Original Scientific Paper

OCHRATOXIN A-INDUCED APOPTOSIS IN RAT KIDNEY TISSUE*

Ana-Marija DOMIJAN[1], Maja PERAICA[1], Željko FERENČIĆ[2], Snježana ČUŽIĆ[2], Radovan FUCHS[1], Ana LUCIĆ[1], and Božica RADIĆ[1]

Unit of Toxicology, Institute for Medical Research and Occupational Health[1], PLIVA Research Institute[2], Zagreb, Croatia

Received July 2004

The aim of our study was to find whether ochratoxin A (OTA) induces the apoptosis and/or necrosis of kidney tissue in rats. In the first experiment, the highest number of apoptotic cells was found in rats sacrificed one day after OTA administration (1.00 mg/kg b.w., i.p.). The number of apoptotic cells reduced gradually and they were not seen nine days after OTA administration. A possible dose-dependence of histological changes was checked in kidney tissue of rats given 0.25, 0.50 or 1.00 mg of OTA/kg b.w., i.p. three times a week for four weeks. The number of apoptotic cells showed a clear dose-dependence, but necrosis was absent even at the highest doses.

The time-dependent appearance of lesions related to OTA administration was checked by administering 0.50 mg OTA/kg body weight to rats, and sacrificing them one day after 1, 3, 6, and 9 doses/administrations, or 6 and 21 day after 12 doses/administrations. Long-term administration is associated with continued and increased apoptosis without necrosis, suggestive of OTA's role in the pathogenesis of progressive renal atrophy.

KEY WORDS: *endemic nephropathy, necrosis, proximal tubules*

Ochratoxin A (OTA) is a mycotoxin with known nephrotoxic and carcinogenic properties in experimental animals. Morphologically, renal damage caused by OTA in animals is characterized by the atrophy of proximal tubules and by interstitial cortical sclerosis (1). OTA is believed to be involved in the aetiology of endemic nephropathy – a human kidney disease associated with some regions of Bulgaria, Croatia, Bosnia and Herzegovina, Romania, and Serbia and Montenegro – and in the aetiology of urothelial tumours which are very frequent in these regions. Renal changes in patients with endemic nephropathy generally involve bilateral, non-inflammatory lesions in various degenerative stages and regeneration processes with an increased number of cells in mitosis (2).

The mechanism of OTA toxicity is such that it affects phenylalanine metabolism and the mitochondrial function. A number of authors have shown that another mechanism of OTA toxicity involves oxidative damage by enhancing the production of free radicals followed by increased peroxidation of lipids. Lipid peroxidation may trigger the mechanism of apoptosis in cultured cells and in experimental animals. However, the reports of OTA-induced apoptotic changes in tubular epithelium of experimental animals are contradictory. *Mantle and et al.* (3) found severe necrosis without apoptotic changes in the tubular epithelium of rats exposed to OTA in feed (0.8 mg/rat/day for 5 days). In contrast, *Maaroufi et al.* (4) found karyomegaly with some necrotic and a few apoptotic-like cells in rats

* Partly presented at the 3rd Croatian Congress of Toxicology, Plitvice, Croatia, 26-29 May 2004

given OTA intraperitoneally (0.4 and 0.8 mg/kg b.w., respectively, every 72 hours for 90 days).

The aim of this work was to see whether apoptotic changes could be induced by a single dose of OTA and whether their appearance is dose and time related. Although such high concentrations of OTA are unrealistic for human exposure, such experimental design was chosen in order to elucidate whether kidney lesions are primarily caused by apoptosis and/or necrosis or not.

MATERIAL AND METHODS

Animals

Our experiments included female adult Wistar rats (240-280 g) kept in macralone cages, fed on a standard rat diet (PLIVA, Zagreb, Croatia). In three separate experiments, rats received intraperitoneal (i.p.) doses of OTA dissolved in TRIS; pH 7.4, as follows:

Study design 1. Twenty-four animals were randomly divided in four control and four exposed groups (3 animals per group). The animals were given a single dose of OTA (1.00 mg/kg b.w.) or a vehicle only. One exposed and one control group was sacrificed on days 1, 2, 6, or 9 after administration.

Study design 2. Nineteen animals were randomly divided in three treated and one control group. Animals in groups of four or five were given multiple doses of OTA (0.25, 0.50 and 1.00 mg/kg b.w.) or a vehicle three times a week for four weeks and sacrificed one day after the last administration.

Study design 3. Twenty-nine animals were given 0.50 mg OTA/kg b.w.. They were divided in groups of four or five to be sacrificed 24 hours after the 1st, 3rd, 6th, or the 9th dose of OTA, or on days 6 and 21 after the 12th dose of OTA. Control animals (24 animals) in groups of four were given vehicle only and sacrificed following the same schedule as designed for the exposed animals.

The kidneys were taken out immediately, washed in saline and immersed into a 4 % buffered formaldehyde solution.

OTA analysis

The extraction of OTA and purification of plasma samples were performed according to the method described earlier (5), and kidney homogenates were made according to the method described by *Bauer et al.* (6). All measurements were performed on a Varian HPLC using a reverse-phase (C-18) column (LiChrospher, Merck; 125.0x4.0 mm). The mobile phase consisted of acetonitrile:water:acetic acid (500:500:5). The flow-rate was 0.5 mL/min.; pressure 22 bars; detector wavelengths 336 nm λ_{ex}, 464 nm λ_{em}; retention time ca. 9 min.; and injection volume 50 μL.

The concentration of OTA was calculated using OTA-spiked plasma and kidney homogenates extracted and purified in the same way as the samples. The detection limits in plasma and kidneys were 0.2 ng OTA/mL, and 0.5 ng OTA/g tissue, respectively.

Histology

Whole kidneys were fixed in formalin, cut perpendicularly in two halves, routinely processed, and embedded with paraplast. Sections of 4 μm were stained with hematoxyllin and eosin (HE). A pathologist looked for evidence of necrosis and fibrosis and counted apoptotic cells using a light microscope according to *Schumer et al.* (7). The number of apoptotic cells was counted in a single section of the whole kidney.

Statistics

Results were expressed as mean ± SD. The statistical differences of results were evaluated using a *t*-test for independent variables. Probability values (P) lower than 0.05 were considered statistically significant.

RESULTS

The gain in weight, the weight of the rat kidneys, and the ratio of these values did not differ between treated and control animals (data not shown). All animals survived until the sacrifice.

Apoptotic cells were formed in the tubular epithelium of the outer stripe zone in the rat kidney of both rats treated with a single and with multiple i.p. doses of OTA. Figure 1 (b and c) shows apoptotic cells formed in the tubular epithelium of OTA-exposed rats. Apoptotic cells were not seen in the renal tissue of control animals (Figure 1 a).

A single i.p. dose of OTA (1.00 mg/kg b.w.) caused the formation of apoptotic cells without necrosis in the epithelium of proximal kidney tubules (Table 1). Rats sacrificed 24 hours after the treatment showed the highest number of apoptotic cells in the kidney,

but it gradually decreased with the decrease in OTA concentration in plasma and kidney. On day six after the administration, apoptotic cells were almost absent.

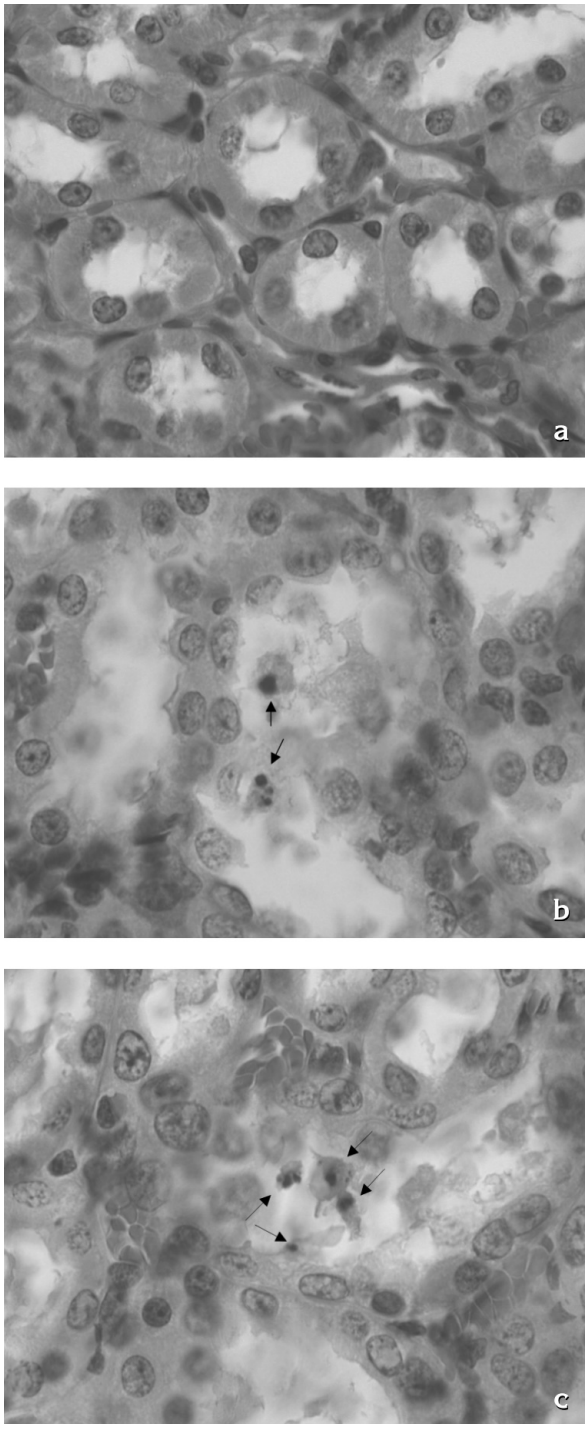

Figure 1 *Lesions of tubular epithelium in rats after exposure to ochratoxin A (OTA). Photomicrographs of HE-stained kidney sections obtained from (a) control animals, (b) two apoptotic cells in OTA-treated rats (0.50 mg /kg b.w./ 3 times a week for 4 weeks), and (c) a number of apoptotic cells in OTA-treated rats (1.00 mg /kg b.w./3 times a week for 4 weeks). Magnification was x 1000*

Table 1 *Concentrations of OTA in plasma and kidney homogenates and the number of apoptotic cells in the epithelium of kidney tubules of rats given a single dose of OTA (1.00 mg/kg b.w., i.p.) and sacrificed 1, 2, 6, or 9 days after OTA administration. All results are presented as the mean and SD from three animals*

Time after administration days[a]	OTA concentration		Number of apoptotic cells (Number of animals)
	Plasma μg/mL	Kidney μg/g tissue	
1	13.9±3.4	2.0±0.3	19.3±13.2 (3)
2	9.5±4.7	1.9±0.4	12.7±11.1 (3)
6	6.7±2.2	1.2±0.4	0.3±0.3 (3)
9	1.7±0.9	0.8±0.1	0 (3)

[a] *Control groups (N=3) treated with a vehicle alone and sacrificed on days 1, 2, 6, or 9 after administration showed no OTA in the plasma and kidney homogenates or apoptotic cells*

Multiple OTA dosing (0.25, 0.50 and 1.00 mg/kg b.w., respectively) caused a dose-dependent increase in its concentration in plasma and kidney homogenate and a significant increase in apoptotic cells in the epithelium of rat kidneys ($P<0.01$) (Table 2). Necrosis was not seen at any applied dose of OTA.

Table 2 *Concentrations of OTA in plasma and kidney homogenates and the number of apoptotic cells in the epithelium of kidney tubules of rats given multiple doses of OTA (0, 0.25, 0.50 and 1.00 mg OTA/kg b.w., three times a week for four weeks) and sacrificed 1 day after the last administration. All results are presented as the mean value and SD of (N) animals*

Dose of OTA mg /kg b.w. x 12	OTA concentration		Number of apoptotic cells (Number of animals)
	Plasma μg/mL	Kidney μg/g tissue	
0[a]	0 (5)	0 (5)	0 (5)
0.25	5.9±1.4(4)	2.2±0.7(4)	40.0±6.8 (4)[b]
0.50	14.2±2.0(5)	5.6±0.8(5)	70.2±15.8 (5)[b]
1.00	n/d	n/d	138.6±38.9 (5)[b]

[a] *Controls treated with a vehicle alone showed no OTA in the plasma and kidney homogenates or apoptotic cells;* [b] *Different from controls (t-test, $P<0.01$); n/d = not done*

In animals administered multiple doses of 0.50 mg OTA/kg b.w., plasma and kidney OTA concentration and the number of apoptotic cells increased gradually up to the six administration ($P<0.05$) (Table 3). Although kidney OTA concentration reached its peak after the 12th administration, plasma OTA concentration, as well as the number of apoptotic cells decreased. The number of apoptotic cells, OTA concentration in plasma and kidney decreased

Table 3 *Concentrations of OTA in plasma and kidney homogenates and the number of apoptotic cells in the kidney epithelium of rats treated with multiple doses of OTA (0.50 mg OTA/kg b.w., i.p.) and sacrificed either on day one after the last administration or on days 6 and 21, respectively. All results are presented as the mean value and SD of (N) animals. No OTA was found in plasma and kidney homogenates or apoptotic cells were seen in six control groups (N=4) given a vehicle alone, and sacrificed on the same days as exposed animals.*

0.50 mg/kg b.w. Number of treatments	Time after last treatment days	OTA concentration		Number of apoptotic cells (Number of animals)
		Plasma μg/mL	Kidney μg/g tissue	
1	1	3.1±2.0 (5)	0.7±0.1 (5)	0.6±0.5[b] (5)
3	1	8.0±3.3 (5)	0.9±0.3 (5)	7.8±12.5 (5)
6	1	16.8±4.6 (5)	2.1±0.5 (5)	112.0±50.4[a] (5)
9	1	n/d	n/d	74.2±34.9[b] (4)
12*	1	14.2± 2.0(5)	5.6±0.8 (5)	70.2±15.8[a] (5)
12	6	n/d	n/d	55.8±23.4[b] (5)
12	21	7.0±3.5(5)	0.9±0.3 (5)	48.0±11.2[b] (5)

[a] Different from controls (t-test, $P<0.05$); [b] $P<0.001$
* from Table 2.; n/d = not done

in rats sacrificed on days 6 and 21 after the 12th administration.

DISCUSSION

OTA increases lipid peroxidation in rat liver or kidney microcosms *in vitro* (8, 9), in bacteria (10) and in Vero cells (11). Lipid peroxidation may contribute to negative effects of OTA *in vivo*, but the effects, measured as concentration of malondialdehyde, vary greatly from species to species (12). The OTA-induced increase in free radicals and lipid peroxidation may trigger apoptosis. The apoptotic changes caused by OTA were demonstrated in cultured hamster kidney and in HeLa cells (13). Apoptotic cells may be distinguished from normal cells by light microscopy, electron microscopy, and several biochemical methods based on the detection of DNA breaks. In our study, light microscopy was used because cell morphology continues to be the "gold standard" for the initial identification of apoptosis (14).

Endemic nephropathy is a disease with insidious initial phase characterised by lesions of the proximal tubules. The most important pathological finding in all advanced cases of the disease is a marked shrinking of the kidney (2). The kidney shrinks progressively, that is, it can be of a normal or just slightly smaller size in the early stage of the disease. *Maaroufi et al.* (4) reported a statistically significant reduction in kidney weight and size in rats treated with 0.4 and 0.8 mg OTA /kg b.w., i.p. every 72 hours for 90 days. We did not notice the described reduction in kidney weight and size in our experiments, which is probably due to shorter exposure to OTA.

In this study, high doses of OTA were applied in order to see whether OTA produced apoptotic cells in the kidney as the target organ of its toxicity. OTA administration, either in a single or multiple doses caused apoptotic changes in proximal tubules of rats' kidney. When a single OTA dose was applied, the number of apoptotic cells decreased with the lag period after OTA administration (Table 1). The administration of different doses of OTA showed a clear dose-dependent appearance of apoptotic cells (Table 2). Other authors described (3) histological changes in the kidney of male rats after a 5-day exposure of to OTA in food. The rats received 0.8 mg of OTA/rat/day (or about 4.00 mg OTA/kg b.w/day) for 5 days. This dose caused an extensive necrosis of the tubular epithelium without apoptotic changes. The necrosis of the tubular epithelium was not seen in our experiments in rats given even the highest OTA dose (1.00 mg/kg b.w. 3 times a week for 4 weeks, i.p.). This difference between the two experiments is either due to differences in OTA doses or in the length of exposure.

The increase in the number of OTA doses (0.50 mg/kg b.w) in our experiments resulted with dose-dependent apoptotic changes until the 6th dose (Table 3). In rats sacrificed 1 day after the 9th and 12th dose, the number of apoptotic cells decreased gradually, although the accumulation of OTA in kidney continued. In animals that received 12 doses and were sacrificed on days 1, 6 and 21, the number of apoptotic cells decreased with the decrease in OTA concentration in plasma and kidney, which is comparable to the results presented in Table 1.

However, it seems that the concentration of OTA in plasma and kidneys is not crucial for the appearance of apoptotic cells, because the concentration of about 7 μg OTA/mL in plasma and about 1 μg OTA/g of kidney tissue in different experiments presented in this paper is associated with a largely varying number of apoptotic cells (Table 1 and Table 3). It seems that the appearance of apoptotic cells is not merely the result of actual OTA concentration in plasma and kidney, but rather of earlier dose and length of exposure.

In our experiments, the apoptotic changes in the rat kidney were the result of very high doses of OTA given intraperitoneally. The tolerable daily intake (TDI) of OTA for humans defined by The WHO Joint Expert Committee of Food Additives is 16.0 ng/kg b.w./day (15, 16), and it is rarely exceeded. Therefore, the human exposure to such high doses of OTA applied in our experiments is highly improbable. However, long-term exposure to small doses could perhaps induce a few apoptotic changes. Detectable apoptosis suggests that there is a large amount of cell deaths because dying cells are rapidly ingested and phagocytised. Chronic toxic lesions can turn into an end-stage renal disease involving the deposition of fibrous tissue in spaces between the tubules, similarly to that seen in kidneys of patients with endemic nephropathy (2).

In conclusion, a single OTA dose can cause apoptosis in rats, but the effect is reversed in nine days. Multiple doses of OTA administered for four weeks maintain tubular epithelial apoptosis and not necrosis, but renal weight as a percentage of body weight does not decrease, which indicates that regenerative processes are counterbalancing the loss of cells due to apoptosis.

Acknowledgement

We wish to thank to Mrs Z. Ihas, Mrs M. Matašin, and Miss M. Škalic for technical assistance. We hereby gratefully acknowledge the financial support by grant No. 0022018 of the Ministry of Science, Education and Sports of the Republic of Croatia.

REFERENCES

1. Krogh P. Role of ochratoxin in disease causation. Food Chem Toxicol 1992;30:213-24.
2. Vukelić M, Šoštarić B, Belicza M. Pathomorphology of Balkan endemic nephropathy. Food Chem Toxicol 1992;30:193-200.
3. Mantle PG, Miljković A, Udupa V, Dobrota M. Does apoptosis cause renal atrophy in Balkan endemic nephropathy? Lancet 1998;352:1118-9.
4. Maaroufi K, Zakhama A, Baudrimont I, Achour A, Abid S, Ellouz F, Dhouib S, Creppy EE, Bacha H. Karyomegaly of tubular cells as early stage marker of the nephrotoxicity induced by ochratoxin A in rats. Hum Exp Toxicol 1999;18:410-4.
5. Peraica M, Domijan A-M, Fuchs R, Lucić A, Radić B. The occurrence of ochratoxin A in blood in general population of Croatia. Toxicol Lett 1999;110:105-12.
6. Bauer von J, Gareis M, Gedek B. Zum Nachweis und Vorkommen von Ochratoxin A bei Schlachtschweinen. Berl Münch Tierärztl Wschr 1984;97:279-83.
7. Schumer M, Colombel MC, Sawczuk IS, Gobe G, Connor J, O'Toole KM, Olsson CA, Wise GJ, Buttyan R. Morphologic, biochemical, and molecular evidence of apoptosis during the reperfusion phase after brief periods of renal ischemia. Am J Pathol 1992;140: 831-8.
8. Rahimtula AD, Bereziat JC, Bussacchini-Griot V, Bartsch H. Lipid peroxidation as a possible cause of ochratoxin A toxicity. Biochem Pharmacol 1988;37: 4469-77.
9. Omar RF, Rahimtula AD, Bartsch H. Role of cytochrome P-450 in ochratoxin A-stimulated lipid peroxidation. J Biochem Toxicol 1991;6,203-9.
10. Hoehler D, Marquardt RR, McIntosh AR, Xiao H. Free radical generation as induced by ochratoxin A and its analogs in bacteria (*Bacillus brevis*). J Biol Chem 1996;271:27388-94.
11. Baudrimont I, Ahouandjivo R, Creppy EE. Prevention of lipid peroxidation induced by ochratoxin A in Vero cells in culture by several agents. Chem Biol Interact 1997;104:29-40.
12. Hoehler D, Marquardt R, Frohlich A. Lipid peroxidation as one mode of action in ochratoxin A toxicity in rats and chicks. Can J Anim Sci 1997;7:287-92.
13. Seegers JC, Bohmer LH, Kruger MC, Lottering ML, de Kock M. A comparative study of ochratoxin A-induced apoptosis in hamster kidney and HeLa cells. Toxicol Appl Pharmacol 1994;129:1-11.
14. Davis MA, Ryan DH. Apoptosis in the kidney. Toxicol Pathol 1998;26:810-25.
15. World Health Organization (WHO). Evaluation of certain food additives and contaminants. Forty-fourth report of the joint FAO/WHO Expert committee on food additives. WHO Technical Report Series 859, Geneva: WHO, 1995, pp. 35-6.
16. World Health Organization (WHO). Evaluation of certain food additives and contaminants. Thirty-seventh report of the joint FAO/WHO Expert committee on food additives. WHO Technical Report Series 806, Geneva: WHO, 1991, pp. 29-31.

Sažetak

APOPTOZA U BUBREŽNOM TKIVU ŠTAKORA UZROKOVANA OKRATOKSINOM A

Okratoksin A (OTA) nefrotoksični je mikotoksin za koji se pretpostavlja da je uzročnik endemske nefropatije i tumora urotela koji se s većom učestalošću javljaju u području endemske nefropatije. Endemska nefropatija je smrtonosna bolest koja zahvaća oba bubrega, a primarno dolazi do oštećenja proksimalnih tubula bubrega. Za sada uzrok bolesti nije poznat. Međutim, niske koncentracije OTA čest su nalaz u krvi ljudi i iz drugih područja. Cilj našeg istraživanja bio je utvrditi da li OTA u bubregu pokusnih životinja uzrokuje apoptozu ili nekrozu. U prvom pokusu štakori su bili tretirani jednokratnom dozom OTA (1,00 mg/kg tj. t., ip.) te su žrtvovani u razdoblju od jedan do 9 dana. Najveći broj apoptotskih stanica nađen je u životinja koje su žrtvovane jedan dan nakon tretmana, njihov se broj postepeno smanjivao te u životinja koje su žrtvovane 9 dana nakon tretmana nisu nađene apoptotske stanice. Ovisnost nastanka histoloških promjena u bubregu štakora o dozi ispitana je u supkroničnom pokusu primjenom triju doza OTA (0,25, 0,50 i 1,00 mg/kg tj. t., ip.) tijekom 4 tjedna (3 puta na tjedan). Koncentracija OTA i nastanak apoptotskih stanica bili su ovisni o primijenjenoj dozi, a u histološkim preparatima nije nađena nekroza. Ovisnost nastanka apoptotskih stanica u bubregu o duljini tretmana s OTA ispitana je na štakorima koji su tretirani višekratno s OTA (0,50 mg/kg tj. t., ip.) i žrtvovani jedan dan nakon prvog, trećeg, šestog, devetog i dvanaestog tretmana, odnosno 6. i 21. dan nakon dvanaestog tretmana. Ovim je pokusom dokazano da je nastanak apoptotskih stanica u bubregu štakora povezan s duljinom tretmana s OTA.

KLJUČNE RIJEČI: *endemska nefropatija, nekroza, proksimalni tubuli*

REQUESTS FOR REPRINTS:

Maja Peraica, M.D., Ph.D.
Institute for Medical Research and Occupational Health
P.O. Box 291, HR-10001 Zagreb, Croatia
E-mail: *mperaica@imi.hr*