

TOXICOLOGICAL PROPERTIES OF CITRININ

Dubravka FLAJS and Maja PERAICA

Institute for Medical Research and Occupational Health, Zagreb, Croatia

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Citrinin (CTN) is a nephrotoxic mycotoxin produced by several fungal strains belonging to the genera *Penicillium*, *Aspergillus*, and *Monascus*. It contaminates various commodities of plant origin, cereals in particular, and is usually found together with another nephrotoxic mycotoxin, ochratoxin A (OTA). These two mycotoxins are believed to be involved in the aetiology of endemic nephropathy. In addition to nephrotoxicity, CTN is also embryocidal and fetotoxic. The genotoxic properties of CTN have been demonstrated with the micronucleus test (MN), but not with single-cell gel electrophoresis. The mechanism of CTN toxicity is not fully understood, especially not whether CTN toxicity and genotoxicity are the consequence of oxidative stress or of increased permeability of mitochondrial membranes. CTN requires complex cellular biotransformation to exert mutagenicity.

Compared with other mycotoxins, CTN contamination of food and feed is rather scarce. However, it is reasonable to believe that humans are much more frequently exposed to CTN than generally accepted, because it is produced by the same moulds as OTA, which is a common contaminant of human food all over the world.

At present, there are no specific regulations either in Croatia or in the European Union concerning CTN in any kind of commodity.

KEY WORDS: *mycotoxin, nephrotoxicity, organic anion, oxidative stress*

Citrinin (CTN, Figure 1) is a secondary product of fungal metabolism, first isolated by Hetherington and Raistrick from a culture of *Penicillium citrinum* Thom (1). Meanwhile, several other fungal species within the three genera, *Penicillium* (*P. expansum*, *P. verrucosum*), *Aspergillus* (*A. terreus*), and *Monascus* (*M. ruber*) were also found to produce this mycotoxin (2, 3). CTN contaminates maize (4), wheat, rye, barley, oats (5), and rice (6). Strains of *Monascus* are traditionally used in China to produce red and yellow pigments for food (7). Western countries limit the use of synthetic food colorants due to their toxicity and mutagenicity. Natural food pigments obtained from *Monascus* were good candidates for their substitution because reports on their toxic effects had been scarce for more than 1000 years. However, in 1981 Wong and Koehler (8) isolated from *Monascus purpureus* a pale yellow pigment monascidin A, which was later

determined to be CTN (9). An analysis of traditional Chinese commercial *Monascus* products obtained by culturing the fungus on steamed rice determined CTN in all samples in the mass fraction range of 0.2 $\mu\text{g g}^{-1}$ to 17.1 $\mu\text{g g}^{-1}$ (10).

CTN is decomposed at 175 °C by dry heating, but decomposition temperature decreases to 140 °C in the presence of a small amount of water (11, 12). Decomposition products obtained by heating CTN with water at 140 °C to 150 °C were as toxic as or more toxic than CNT. These new toxins are CTN H₁ and CTN H₂ (12). The concentration of CTN in the extract of *Monascus* decreases by 50 % after boiling in water for 20 minutes, which proves that CTN is thermally unstable in aqueous solution (13).

As there are no legal requirements to measure CTN in food and feed and as probably citrinin H₁ is not detectable by usual methods due to its dimeric

structure, data on food contamination with CTN are scarce. Table 1 shows data for CTN contamination of some common foodstuffs.

CTN has antibiotic properties against gram-positive bacteria, but it has never been used as a drug due to its high nephrotoxicity. The kidney is the major target organ of CTN toxicity, but other target organs such as liver and bone marrow have also been reported (21). Historically, CTN is one of the first isolated mycotoxins; however, the data on the mechanism of its toxicity are still controversial and most have been obtained *in vitro*. Like other mycotoxins, CTN could be implicated in porcine nephropathy (14). It is frequently found in food and feed in combination with ochratoxin A (OTA), and these two nephrotoxic mycotoxins are suspected to be involved in the aetiology of a human kidney disease called Balkan endemic nephropathy. In the endemic area in Bulgaria, CTN was more common and had higher concentrations in maize and beans intended for human consumption than in the non-endemic area (22). CTN was also found to increase the toxicity of OTA either additively or synergistically (23).

The International Agency for Cancer Research (IARC) classified CTN in Group 3 of carcinogens because of the limited evidence of its carcinogenicity to experimental animals and no evidence for humans (24).

Analytical methods for CTN determination were recently reviewed (12). Common methods for CTN analysis are thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) with UV or fluorescence detection, and enzyme immunoassays. For qualitative and quantitative determination of CTN LC-MS and GC-MS techniques are used.

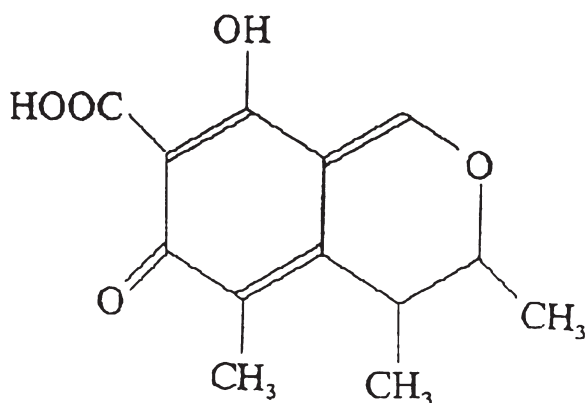


Figure 1 Structural formula of citrinin

TOXICITY, CARCINOGENICITY, AND TERATOGENICITY

The cytotoxicity of CTN varies remarkably from one cell culture to another (Table 2). CTN applied on cultured hepatoma cells at doses up to 25 $\mu\text{mol L}^{-1}$ was cytostatic, while at concentrations from 50 $\mu\text{mol L}^{-1}$ to 200 $\mu\text{mol L}^{-1}$ it was cytotoxic (29). The effect of CTN on cell viability was tested with the MTT assay on Vero cells from the green monkey kidney treated with increasing CTN concentrations from 0 $\mu\text{mol L}^{-1}$ to 250 $\mu\text{mol L}^{-1}$ (28). Up to the concentration of 60 $\mu\text{mol L}^{-1}$ no significant change in cell viability was observed, and the estimated IC_{50} of citrinin was about 220 $\mu\text{mol L}^{-1}$ after 48 hours of exposure. With the same exposure time, the IC_{50} of CTN for human embryonic cell line was 120 $\mu\text{mol L}^{-1}$. At 24 hours of exposure, the IC_{50} of CTN for human promyelocytic leukaemia (HL-60) cells and porcine kidney PK15 cells was 50 $\mu\text{mol L}^{-1}$ and 68 $\mu\text{mol L}^{-1}$, respectively (26, 27).

Acute LD_{50} of CTN varies with the route of administration, physiological conditions, and animal species (Table 3). Oral LD_{50} for rats is 50 mg kg^{-1} b.w. (30), while subcutaneous LD_{50} is 67 mg kg^{-1} b.w. (31). The subcutaneous treatment of pregnant rats with 35 mg kg^{-1} on days 6, 9, and 10 of pregnancy resulted in 50 % or higher maternal mortality (32). In the Dutch Belted rabbit, oral LD_{50} is 134 mg kg^{-1} , and in the New Zealand White rabbit it is about 120 mg kg^{-1} (33, 34). Acute lethal doses administered to rabbits, guinea pigs, rats, and swine caused swelling of the kidneys and acute tubular necrosis (36-38).

Subchronical oral treatment of rats with water suspension isolated from a strain of *Penicillium viridicatum* Westling caused CTN-induced kidney damage characterised by enlarged kidney, hydropic degeneration, loss of brush border, and pyknotic nuclei in the proximal tubules (36). Treatment of mice with weekly injections of CTN (20 mg kg^{-1}) for six weeks resulted in a significant decrease in total bone marrow cells, red blood cell precursors, white blood cell precursors, megakaryocytes, decrease in spleen weight, and decrease in the total spleen cell count (39).

Data on CTN carcinogenicity in the available literature are rather scarce. Kanisawa (40) did not find that CTN caused tumours in mice exposed for to CTN (0 mg kg^{-1} , 100 mg kg^{-1} , and 200 mg kg^{-1}) through diet for 70 weeks, while Arai and Hibino (41) reported benign tumours in the kidney of male Fisher 344 rats after CTN treatment for 60 and 80 weeks.

Table 1 Natural occurrence of citrinin in commodities intended for human consumption

Commodity contaminated	Mass fraction of citrinin $\mu\text{g kg}^{-1}$	Country	References
Barley	160 to 2000	Denmark	14
	30 to 480	Sweden	15
Apples	320 to 920	Portugal	16
Rice	49 to 92	India	6
	700 to 1130	Japan	
Corn flour	27 to 73	Japan	
	10 to 98	Thailand	17
	212	Burma	
Tomatoes	70 to 760	Canada	18
Maize	12	India	19
Fermented maize	580	Ghana	20

CTN is embryocidal and foetotoxic in mice (42). In pregnant Sprague-Dawley rats, CTN given subcutaneously ($35 \text{ mg kg}^{-1} \text{ b.w.}$) on gestation day 3 to 15 did not decrease the number of implants and no gross or skeletal malformations were found, but the foetuses were about 22 % smaller than control (32). CTN injected to pregnant rats of the same strain at a dose of 30 mg kg^{-1} on gestation days 5 to 14 resulted in a few foetal resorptions and minimal malformations (43). The LD_{50} of CTN in a 4-day old chicken embryo is $80.5 \mu\text{g}$ per egg (upper limit of $131 \mu\text{g}$, lower limit of $54.3 \mu\text{g}$) (2). CTN doses of $50 \mu\text{g}$, $100 \mu\text{g}$, and $150 \mu\text{g}$ per egg, were teratogenic in 46 %, 48 %, and 73 % of surviving chicken embryos, respectively.

EFFECTS ON RENAL FUNCTION AND STRUCTURE

In a study by Jordan et al., a single intraperitoneal (i.p.) dose of 50 mg kg^{-1} CTN caused nephrosis in Sprague-Dawley rats (44). Over the first 48 hours, urine glucose and blood concentrations increased, reaching peak three hours after treatment. Kanisawa found that exposure of male mice to 200 mg kg^{-1} CTN through feed for 70 weeks caused very mild renal lesions, but not renal tumours (40).

Citrinin given for six weeks in a daily dose of 2.5 mg kg^{-1} and 5.0 mg kg^{-1} in gelatine capsules did not cause clinical or pathological changes in the kidney of young beagle dogs (45). Slow intravenous injection of $20 \mu\text{mol kg}^{-1}$ CTN to dogs did not affect renal tissue ultrastructure or any of 23 whole blood, plasma, or renal function parameters that were monitored for six hours

after treatment (46). On the other hand, $80 \mu\text{mol kg}^{-1}$ of CTN significantly increased hematocrit and renal excretion rates of proteins and glucose while modest reductions were noted in C_{in} (clearance of inulin), renal blood flow, and elimination of inorganic phosphorus. This dose induced ultrastructural lesions in the cells of the S2 proximal tubular segment, the thick ascending limb, the distal convoluted tubule, and the collecting ducts. The glomeruli, S1 and S3 cells of the proximal tubule, and the thin descending and ascending limbs of Henle's loop remained unaffected.

In male New Zealand white rabbits, oral CTN dose of $120 \text{ mg kg}^{-1} \text{ b.w.}$ produced azotaemia and metabolic acidosis with haemoconcentration and hypokalaemia within 4 to 12 h (34). In surviving rabbits, oral doses of 80 mg kg^{-1} and 100 mg kg^{-1} decreased creatinin clearance which reached the peak on days 2 to 4, and returned to normal or near normal by day 7. In a more recent ultrastructural assessment of young growing New Zealand white rabbits exposed to 15 mg kg^{-1} of CTN through feed, most lesions were observed in the proximal convoluted tubule lining cells, but distal convoluted tubules were unaffected (47). CTN treatment induced nucleus crenation, loss of nucleolus, depletion of cytoplasmic organelles, mitochondrial pleomorphism, nuclear fragmentation, uniform folding of cell membrane, and cytoplasmic vacuolation in proximal convoluted tubules.

In rat liver catalase, butylhydroxitoluene, and dithiothreitol did not protect against swelling and against increased mitochondrial permeability caused by Ca^{2+} plus citrinin (48). Protection conferred by ATP- Mg^{2+} and cyclosporine A indicates pore formation.

DISTRIBUTION, METABOLISM, AND MECHANISMS OF TOXICITY

The target organ of CTN toxicity is the kidney, but hepatotoxicity has also been reported (37, 49). The distribution of ^{14}C -CTN (3 mg kg^{-1}) was studied in the liver, kidney, and plasma of rats sacrificed at 10 time points after treatment (50). At 0.5 h after treatment, 14.7 % and 5.6 % of total radioactivity was found in the liver and kidney, respectively. At 6 h, these values decreased to 7.5 % in the liver and 4.7 % in the kidney. Plasma half-lives were 2.6 h and 14.9 h, respectively. Most of the radioactivity (approx. 80 %) was found in the urine (74 %) and faeces (4 %) in the first 24 h after treatment. At 72 h after treatment, 95 % of radioactivity was excreted via urine and faeces.

The major urinary metabolite of CTN is dihydrocitrinone (51). However, CTN as parent compound and not its metabolite was responsible for nephrotoxicity (52). CTN is an organic anion and its effect on renal transport was studied on renal cortical slices of animals treated with a single i.p. injection of $55 \text{ mg kg}^{-1} \text{ }^{14}\text{C}$ -CTN. Animals were killed 72 h after dosing, when the effect on renal tubular transport was expected to be the highest. The importance of anion transport system was demonstrated by the reduced uptake of ^{14}C -CTN in the presence of probenecid, a specific inhibitor of anion transport (53).

In vitro studies on various cultured cells gave contradictory results about the involvement of oxidative stress in CTN toxicity. Ribeiro et al. reported that CTN modified antioxidative enzymatic defences of rat liver cells by inhibiting GSSG-reductase and transhydrogenase (54). However, no effect was observed on GSH-peroxidase, catalase, glucose 6-phosphate, 6-phosphogluconate dehydrogenase, and superoxide dismutase. CTN increased formation of reactive oxygen species, stimulating the production of superoxide anion in the respiratory chain. The authors concluded that oxidative stress was an important mechanism in CTN-induced cytotoxicity and cellular death in several tissues.

The importance of oxidative stress in CTN toxicity was confirmed by microarray studies of CTN effects of citrinin in yeast cells (55) and of the viability of Vero cells exposed to CTN and antioxidant vitamin E (56). Aleo et al. (57) used suspensions of renal proximal tubules (RPT) and found that CTN increased lipid peroxidation and that deferoxamine (which prevents iron-mediated lipid peroxidation) did not protect RPT from CTN-induced cell death. The possibility that CTN may alter mitochondrial function, put forward by this group of authors, was corroborated by studies with baby hamster kidney cells (58). Electron microscopy showed that CTN significantly affected normal mitochondria with swelling and cell death. Chagas et al. have suggested that CTN decreases Ca^{2+} accumulation in the matrix by inhibiting its influx and increasing its efflux (59).

CTN was found to cause apoptosis in human promyelocytic leukemia (HL-60) cells and porcine kidney PK15 cells (26, 27). The number of apoptotic cells increased dramatically at doses higher than LC_{50} ($50 \mu\text{g}$). CTN induced dose-dependent formation of caspases 3, 6, 7, and 9, but not caspase 8. Furthermore, the authors noted increased cytochrome c release from mitochondria into the cytoplasm, and cytochrome c is known to activate apoptosis-regulating caspases 3, 6, 7, and 9. This suggests that CTN induces apoptosis through cytochrome c and not through oxidative stress. This has been corroborated by the finding that CTN-induced increase in caspase 3 catalytic activity could not be suppressed by antioxidants.

CTN reduces GSH levels in a dose- and time-dependent manner in human alveolar epithelial cells A549 at non-toxic concentrations (60). This effect is the sign of oxidative stress and may contribute to inflammation in people exposed to moulds.

GENOTOXICITY

Genotoxicity of CTN has not been unequivocally established because various test systems gave

Table 2 Citrinin IC_{50} values in different cell cultures

Cell culture	$\text{LC}_{50} / \mu\text{mol L}^{-1}$			Reference
	24 h	48 h	72 h	
Human embryonic cell line (HEK293)	-	120	80	25
Human promyelocytic leukemia (HL-60) cells	50			26
Porcine kidney PK15	68			27
Vero cells		220		28

Table 3 Citrinin LD₅₀ values by way of administration and by experimental animal

Species	Sex and age	Route of administration	LD ₅₀ / mg kg ⁻¹ b.w.	Reference
Rat	M	oral	50	30
Rat	-	s.c.	67	31
Rat	-	i.p.	67	31
Rat	F, pregnant	s.c.	<35	32
Mouse	M, BALB/C	i.p.	45	28
Guinea-pig	-	i.p.	37	31
Rabbit	Dutch Belted rabbit	oral	134	33
Rabbit	New Zealand White rabbit	oral	» 120	34
Rabbit	-	i.v.	19	31
Turkey	M, 7 day-old	oral	56	35
Pekin duck	M, 7 day-old	oral	57	35

both positive and negative results. An increase in DNA damage was detected using single cell gel electrophoresis (comet test) in Vero cells exposed 24 h to CTN (28). However, the same method gave negative results in human-derived liver cells (HepG2) (61) and human embryonic kidney cells (HEK293) (25) no matter if Fpg was present or not. This suggests that CTN-induced oxidative stress did not affect DNA.

In contrast to negative results, various cell cultures exposed to CTN showed a significant increase in micronucleus (MN) frequency. Twenty-four-hour exposure of PK15 cells to 30 µmol L⁻¹ of CTN resulted in a significant increase in MN frequency (9.5 %) over control (2.75 %) (27). This increase was also noticed in HEPG2 cells, human lymphocytes, and Chinese hamster V79 cells, but CTN concentrations showing genotoxicity differed between cell cultures (61-63).

MUTAGENICITY

CTN mutagenicity testing is also inconclusive. CTN was not mutagenic when tested with or without S9-mix (HepG2-derived enzyme homogenate) activation in TA-98 and TA-100 strains of *Salmonella typhimurium* (61). In another study, three additional strains (TA-1535, TA-1538 and TA-97) of *S. typhimurium* were used to test CTN mutagenicity (10), but no mutagenic effect was observed. However, when a primary hepatocyte culture was added, strain TA-98 showed a significant dose-dependent mutagenic response, and strain TA-100 a slight positive response. These results indicate that CTN requires a complex cellular biotransformation to become mutagenic.

Several studies have shown clastogenic CTN activity *in vitro* and *in vivo*, including a variety of chromosomal aberrations, save for sister chromatid exchange (SCE). In a study of Chinese hamster ovary cells and HEK293, CTN did not produce any significant difference in either SCE frequency or DNA gaps and breaks (25).

Thust and Kneist established CTN-induced SCEs in Chinese hamster V79-E cells in the presence of S9-mix (64). They also observed the aneuploidic potential of CTN. CTN was found to be aneugenic because it caused concentration-dependent mitotic arrest, regardless of incubation time. This effect was reversible after the removal of CTN (64).

Jeswal (65) has found that CTN induces chromosome abnormalities and breaks in bone marrow cells in young weanling mice. The most frequent CTN-induced chromosome aberrations found in another study by Bouslimi et al. in bone marrow cells of adult mice included breaks, centric fusions, rings, and gaps (28).

CONCLUSIONS

Although CTN is a nephrotoxic compound in experimental animals, the mechanism of its toxicity is not fully understood. Combined with OTA, it may be involved in the aetiology of endemic nephropathy. However, little is known about human exposure to this mycotoxin through food because there is no legal obligation to measure it in Croatia or in the European Union.

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Sažetak**TOKSIKOLOŠKA SVOJSTVA CITRININA**

Citrinin (CTN) nefrotoksičan je mikotoksin koji proizvode različiti sojevi plijesni iz rodova *Penicillium*, *Aspergillus* i *Monascus*. CTN se može naći u različitim namirnicama biljnog podrijetla, osobito u žitaricama i obično se nalazi zajedno s drugim nefrotoksičnim mikotoksinom, okratoksinom A (OTA). Pretpostavlja se da je izloženost ovim mikotoksinima povezana s nastankom endemske nefropatije. Osim što je nefrotoksičan, CTN je još i embricidan i fetotoksičan. Na genotoksičnost citrinina upućuje pozitivan mikronukleusni test na različitim vrstama staničnih kultura, iako je kometski test negativan. Mutagenost CTN-a očituje se na različitim vrstama stanica samo ako se pridodaju stanični aktivatori kao npr. S9-mix. Mehanizam toksičnosti CTN-a nije potpuno razjašnjen pa još uvijek traje znanstvena rasprava je li njegova toksičnost i genotoksičnost posljedica oksidacijskog stresa ili povećane permeabilnosti mitohondrijskih membrana. U dostupnoj literaturi podaci o kontaminiranosti hrane i krmiva ovim mikotoksinom mnogo su rjeđi od onih za druge mikotoksine. Može se pretpostaviti da su ljudi često izloženi ovom mikotoksinu zato što ga proizvode iste plijesni koje proizvode i OTA, a one kontaminiraju hranu po cijelom svijetu. U Hrvatskoj i u zemljama Europske Unije ne postoje zakonske odredbe o dopuštenim granicama CTN-a u bilo kojoj vrsti hrane.

KLJUČNE RIJEČI: *mikotoksin, nefrotoksičnost, oksidacijski stres, organski anion*

CORRESPONDING AUTHOR:

Dubravka Flajs, BSc
Institute for Medical Research and Occupational Health
P. O. Box 291, HR-10001 Zagreb, Croatia
E-mail: dflajs@imi.hr