% of maize samples collected in Croatian maize producing counties [12]. The mean OTA and FB_1 concentrations in positive samples were 1.47 ± 0.38 and 459.8 ± 310.7 μg/kg, respectively. Several studies found OTA in higher concentrations or more frequently in samples of food collected from endemic than from nonendemic regions [13, 14]. In a two-year follow-up of OTA and FB_1 contamination of maize, Jurjević *et al.* [15] did

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Abbreviations: FB_1, fumonisin B_1; **MDA,** malondialdehyde; **OTA,** ochratoxin A; **PC,** protein carbonyls; **SOD,** superoxide dismutase

not find significant difference in FB_1 contamination of maize, but in 1997 OTA concentration was much higher in samples collected from the endemic region than in control regions.

The estimated mean daily intake of OTA and FB_1 in the European-type diet ranges from 0.7 to 4.7 and 200 ng/kg b.w., respectively [16, 4]. These doses are considered not to be harmful to humans. The aim of this study was to find out whether these doses of OTA and FB_1 could cause oxidative damage of proteins and lipids and whether they inhibited antioxidative enzymes in the kidney and liver of experimental animals. We also wanted to see whether a combination of both mycotoxins increased parameters of oxidative damage.

2 Materials and methods

2.1 Chemicals

Mycotoxins OTA and FB_1 (98% purity), guanidine hydrochloride, 1,1,3,3-tetramethoxypropane, superoxide dismutase (SOD), nitrilotriacetic acid, and Na_2EDTA were purchased from Sigma Chemicals (St. Louis, MO, USA). All other chemicals were from Kemika (Zagreb, Croatia) and were of *pro analysi* grade.

2.2 Animal study

Adult male Wistar rats (190 g of weight) were kept in macrolone cages, were fed on a standard diet for laboratory rodents (Pliva, Zagreb), and had free access to water. The rats were randomized into ten groups of six animals.

OTA and FB_1 were dissolved in 51 mM $NaHCO_3$ (pH 7.4), and given orally once a day.

Two groups of animals were treated with OTA (5 ng/kg b.w. and 50 μg/kg b.w., respectively) for 15 days.

Two groups of animals were treated with FB_1 (200 ng/kg b.w. and 50 μg/kg b.w., respectively) for 5 days.

Three groups of animals were treated with OTA for 15 days and with FB_1 for the last 5 days of OTA treatment. The doses were 5 ng of OTA/kg b.w. + 200 ng of FB_1/kg b.w. for the first group, 5 ng of OTA/kg b.w. + 50 mg of FB_1/kg b.w. for the second group, and 50 μg of OTA/kg b.w. + 50 μg of FB_1/kg b.w. for the third group.

Control groups were given 10 mL/kg b.w. of solvent (51 mM $NaHCO_3$, pH 7.4) or redistilled water for 15 days. Positive controls were given a single dose of ferric nitrilotriacetate (Fe-NTA, 15 mg Fe/kg b.w., i.p.), which is known to be a potent oxidant and renal carcinogen. This group of animals was sacrificed by cervical dislocation, 4 h after treatment.

All other animals were sacrificed 24 h after the last dose. Kidneys and liver were collected on ice, washed in saline (0.9% NaCl), and frozen (−80°C) until analyzed.

The principles of good laboratory animal care were followed throughout as well as the Croatian Animal Welfare Act. The Institute's Ethical Committee approved the study.

2.3 Markers of oxidative stress

Liver and kidney tissue homogenates (10%) were prepared in chilled 0.05 M potassium phosphate buffer (pH 7.4). The Na_2EDTA (3 mM) was added to the phosphate buffer to prevent further oxidative damage. Part of homogenates were immediately centrifuged at 4°C and $10\,000 \times g$ for 15 min to obtain a supernatant for enzyme determination.

The concentration of malondialdehyde (MDA) was measured in liver and kidney homogenates spectrophotometrically at 532 nm, using the method of Drury *et al.* [17]. The concentrations were determined using standard curves of MDA and results expressed as nmol MDA/mg of proteins.

Protein carbonyl (PC) content was determined in liver and kidney homogenates by measuring the reactivity of carbonyl groups with 2,4-dinitrophenylhydrazine (2,4-DNPH) as described earlier [18]. PC concentration was determined from the absorbance at 370 nm, applying the molar extinction coefficient of 22.0/mM/cm. The results were expressed as nmol of 2,4-DNPH bound to a milligram of protein.

Catalase activity was measured in the supernatant of the same liver and kidney tissue homogenates according to Aebi [19] at 240 nm, 25°C, and pH 7.0. It was calculated using the molar extinction coefficient (43.6/mM/cm).

The catalytic activity of SOD in liver and kidney supernatants was measured according to Flohé and Ötting [20] spectrophotometrically measuring the increase of absorbance at 550 nm. The concentration was determined using a calibration curve prepared from SOD standards.

The protein concentration was determined according to Bradford [21].

2.4 Statistics

Differences in biochemical parameters (MDA, PCs, and catalytic activity of catalase and SOD) between control and treated animals and between different treatments were tested with the Student's *t*-test for independent samples using Statistica 5.0.

Probability values of $p < 0.05$ were considered statistically significant.

3 Results

During the experiment, there were no changes in body and kidney weight of OTA, FB_1, and OTA + FB_1-treated rats as compared to the negative control group treated with $NaHCO_3$.

Kidney and liver concentrations of MDA and PCs as well as the catalytic activity of catalase and SOD were significantly different between positive (Fe-NTA-treated) and negative controls (Tables 1 and 2) ($p < 0.05$).

The concentration of MDA was significantly higher in the liver and kidney when the higher OTA dose was applied

Table 1. Concentrations of MDA, PCs, and activity of catalase and SOD in the liver of rats treated with OTA (15 days) and FB_1 (5 days) alone and in combination

Treatment	MDA (nmol/mg proteins)	PC (nmol/mg proteins)	Catalase (mM/mg protiens)	SOD (mM/mg proteins)
Controls, rewater	1.03 ± 0.04	1.01 ± 0.04	2.96 ± 0.14	56.55 ± 3.87
Controls, $NaHCO_3$	1.00 ± 0.03	0.99 ± 0.04	2.89 ± 0.26	57.20 ± 4.17
Controls, Fe-NTA	1.34 ± 0.07[a)]	1.36 ± 0.07[a)]	2.57 ± 0.29[a)]	48.89 ± 3.12[a)]
5 ng OTA/kg b.w.	1.02 ± 0.02	1.04 ± 0.02	2.80 ± 0.10	56.91 ± 3.70
50 mg OTA/kg b.w.	1.21 ± 0.06[a, b)]	1.13 ± 0.03[a, b)]	2.77 ± 0.21	56.23 ± 1.20
200 ng FB_1/kg b.w.	1.01 ± 0.03	0.99 ± 0.03	3.02 ± 0.19	55.41 ± 6.50
50 mg FB_1/kg b.w.	1.01 ± 0.03	1.00 ± 0.05	3.04 ± 0.19	58.45 ± 2.65
5 ng OTA/kg b.w. + 200 ng FB_1/kg b.w.	1.12 ± 0.06[a, e, f)]	1.10 ± 0.05[a, e, f)]	2.78 ± 0.13	56.27 ± 3.22
5 ng OTA/kg b.w. + 50 μg FB_1/kg b.w.	1.17 ± 0.04[a, e, f)]	1.29 ± 0.06[a, c, e, f)]	2.83 ± 0.22	56.16 ± 3.48
50 mg OTA/kg b.w. + 50 μg FB_1/kg b.w.	1. 29 ± 0.08[a, c, d, f)]	1.55 ± 0.05[a, c, d, e, f)]	2.82 ± 0.17	52.15 ± 3.74

a) Different from two negative controls ($p < 0.05$).
b) Different from OTA (5 ng/kg b.w.) or FB_1 (200 ng/kg b.w.) ($p < 0.05$).
c) Different from the OTA + FB_1 (5 + 200 ng/kg b.w.) ($p < 0.05$).
d) Different from the OTA + FB_1 (5 ng/kg b.w. + 50 mg/kg b.w.) ($p < 0.05$).
e) Different from the corresponding dose of OTA ($p < 0.05$).
f) Different from the corresponding dose of FB_1 ($p < 0.05$).

Table 2. Concentrations of MDA, PCs, and activity of catalase and SOD in the kidney of rats treated with OTA (15 days) and FB_1 (5 days) alone and in combination

Treatment	MDA (nmol/mg proteins)	PC (nmol/mg proteins)	Catalase (mM/mg proteins)	SOD (mM/mg proteins)
Controls, rewater	1.07 ± 0.08	1.04 ± 0.10	0.95 ± 0.08	54.17 ± 4.17
Controls, $NaHCO_3$	1.12 ± 0.12	1.08 ± 0.06	0.92 ± 0.07	54.23 ± 4.30
Controls, Fe-NTA	2.00 ± 0.15[a)]	1.70 ± 0.14[a)]	0.65 ± 0.09[a)]	43.51 ± 5.00[a)]
5 ng OTA/kg b.w.	1.26 ± 0.07[a)]	1.39 ± 0.21[a)]	0.92 ± 0.08	56.38 ± 5.28
50 mg OTA/kg b.w.	1.62 ± 0.05[a, b)]	1.37 ± 0.09[a)]	0.94 ± 0.07	52.38 ± 2.80
200 ng FB_1/kg b.w.	1.61 ± 0.11[a)]	1.26 ± 0.05[a)]	0.92 ± 0.08	56.31 ± 3.90
50 mg FB_1/kg b.w.	1.53 ± 0.10[a)]	1.57 ± 0.16[a, b)]	0.95 ± 0.06	53.69 ± 4.70
5 ng OTA/kg b.w. + 200 ng FB_1/kg b.w.	1.25 ± 0.05[a)]	1.62 ± 0.08[a, e, f)]	0.85 ± 0.06[a)]	52.99 ± 5.00
5 ng OTA/kg b.w. + 50 μg FB_1/kg b.w.	1.81 ± 0.05[a, c, e, f)]	1.59 ± 0.07[a)]	0.75 ± 0.08[a, c, d, e)]	51.53 ± 5.23
50 mg OTA/kg b.w. + 50 μg FB_1/kg b.w.	1.72 ± 0.12[a, c, f)]	2.17 ± 0.24[a, c, d, e, f)]	0.82 ± 0.11[a, d)]	50.28 ± 3.67

a) Different from two negative controls ($p < 0.05$).
b) Different from OTA (5 ng/kg b.w.) or FB_1 (200 ng/kg b.w.) ($p < 0.05$).
c) Different from the OTA + FB_1 (5 ng/kg b.w. + 200 ng/kg b.w.) ($p < 0.05$).
d) Different from the OTA + FB_1 (5 ng/kg b.w. + 50 mg/kg b.w.) ($p < 0.05$).
e) Different from the corresponding dose of OTA ($p < 0.05$).
f) Different from the corresponding dose of FB_1 ($p < 0.05$).

(Tables 1 and 2) ($p < 0.05$). In the kidney, however, this effect was obtained even with the lower dose. The kidney was also affected by FB_1, judging by the significant increase in MDA concentration (Table 2) ($p < 0.05$). The combination of the lower doses of OTA + FB_1 increased the MDA concentration in both the liver and kidney, as compared to controls. MDA concentration in the liver of animals treated with both mycotoxins was higher than when the same doses of OTA and FB_1 were given separately (Table 1) ($p < 0.05$).

These mycotoxins had similar effects on PCs concentrations. Namely, the kidney was more susceptible to OTA and FB_1 toxicity, and the concentration of PCs in the liver and kidney of rats treated with the combination of the lower doses of OTA + FB_1 was higher than in rats treated with OTA or FB_1 alone (Tables 1 and 2) ($p < 0.05$).

The activity of catalase and SOD was not reduced in either organ when mycotoxins were applied alone (Tables 1 and 2). However, the combination of the lower doses of

OTA + FB_1 significantly reduced the catalase activity in kidney as compared to controls (Table 2) ($p < 0.05$).

4 Discussion

Male rats are more susceptible to OTA and FB_1 carcinogenicity than female, and the kidney is the main target organ [6, 10]. In our experiment, male rats were treated with OTA for 15 days and with FB_1 for 5 days because of the differences in toxicokinetics properties and exposure period needed for the expression of their toxicity [4].

It is known that OTA causes peroxidation of lipids in experimental animals and in cell cultures [3, 22, 23]. However, Gautier *et al.* [24] did not find any increase in lipid peroxidation using kidney, liver, and plasma MDA concentration as its markers in rats receiving a single oral dose (0.3, 1, and 2 mg/kg b.w., respectively) and sacrificed 4, 8, 24, and 48 h afterwards. The lack of the effects of OTA on MDA concentration was probably due to the application of low doses. Daily doses of 250 μg OTA/kg b.w. given for 1 month in one study or of 120 μg OTA/kg b.w. given for 2 months in another led to a significant increase in kidney MDA concentration [25, 26]. Increased MDA concentration was seen in the liver after 1 month of dosing with either of 250 or 289 μg OTA/kg b.w./day [25, 27]. Our study has confirmed this OTA effect on MDA concentration in both the kidney and the liver. However, it seems that the rat kidney is more sensitive to OTA, because its effects were observed after 15 days of treatment with a dose as low as 5 ng of OTA/kg b.w.

An increase in MDA concentration due to exposure to FB_1 was reported on various cultured cells (Vero Cells, primary rat hepatocytes, green monkey kidney cells, glioblastoma cells, mouse embryo fibroblasts, porcine kidney cells) [9, 28–31]. The only available study on the effects of FB_1 on MDA *in vivo* showed a significant, dose-related increase in MDA in the liver of rats fed with FB_1-contaminated feed for 21 days [9]. These results cannot be directly compared with our results, because the two studies differed in the way of exposure (contaminated feed *vs.* gavage) and the length of treatment (21 *vs.* 5 days). Although neither dose of FB_1 applied in our study caused an increase in MDA concentration in the liver, its kidney concentration increased significantly.

Apart from lipids, possible major target of oxidative damage are proteins that can be transformed into PCs. The effect of OTA and FB_1 on the concentration of PCs either in cell cultures or in laboratory animals was not studied until now. The effect of OTA and FB_1 on PC concentration in the kidney and liver was similar to their effect on MDA concentration, indicating that proteins are also more susceptible to oxidative lesions in the kidney than in the liver.

The activities of antioxidative enzymes catalase and SOD decrease in the presence of pro-oxidants. Studies on OTA effects on catalase and SOD activity in rat liver and kidney are not conclusive. Namely, Soyoz *et al.* [27] found no decrease in liver catalase or SOD activity after 1 month of OTA (289 μg/kg b.w.) treatment, while Meki and Hussein [25] found a significant decrease in the activity of both enzymes both in the liver and the kidney, using a slightly lower dose (250 μg/kg) for the same amount of time. In another study, kidney SOD activity decreased in rats receiving oral doses of 120 μg OTA/kg for 60 days [26]. With lower OTA doses and shorter treatment period, we did not obtain any effect on the activity of these enzymes either in the liver or in the kidney.

The single available study of FB_1 effects on SOD and catalase activity was performed *in vitro* with pig kidney cells ($LLC\text{-}PK_1$) [32]. The activity of both enzymes did not change after 24 h of exposure to 50 μM FB_1. In our study, the liver and kidney activity of SOD and catalase also did not change.

This is the first report on the combined effects of OTA and FB_1 on the concentrations of MDA and PCs as well as on catalase and SOD activities. Although the lowest doses of OTA and FB_1 given separately did not increase the concentration of MDA and PCs in the liver, their combination produced a significant effect. In the kidney, even the lowest OTA and FB_1 doses increased MDA and PC concentration, but their combination further increased only the PC concentration. Liver or kidney catalase and SOD activity were not affected by separate OTA or FB_1 treatment, and their combination decreased only the catalase activity in rat kidney.

Our results show that some parameters of oxidative stress, such as MDA and PC in the kidney, can be affected even with very low doses of nephrotoxic mycotoxins that humans in Europe are exposed to. The antioxidative enzyme catalase is affected in the kidney only when both mycotoxins are applied simultaneously. In general simultaneous exposure to OTA and FB_1 affected the measured parameters indicating their potential in producing oxidative damage.

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5 References

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