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Ochratoxin A potentiates citrinin accumulation in kidney and liver of rats

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Ochratoxin A (OTA) and citrinin (CTN) are nephrotoxic mycotoxins often found together in grain. The aim of this study was to measure their accumulation in the kidney and liver of adult male Wistar rats, see how it would be affected by combined treatment, and to determine if resveratrol (RSV) would decrease their levels in these organs. The rats received 125 or 250 μg/kg bw of OTA by gavage every day for 21 days and/or 20 mg/kg bw of CTN a day for two days. Two groups of rats treated with OTA+CTN were also receiving 20 mg/kg bw of RSV a day for 21 days. In animals receiving OTA alone, its accumulation in both organs was dose-dependent. OTA+CTN treatment resulted in lower OTA but higher CTN accumulation in both organs at both OTA doses. RSV treatment increased OTA levels in the kidney and liver and decreased CTN levels in the kidney. Our findings point to the competition between CTN and OTA for organic anion transporters 1 and 3.

KEY WORDS: experimental rats; mycotoxins; organic anion transporters; resveratrol; toxicity

Mycotoxins ochratoxin A (OTA) and citrinin (CTN) are produced by *Aspergillus* and *Penicillium* strains and are often found together in grain. Their co-occurrence in food and feed may be the consequence of contamination with several moulds or one mould species producing both mycotoxins such as *P. verrucosum* (1–4). Mandatory OTA monitoring in various commodities in the EU has given a rather good insight into human exposure to this mycotoxin (5). CTN monitoring, in contrast, is not mandatory, despite the recommendation issued by the European Food Safety Authority (6), and data on its levels in food and feed are limited. Grain contamination with CTN is usually low (<10 % of positive samples), but its levels tend to be high (1.1–33 times higher than those of OTA) (7). In Croatia contamination is high (50–75 % samples), with a considerably large level range (0–400 μg/kg) (8, 9). In Bangladesh (10) and Germany (11) a very high percentage of CTN-positive samples was found in human urine, indicating substantial exposure to this mycotoxin (10, 11).The biological half-life of OTA is long, which increases the possibility of OTA interaction with other mycotoxins. In rat plasma it is 120 h and in human plasma 35.5 days (12, 13). Being a small molecule, OTA enters the enterohepatic circulation, gets redistributed across organs, and accumulates in the kidney (14). Its renal excretion by glomerular filtration is limited and involves organic anion transporters (OATs) either on the basolateral or apical side of cells in kidney tubules (15).

Target organs of OTA toxicity are the kidney and liver, but it is also immunotoxic, teratogenic, and carcinogenic.

The toxicological properties of CTN have been summarised elsewhere (16), but, generally, it is considered less nephrotoxic than OTA (8). However, CTN could become equally toxicologically important as OTA if the climate change increases CTN production by *P. verrucosum*. Studies of its mutagenicity are inconclusive and studies of its carcinogenicity are lacking. Some have found it genotoxic (17–19), and others not (20, 21).

The aim of our study was to establish the accumulation of OTA and CTN in the kidney and liver of rats and see how it would be affected in combined exposure. We also wanted to see how resveratrol (RSV) would affect organ accumulation of these mycotoxins, as this antioxidant is known to inhibit the expression of organic anion transporters (OATs) 1 and 3 (22, 23). This study was a part of a larger study investigating the effects of these two mycotoxins on oxidative stress in rat kidney, liver, and plasma (24).

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MATERIALS AND METHODS

Ketamine hydrochloride and xylazine hydrochloride used in combination to anaesthetise the rats were purchased under brand names Narketan and Xylapan from Chassot AG (Bern, Switzerland). OTA, CTN, and methanol were purchased from Sigma (St. Louis, MO, USA). Ultrapure water (18 MW) was obtained from a Milli-Q Smart2pure 3 UV/UF gradient water purification system (Thermo Fisher Scientific, Waltham, MA, USA). Acetic acid (p. a.) was obtained from Merck (Darmstadt, Germany). Other chemicals and reagents were of analytical grade, and their commercial source is indicated with the description of specific methods.

Adult male Wistar rats (10 weeks old, 230–270 g bw) were kept in makrolon cages at room temperature of 22 °C and 12-hour day/night cycles and had free access to tap water and standard pelleted food (Mucedola, Settimo Milanese, Italy). Animals were divided into eight groups (N=6 each) as follows: controls (receiving 51 mmol/L $NaHCO_3$), OTA_{125} (receiving 125 μg/kg bw of OTA alone), OTA_{250} (receiving 250 μg/kg bw of OTA alone), CTN (receiving 20 mg/kg bw of CTN alone), OTA_{125}+CTN, OTA_{250}+CTN, OTA_{125}+CTN+RSV (20 mg/kg bw), and OTA_{250}+CTN+RSV. OTA was given dissolved in 51 mmol/L $NaHCO_3$ by gavage every day for 21 days. CTN was dissolved in 50 mmol/L Na_2CO_3 and given by gavage for two days (CTN alone group), which in combined treatment coincided with the last two days of treatment with OTA and RSV every day between 8 and 9 AM. Animals were sacrificed under general anaesthesia with ketamine and xylazine.

Animal experiments were approved by the Ethics Committee of the Institute for Medical Research and Occupational Health in accordance with the EC Council Directive 2010/63/EU (25).

Organs were taken and kept at -80 °C until analysis. Samples were prepared according to the method described previously and modified for these samples accordingly (26).

Chromatographic analysis of OTA and CTN was run on a tandem quadrupole ultra performance liquid chromatography/tandem mass spectrometry system (ACQUITY TQD UPLC-MS/MS, Waters, Milford, MA, USA). Separation was done on a Hibar™ Purospher STAR HR 50x2.1 mm column (Merck, Darmstadt, Germany), 2 μm particle size, and flow rate of 0.53 mL/min. Gradient elution was applied (eluent A – 0.1 % acetic acid; eluent B – methanol) according to the following program: 0–0.61 min – 95 % A; 0.61–4.5 min – 5 % A; 4.5–5 min – 95 % A. The chromatographic run was 7 min per sample. Molecular ions were obtained with electrospray ionisation (positive mode for OTA and negative for CTN). The temperature of the ionisation source was maintained at 115 °C and the temperature of the desolvation gas at 350 °C. Cone gas flow was 60 L/h, and desolvation gas flow 750 L/h. Capillary and cone voltages were maintained at 3.5 kV and ±40 V respectively. Quadrupoles were set to the multiple reaction monitoring (MRM) mode. Each compound was confirmed by the presence of the parent ion and two transitional products. Specific transitions of the precursor ion and product ion were as follows: 249.1 ->177.3 and 249.1->205.4 m/z for CTN and 404->221 and 404->239 m/z for OTA, respectively. Quantification transitions were 249.1->205.4 m/z for CTN and 404->239 m/z for OTA. Optimised collision energy (CE) was 22 and 15 eV for CTN and 20 eV for OTA. Dwell times for each MRM were 0.15 s. Retention times were 3.3 min for CTN and 3.4 min for OTA.

For calibration tissue extracts were spiked with OTA and CTN as follows: 0.1, 1, 10, and 20 μg/kg of the sample for OTA and 0.1, 1, 2, and 10 μg/kg of the sample for CTN. The resulting calibration curves were used for quantification. The established quantification limits of the analytical method were 0.5 μg/kg for OTA, and 0.8 μg/kg for CTN, with a relative standard deviation of reproducibility below 5 % for both compounds. Coefficients of determination (R^2) were 0.997 and 0.996 for OTA and CTN, respectively.

Statistical analysis

Data were analysed and plotted with the GraphPad Prism for Windows version 5 (San Diego, CA, USA) and R statistical software version 3.3.1 (The R Foundation for Statistical Computing, Vienna, Austria). OTA and CTN values are presented as medians and interquartile ranges, and were analysed using a nonparametric version of Tukey's multiple comparison test. All applied tests were two-tailed. P values of less than or equal to 0.05 were considered statistically significant.

RESULTS

OTA and CTN concentrations

The increase in OTA concentrations in both organs was dose-dependent (Figures 1 and 2). Kidney OTA levels in the OTA_{125}+CTN and OTA_{250}+CTN were significantly lower than in the groups receiving respective doses of OTA alone. This effect was also observed in the liver of animals receiving the higher OTA dose.

RSV did not lower OTA levels in animals receiving OTA+CTN+RSV compared to those receiving OTA+CTN regardless of the OTA dose. In fact, it increased kidney OTA in the OTA_{250}+CTN+RSV group and liver OTA in the OTA_{125}+CTN+RSV group.

Kidney and liver CTN levels in animals receiving OTA+CTN were three to six times higher than in respective tissues of animals receiving CTN alone (Figures 3 and 4).

RSV lowered kidney CTN in the OTA_{250}+CTN+RSV group compared to the OTA_{250}+CTN treatment.

DISCUSSION

We found that OTA accumulation in the kidney was dose-dependent and comparable with our previous studies (27). Our main finding that OTA levels significantly dropped in both organs in the

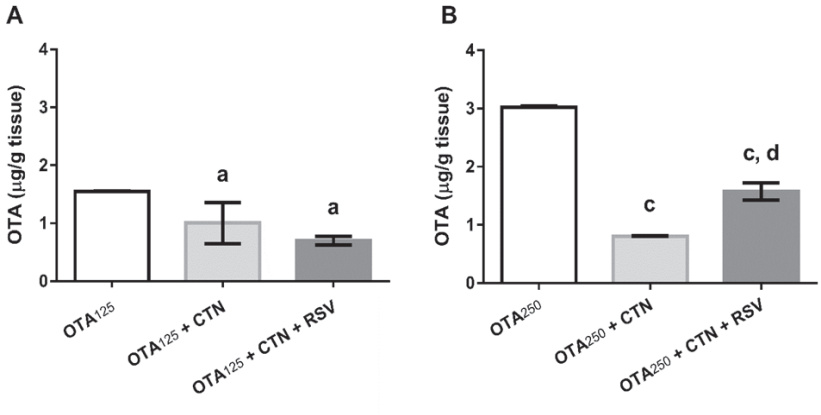

Figure 1 Kidney ochratoxin A levels in rats treated with OTA doses of 125 μg/kg bw (OTA_{125}) or 250 μg/kg bw (OTA_{250}) alone or in combination with citrinin (CTN) and resveratrol (RSV), both in the dose of 20 mg/kg bw. [a] different from OTA_{125} alone; [b] different from OTA_{125}+CTN; [c] different from OTA_{250} alone; [d] different from OTA_{250}+CTN ($P<0.05$)

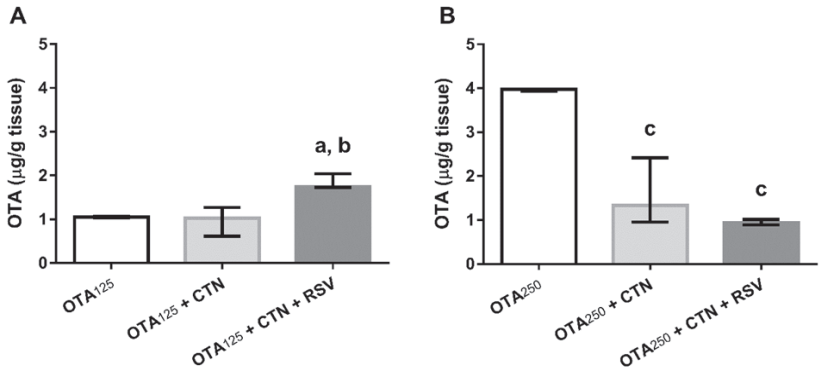

Figure 2 Liver ochratoxin A levels in rats treated with OTA doses of 125 μg/kg bw (OTA_{125}) or 250 μg/kg bw (OTA_{250}) alone or in combination with citrinin (CTN) and resveratrol (RSV), both in the dose of 20 mg/kg bw. [a] different from OTA_{125} alone; [b] different from OTA_{125}+CTN; [c] different from OTA_{250} alone ($P<0.05$)

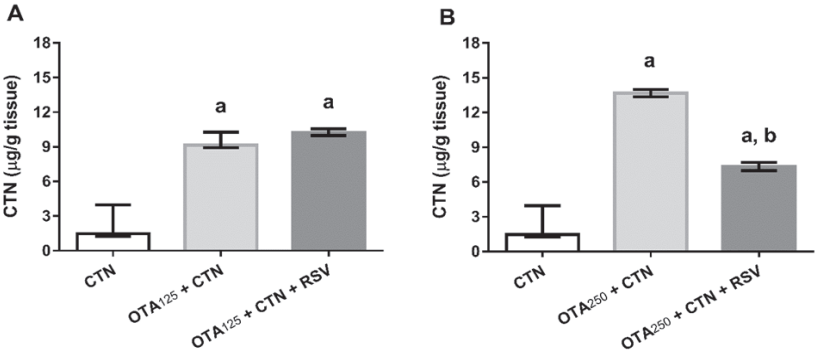

Figure 3 Kidney citrinin levels in rats treated with OTA doses of 125 μg/kg bw (OTA_{125}) or 250 μg/kg bw (OTA_{250}) alone or in combination with citrinin (CTN) and resveratrol (RSV), both in the dose of 20 mg/kg bw. [a] different from CTN alone; [b] different from OTA_{250}+CTN ($P<0.05$)

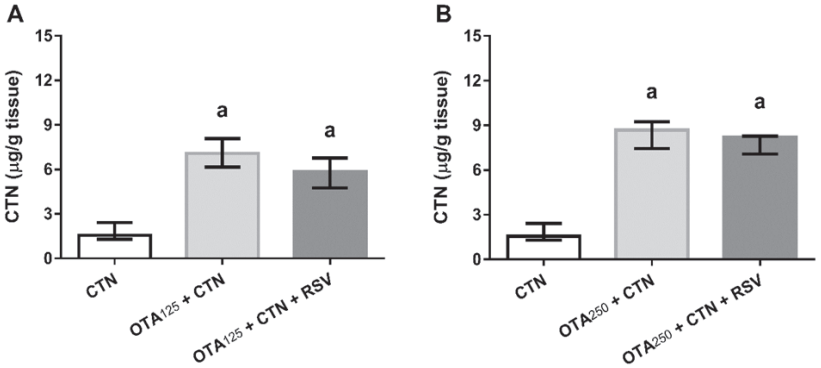

Figure 4 Liver citrinin levels in rats treated with OTA doses of 125 μg/kg bw (OTA_{125}) or 250 μg/kg bw (OTA_{250}) alone or in combination with citrinin (CTN) and resveratrol (RSV), both in the dose of 20 mg/kg bw. [a] different from CTN alone ($P<0.05$)

presence of CTN (Figures 1 and 2) supports *in vitro* findings that CTN competes with OTA for human OAT1 and 3 with different K_i values (3080 and 15.4 μmol/L, respectively) and that these two transporters have higher affinity for CTN than for OTA (28). Similar findings have also been reported in immortalised human proximal tubule cells in the presence of CTN (29) in which OTA dropped by over 60 %. In another study (30) in which rats received ten times lower doses than in ours for 21 days, CTN levels did not change and CTN did not affect Oat1 and Oat3 protein expression, but OTA_{250} significantly downregulated Oat2 protein expression in the kidney. In addition, both mycotoxins downregulated Oat5 protein expression. All this suggests that high doses of OTA or both mycotoxins together inhibit kidney transporters involved in the excretion of CTN. There are no reports of transporters responsible for CTN excretion from the liver.

RSV lowered kidney CTN levels in animals treated with OTA_{250}+CTN+RSV compared to OTA_{250}+CTN treatment but increased liver OTA in animals receiving OTA_{125}+CTN. Reports on RSV transport suggest that RSV and its conjugates are the substrates of OAT transporters involved in the transport of OTA and CTN in the kidney (15, 31, 32). RSV was also reported to inhibit OAT1 and 3 when combined with methotrexate (MTX) (33). The same mechanism probably regulates OTA and CTN accumulation in the kidney. However, further mycotoxin interaction studies in doses closer to natural exposure are needed to pinpoint the exact mechanisms of toxicity and their transport through membranes.

Conflicts of interest

None to declare.

Acknowledgements

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Okratoksin A pospješuje nakupljanje citrinina u bubrezima i jetri štakora

Okratoksin A(OTA) i citrinin (CTN) nefrotoksični su mikotoksini koji zajednički kontaminiraju žitarice. Cilj ovoga istraživanja bio je izmjeriti koncentraciju OTA-e i CTN-a u bubrezima i jetri štakora, tretiranih tim mikotoksinima, te provjeriti hoće li tretman resveratrolom (RSV) smanjiti koncentraciju mikotoksina u tkivima. Istraživanje je provedeno na mužjacima štakora soja Wistar, koji su 21 dan bili tretirani OTA-om (0,125 i 0,250 mg/kg t. m.), a dva dana CTN-om (20 mg/kg t. m.) ili kombinacijama tih mikotoksina. Dvije skupine štakora koje su tretirane mikotoksinima OTA+CTN dobivale su 21 dan RSV (20 mg/kg t. m.). Povećanje koncentracija OTA-e u bubrezima i jetri bio je u skladu s povećanjem doze. Tretman mikotoksinima OTA+CTN smanjio je nakupljanje OTA-e u bubrezima i jetri, a povećao je koncentraciju CTN-a. Tretman RSV-om povećao je koncentraciju OTA-e u bubrezima i jetri, ali je smanjio koncentraciju CTN-a u bubrezima tretiranih štakora. Koncentracija OTA-e značajno se smanjila u prisutnosti CTN-a, vjerojatno zbog kompeticije CTN-a i OTA-e za prijenosnike OAT1 i 3, koji služe za prijenos tih toksina kroz membrane u bubrezima.

KLJUČNE RIJEČI: mikotoksini; organski anionski prijenosnici; pokusne životinje; resveratrol; toksičnost