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Source / Izvornik: **Croatica Chemica Acta, 2005, 78, 447 - 453**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:163:960659

CROATICA CHEMICA ACTA
CCACAA **78** (3) 447–453 (2005)

ISSN-0011-1643
CCA-3034
Original Scientific Paper

Ochratoxin A Induces Apoptotic and Necrotic Renal Cell Death*

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RECEIVED FEBRUARY 12, 2005; REVISED JUNE 17, 2005; ACCEPTED JUNE 28, 2005

MDCK, LLC-PK1 and RK 13 renal cells were exposed to different OTA concentrations (2.5 to 25 μg OTA/mL of medium) for 24 hours. 2.5 μg OTA/mL significantly reduced the number of living RK 13 cells to 69.4 %, LLC-PK1 cells to 34.5 % and MDCK cells to 27.5 %, as compared to untreated control cells. Effects of OTA on cell morphology were examined by haematoxylin/eosin or phalloidin-FITC/propidium iodide staining. 12 %, 11 % and 8.2 % apoptotic cells were observed in MDCK, LLC-PK1 and RK 13 cells treated with 2.5 μg OTA/mL, respectively. The structure of actin cytoskeleton showed significant changes in OTA-treated cells. Release of LDH into the culture medium was more abundant in MDCK and LLC-PK1 cells. OTA induced apoptotic and/or necrotic cell death in a cell type- and concentration-dependent manner. The results suggest that MDCK and LLC-PK1 cells are more sensitive to OTA than RK 13 cells.

Keywords
ochratoxin A
apoptosis
necrosis
MDCK cells
LLC-PK1 cells
RK 13 cells

INTRODUCTION

Ochratoxin A (OTA) is a widespread mycotoxin produced by several *Aspergillus* and *Penicillium* species.[1–3] Ingestion of OTA-contaminated feed or food has been associated with nephrotoxic,[4] genotoxic[5,6] and carcinogenic[7–10] effects, perturbation of normal blood coagulation[11] and immune response.[12] OTA is considered as one of the etiologic agents involved in the development of endemic nephropathy[10,13,14] in humans as well as in tubular-interstitial nephropathy in humans and animals. The toxin affects different nephron functions, including renal blood flow, glomerular and tubular function.[4,15–18] The main targets of OTA are epithelial cells of proximal and distal tubules and interstitial cells. OTA induces defects of the organic anion transport mechanism located on the brush border membranes of the proximal convoluted tubule cells and basolateral membrane.[19] It has been found that the middle (S2) and the terminal (S3) segments of the proximal tubule are the most sensitive to the toxic ef-

* Dedicated to Professor Željko Kućan on the occasion of his 70th birthday. Presented at the Congress of the Croatian Society of Biochemistry and Molecular Biology, $HDBMB_{2004}$, Bjelolasica, Croatia, September 30 – October 2, 2004.

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fects of OTA. These effects are observed through a significant decrease in mitochondrial and cellular ATP contents after treatment with 0.05 mmol/L OTA.[20]

Long-term effects of OTA on kidneys are usually primarily related to lesions of proximal tubules, followed by spontaneous damage of the glomeruli and involution of the interstitia.[4] On the other hand, it was shown that acute OTA intoxication resulted in massive acidophilic degeneration with necrosis and desquamation of epithelium in proximal tubules.[21]

The mechanism of OTA cytotoxicity mainly depends on the toxin concentration and on the period of organism/cell exposure to OTA. Lower toxin concentrations affect a cell in a specific way through interactions with secondary messengers and other cell targets, which subsequently activate mitogen-activated protein kinases (MAPKs)[22] or inhibit certain carriers and disturb normal cell homeostasis.[17] At relatively high concentrations severe disturbances in the cell metabolism are observed, such as inhibition of the synthesis of macromolecules,[23] stimulation of lipid peroxidation[24,25] or disintegration of mitochondria[26] and necrosis of epithelial tubule cells.[21] In addition, previous studies implicate stimulation of oxidative stress in OTA-induced cytotoxicity.[25,27–31]

OTA-dependent induction of cell death has been reported both *in vivo* and *in vitro*: in rat kidney,[29,32] in mice liver,[28] in human lymphocytes,[33] in MDCK and HeLa cells,[34–36] in LLC-PK1 cells,[30] in V79 and CV-1 cells, respectively.[31]

Cell death can generally proceed via necrosis or apoptosis. Necrosis is a deregulated form of cell death induced by strong pathological stimuli. On the other hand, apoptosis is an organized, highly regulated physiological and pathological form of cell death. In contrast to the swelling and membrane rupture being typical of necrosis, a cell undergoing apoptosis rapidly condenses into small enclosed apoptotic bodies, which can be consequently removed by phagocytosis. Apoptosis can be induced by multiple stimuli, including radiation, heat, cytokines, free radicals and/or toxins.[37–39] Apoptosis is an active mode of cell death that promotes cell loss during both acute and chronic renal damage. Numerous reports have indicated that alterations in the regulation of cell death/survival contribute to the pathogenesis of many human diseases.[40,41] Mechanisms of cell death induced by OTA have not been fully characterized.

The aim of the present study was to examine the toxicity of relatively high OTA concentrations on renal cells, as well as to establish the mode and the extent of cell death provoked by OTA. We performed our research on three different cell lines: LLC-PK1 (pig kidney cell line, corresponding to the proximal tubule epithelial cells), MDCK (Madin-Darby canine kidney cell line, corresponding to the distal tubule epithelial cells) and RK 13 (rabbit kidney cell line).

EXPERIMENTAL

Cell Culture

Kidney cell lines were maintained in Eagle´s MEM culture medium supplemented with 10 % foetal bovine serum (FBS), 100 IU/mL (≈ 60 μg/mL) penicillin, 100 μg/mL streptomycin and 2.5 μg/mL amphotericin B in a humidified atmosphere containing 5 % CO_2 at 37 °C. MDCK (Madin-Darby canine kidney cell line), LLC-PK1 (pig kidney cell line) and RK 13 (rabbit kidney cell line) cells were plated in 6 or 12 well-plates at a plating density of 10^5–10^6 cells/mL.

Treatment

MDCK, LLC-PK1 and RK13 cell lines were treated with 2.5, 5, 15 and 25 μg OTA/mL for 24 hours. Stock solution (10 mg/mL) was prepared by dissolving OTA in ethanol and was protected from light. The final OTA concentration was obtained by appropriate dilution of the stock solution in the respective culture medium. The final ethanol concentration in the medium of treated or control cells was 0.25 % for each sample.

Morphological Characterization

Approximately 5×10^4/mL MDCK, LLC-PK1 and RK 13 cells were seeded onto rounded glass coverslips placed in 6 or 12 well-plates. 80–90 % confluence was reached after 24–48 hours of incubation. The cells were then exposed to different OTA concentrations. The cells were washed with phosphate-buffered saline (PBS, 20 mmol dm^{-3} phosphate buffer, 150 mmol dm^{-3} NaCl, pH = 7.2) and the adherent cells were fixed and stained with haematoxylin and eosin (H + E) using the Kiernan method.[42] The effects of OTA in MDCK, LLC-PK1 and RK 13 cells, including apoptotic and mitotic changes, were quantified after 24 h treatment.

TUNEL Assay

TUNEL was carried out using dUTP–fluorescein isothiocyanate (dUTP–FITC), according to the instructions of the manufacturer (In Situ Cell Death Detection Kit, Boehringer Mannheim, Germany). Adherent cells were fixed onto glass slides with 4 % paraformaldehyde in PBS, and the fragmented DNA of apoptotic cells was measured by catalytic incorporation of FITC-labelled dUTP at 3'-OH ends using the terminal deoxynucleotidyl transferase. The dUTP-FITC labelled DNA was visualized using fluorescence microscopy (Olympus BX50).

Actin Cytoskeleton Stained with Phalloidin-FITC

Adhered cells were fixed onto coverslips with 3.5 % paraformaldehyde, washed in PBS, permeabilized with 0.1 % Triton X-100 for 15 min and washed again in PBS. The cytoskeleton structure was studied by staining actin filaments with 50 μL of phalloidin-FITC solution (Sigma) for 15 min (24 μL of 0.05 mg/mL phalloidin-FITC in ethanol dissolved in 1.2 mL PBS). Cell nuclei were simultaneously labelled with 50 μL of propidium iodide solution for 5 min (20 μg

TABLE I. The number of total adherent, apoptotic and mitotic cells in untreated and treated MDCK, LLC-PK1 and RK 13 stained with haematoxylin and eosin[a]

Cell lines	Total cell number	Apoptotic cells	Mitotic cells	Mitosis/1000 stained cells
MDCK				
Control	385 ± 19	0.6 ± 0.1	7.0 ± 1.1	18 ± 2.9
2.5 μg OTA/mL	106 ± 16	12.7 ± 2.3	0,3 ± 0.1	3 ± 0.9
5 μg OTA/mL	17 ± 3.1	4.8 ± 0.7	0	0
15 μg OTA/mL	1 ± 0.2	0	0	0
25 μg OTA/mL	0	0	0	0
LLC-PK1				
Control	336 ± 21	0,7 ± 0.1	6,3 ± 0.9	19 ± 2.7
2.5 μg OTA/mL	116 ± 18	13,1 ± 1.3	0.2 ± 0.1	1.7 ± 0.9
5 μg OTA/mL	26 ± 3	5,3 ± 0.3	0	0
15 μg OTA/mL	2 ± 0.1	2 ± 0.1	0	0
25 μg OTA/mL	1 ± 0.1	1 ± 0.1	0	0
RK 13				
Control	385 ± 24	5,7 ± 1.1	0,7 ± 0.1	1.8 ± 0.3
2.5 μg OTA/mL	267 ± 19	21.9 ± 3.9	0	0
5 μg OTA/mL	190 ± 12	25,2 ± 2.6	0	0
15 μg OTA/mL	153 ± 13	66,2 ± 5.1	0	0
25 μg OTA/mL	119 ± 8	9,4 ± 1.3	0	0

[a]Values represent the means ± SD of total, apoptotic and mitotic cells calculated in 15 optical fields (magnification 200×)

propidium iodide in 1 mL PBS). The phalloidin-FITC and propidium iodide stained cells were visualized using fluorescence microscopy (Olympus BX50).

Lactate Dehydrogenase (LDH) Assay

Catalytic activity of LDH was determined by the kinetic UV test using 50 μL of culture supernatant, 2 mL of working reagent (61.43 mmol dm^{-3} Tris buffer, 0.20 mmol dm^{-3} NADH, pH = 7.4) and 10 μL of substrate (21.5 mmol dm^{-3} pyruvate). Absorbances were measured at 339 nm every 30 s during a 5-min time period with a Pye-Unicam SP8-100 UV/Vis spectrophotometer.

Statistical Analysis

Obtained data were analyzed by the one-way analysis of variance (ANOVA) followed by a multiple comparison method (Dunnett's test). The level of $P<0.05$ was considered statistically significant for all the tests performed.

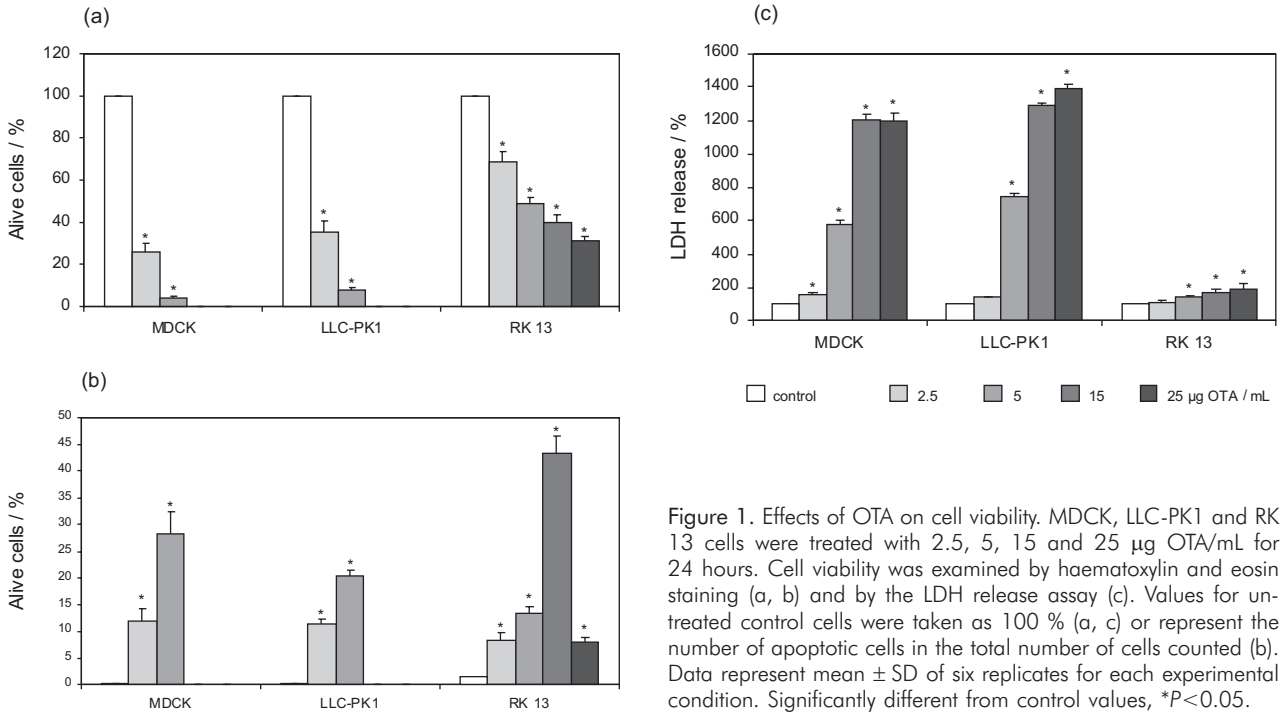

Figure 1. Effects of OTA on cell viability. MDCK, LLC-PK1 and RK 13 cells were treated with 2.5, 5, 15 and 25 μg OTA/mL for 24 hours. Cell viability was examined by haematoxylin and eosin staining (a, b) and by the LDH release assay (c). Values for untreated control cells were taken as 100 % (a, c) or represent the number of apoptotic cells in the total number of cells counted (b). Data represent mean ± SD of six replicates for each experimental condition. Significantly different from control values, *$P<0.05$.

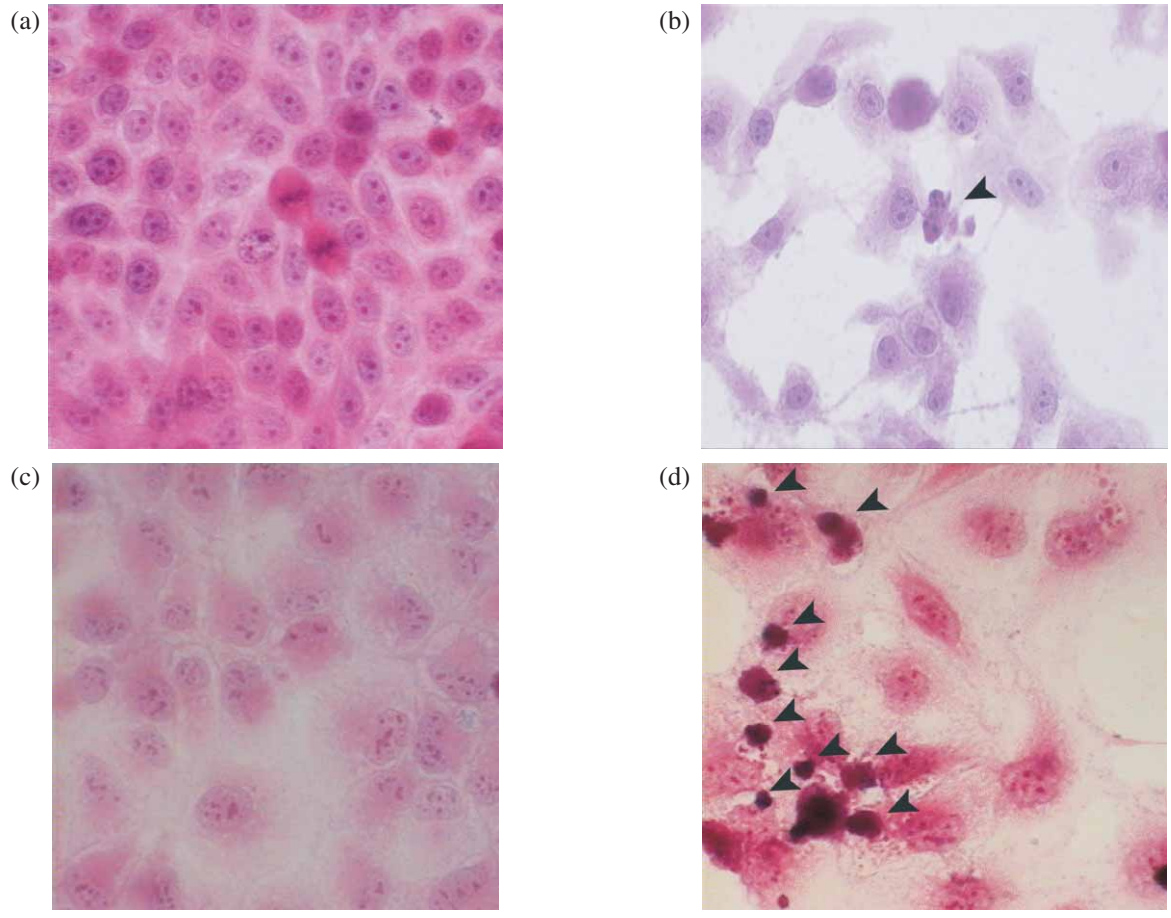

Figure 2. OTA induces apoptosis in MDCK and RK 13 cells. MDCK (a, b) and RK 13 (c, d) cells were treated with 2.5, 5, 15 and 25 μg OTA/mL for 24 hours. Cells were stained with haematoxylin and eosin. Representative photomicrographs for untreated control cells (a, c) and cells treated with 2.5 μg OTA/mL (b) or 15 μg OTA/mL (d) are shown (magnification 630 x). Apoptotic cells and bodies are more intensively stained (arrows).

RESULTS AND DISCUSSION

We have examined the effects of different OTA concentrations (2.5, 5, 15 and 25 μg OTA/mL) on the viability of MDCK, LLC-PK1 and RK 13 cells after 24 hours of treatment. The impairments of the renal cell function observed after exposure to OTA depended significantly on the toxin concentration, as described in many studies. Concentrations of OTA below 10^{-6} mol dm^{-3} (<0.403 μg/mL) are considered as low, and those above 10^{-6} mol dm^{-3} are considered as high OTA concentrations for the majority of cell lines.[15]

We found that the number of living cells significantly decreased with increasing the concentration of OTA. 2.5 μg OTA/mL reduced the number of living RK 13 cells to 69.4 %, LLC-PK1 cells to 34.5 % and MDCK cells to 27.5 %, as compared to untreated control cells (Table I, Figure 1a). This means that the 2.5 μg OTA/mL concentration reduced the number of living MDCK and LLC-PK1 cells to less than 50 % of the control samples. Kitabatake and co-workers found that the LD_{50} value for OTA was 2 ppm (2 μg/mL) in MDCK cells exposed to OTA for 72 hours.[34] On the other hand, the percentage of living RK 13 cells was relatively high (31 %) even after treatment with a ten times higher OTA concentration (25 μg/mL).

In a subsequent series of experiments we examined the extent of apoptosis and necrosis, and also the alterations of cell morphology after OTA treatment by haematoxylin and eosin or phalloidin-FITC and propidium iodide staining. Apoptotic cells were also visualized using the TUNEL assay. Further, the LDH release into the culture medium, as an indicator of necrotic and/or late apoptotic processes, was determined. In MDCK, LLC-PK1 and RK 13 cells treated with 2.5 μg OTA/mL for 24 hours, 12 %, 11 % and 8.2 % apoptotic cells were observed, respectively (Figures 1b, 2 and 3). The number of apoptotic cells showed a concentration dependency with the highest number of apoptotic cells detected after incubation with 5 μg OTA/mL for MDCK and LLC-PK1 cells or with 15 μg OTA/mL for RK 13 cells. Higher OTA concentrations provoked predominantly necrotic cell death. This was confirmed by increased LDH release into the culture me-

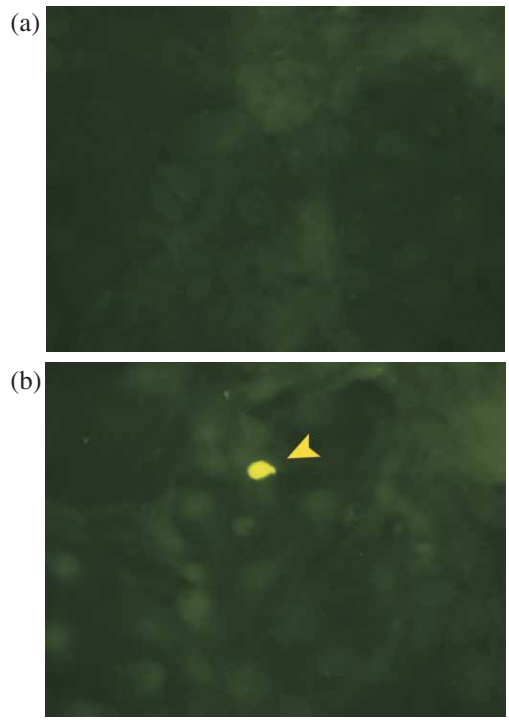

Figure 3. OTA induces apoptosis in LLC-PK1 cells. LLC-PK1 cells were treated with 2.5, 5, 15 and 25 μg OTA/mL for 24 hours. Cells were stained with FITC-dUTP using the TUNEL technique. Representative photomicrographs for untreated control cells (a) and cells treated with 2.5 μg OTA/mL (b) are shown (magnification 500 x). The TUNEL positive nuclei indicating apoptotic cells are stained yellow-green (arrow).

dium. The release of LDH after treatment with OTA was more abundant in MDCK and LLC-PK1 cells than in RK 13 cells, indicating a more intensive necrotic process (Figure 1c). Haematoxylin and eosin staining confirmed a severe loss of MDCK cells treated with 2.5 μg OTA/mL, while RK 13 cells treated with a six times higher OTA concentration still showed a large number of adherent cells and greater resistance to OTA-induced cytotoxicity (Figure 2). Our results suggest that the effects of OTA are not exclusively concentration dependent but cell type-specific as well.

Gekle and colleagues investigated the effects of OTA in two clones of MDCK kidney cells, with MDCK-C7 cells resembling the principal cells and MDCK-C11 cells resembling the intercalated cells.[36] Apoptosis (measured by caspase-3 activity and DNA fragmentation), but not necrosis (measured by LDH leakage), was observed in MDCK-C7 cells treated with 100 nmol/L OTA. The authors suggested that the c-Jun N-terminal kinase (JNK) signalling pathway might play a role in OTA-induced MDCK-C7 cell apoptosis. In contrast, OTA provoked primarily necrosis in MDCK-C11 cells, which confirms once again the different susceptibility of cells to OTA.

It has been reported that OTA increased the rate of both CV-1 cell apoptosis and necrosis within a quite narrow concentration range (1–10 μmol/L), which underlines that OTA mediated cell death cannot be ascribed exclusively to the induction of apoptosis.[31] A research on cell death induced by ischemic and toxic injuries in renal tissue also suggests that tubular epithelial cells may die by either necrosis or apoptosis, depending upon the severity of the injury to which the cells are exposed.[41]

In our study, OTA induced significant changes of the cytoskeleton structure, especially in LLC-PK1 cells (Figure 4). These changes could be caused by a decrease in intracellular ATP levels,[43] activation of caspases[44] and/or activation of calpain.[45] Cytoskeletal and cell-membrane proteins that anchor cytoskeletal elements to the plasma membrane are excellent substrates for calpain, a Ca^{2+}-dependent cytosolic cysteine protease. Calpain cleaves actin-binding proteins ankyrin and spectrin along the basolateral side of the proximal tubule epithelial cells, leading to membrane damage and internalization of Na^+/K^+-ATPase, and consequently to decreased reabsorption of Na^+ and to loss of cell polarity.[44] The loss of cell-cell or cell-matrix connections as well as shrinkage of cells and apoptosis could also be observed.[46] Dopp *et al.* showed that the OTA-induced intracellular Ca^{2+} increase is caused by Ca^{2+}-release from intracellular stores, as well as by Ca^{2+} influx from the extracellular space. It has been suggested that the disruption of actin filaments after OTA

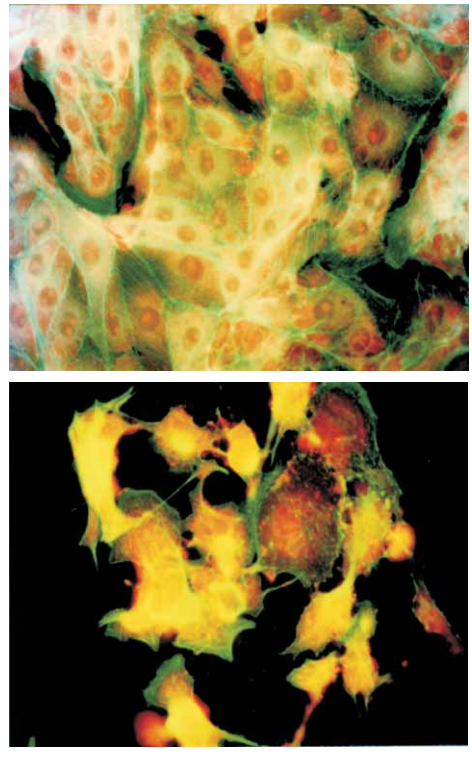

Figure 4. OTA induces structural changes of actin cytoskeleton. LLC-PK1 cells were treated with 2.5, 5, 15 and 25 μg OTA/mL for 24 hours. Cells were stained simultaneously with phalloidin-FITC and propidium iodide. Representative photomicrographs for untreated control cells (a) and cells treated with 2.5 μg OTA/mL (b) are shown (magnification 400 x). Phalloidin-FITC-labeled actin cytoskeleton is changed in OTA-treated cells.

treatment does not correlate with the increased intracellular Ca^{2+}. The authors have concluded that OTA disrupts actin filaments by direct irreversible binding to actin.[47]

In general, OTA-provoked cytotoxicity can occur with different scenarios, mostly depending on OTA concentration: low concentrations induce reversible changes with preserved homeostasis, then activation of the apoptotic process occurs, and stimulation of both apoptosis and necrosis, and finally, at higher OTA concentrations the processes end in induction of necrotic cell death. Our results show that 2.5 to 25 μg OTA/mL concentrations provoked primarily necrosis, but apoptosis also occurred. The highest relative ratio of apoptotic to adherent cells was detected after incubation with 5 μg OTA/mL for MDCK and LLC-PK1 cells or with 15 μg OTA/mL for RK 13 cells. Our results suggest that apoptosis and necrosis occur in a cell type dependent manner.

CONCLUSIONS

OTA induced apoptotic and necrotic processes in MDCK, LLC-PK1 and RK 13 kidney cells. 2.5 and 5 μg/mL OTA significantly stimulated apoptosis in both LLC-PK1 and MDCK (morphologically related to proximal and distal tubule epithelial cells, respectively), and to a lesser extent in RK 13 cells. Due to the observed anti-mitotic effect of OTA, the apoptotic process was accelerated and could play an important role in the development of chronic tubular-interstitial kidney damages. In our study, necrosis occurred primarily when higher OTA concentrations were applied, with the necrotic process being more intensive in MDCK and LLC-PK1 cells. The results suggest that MDCK and LLC-PK1 cell lines are more sensitive to OTA than the RK 13 cell line and represent a better *in vitro* model for studying the mechanisms of OTA nephrotoxicity.

Acknowledgements. – This study was supported by the Croatian Ministry of Science, Education and Sports (grant No. 0006631).

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SAŽETAK

Okratoksin A inducira apoptotičko i nekrotičko umiranje bubrežnih stanica

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MDCK, LLC-PK1 i RK 13 bubrežne stanice tretirane su različitim koncentracijama OTA (od 2,5 do 25 μg OTA/mL medija) tijekom 24 sata. Koncentracije toksina od 2,5 μg/mL uzrokuju značajno smanjenje broja živih stanica u odnosu na netretirane kontrolne uzorke: kod RK 13 stanica broj je smanjen na 69,4 %, kod LLC-PK1 na 34,5 %, a kod MDCK na 27,5 %. Ispitan je učinak OTA na morfologiju stanica koje su bojane hematoksilinom/eozinom odnosno faloidin-FITC/propidijevim jodidom. Kod MDCK, LLC-PK1 i RK 13 stanica tretiranih s 2,5 μg OTA/mL opaženo je 12 %, 11 % odnosno 8,2 % stanica u apoptozi. OTA je uzrokovao značajne promjene u strukturi aktinskoga citoskeleta u tretiranim stanicama. OTA je potaknuo istjecanje LDH iz stanica u okolni medij, što je osobito izraženo u MDCK i LLC-PK1 stanicama. Možemo zaključiti da način umiranja stanica (apoptoza ili nekroza) ovisi o vrsti samih stanica i o primijenjenoj koncentraciji toksina. Naši rezultati ukazuju da su MDCK i LLC-PK1 stanice osjetljivije na djelovanje OTA od RK 13 stanica.