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Ochratoxin A Induces Apoptotic and Necrotic Renal Cell Death*

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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 carcinogenic7–10 effects, perturbation of normal blood coagulation11 and immune response.12 OTA is considered as one of the etiologic agents involved in the development of endemic nephropathy10,13,14 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.3,15–18 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-

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

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.


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fects of OTA. These effects are observed through a signif-
ificant decrease in mitochondrial and cellular ATP con-
tents after treatment with 0.05 mmol/L OTA.20

Long-term effects of OTA on kidneys are usually pri-
marily related to lesions of proximal tubules, follow-
ed by spontaneous damage of the glomeruli and involu-
tion of the interstitia.4 On the other hand, it was shown
that acute OTA intoxication resulted in massive acido-
philic degeneration with necrosis and desquamation of
epithelium in proximal tubules.21

The mechanism of OTA cytotoxicity mainly de-
pend on the toxin concentration and on the period of or-
ganism/cell exposure to OTA. Lower toxin concentra-
tions 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 se-
vere disturbances in the cell metabolism are observed,
such as inhibition of the synthesis of macromolecules,23
stimulation of lipid peroxidation24,25 or disintegration of
mitochondria26 and necrosis of epithelial tubule cells.21
In addition, previous studies implicate stimulation of ox-
dative stress in OTA-induced cytotoxicity.25,27–31

OTA-dependent induction of cell death has been re-
ported 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 physio-
logical and pathological form of cell death. In contrast to
the swelling and membrane rupture being typical of ne-
crosis, a cell undergoing apoptosis rapidly condenses
into small enclosed apoptotic bodies, which can be con-
sequently 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 dur-
ing both acute and chronic renal damage. Numerous re-
ports 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 in-
duced 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 atmos-
phere containing 5 % CO2 at 37 °C. MDCK (Madin-Darby
canine kidney cell line), LLC-PK1 (pig kidney cell line) and
RK 13 (rabbit kidney cell line) were plated in 6 or 12
well-plates at a plating density of 105–106 cells/mL.

Treatment

MDCK, LLC-PK1 and RK 13 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 5×103/mL MDCK, LLC-PK1 and RK 13 cells
were seeded onto round 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 differ-
ent OTA concentrations. The cells were washed with phos-
phate-buffered saline (PBS, 20 mmol dm–3 phosphate buf-
ter) 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 isothio-
cyanate (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 frag-
mented 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 micro-
scopy (Olympus BX50).

Actin Cytoskeleton Stained with Phalloidin-FITC

Adhered cells were fixed onto coverslips with 3.5 % para-
formaldehyde, 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
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.

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**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\(^{(a)}\)

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Total cell number</th>
<th>Apoptotic cells</th>
<th>Mitotic cells</th>
<th>Mitosis/1000 stained cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDCK</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>385 ± 19</td>
<td>0.6 ± 0.1</td>
<td>7.0 ± 1.1</td>
<td>18 ± 2.9</td>
</tr>
<tr>
<td>2.5 µg OTA/mL</td>
<td>106 ± 16</td>
<td>12.7 ± 2.3</td>
<td>0.3 ± 0.1</td>
<td>3 ± 0.9</td>
</tr>
<tr>
<td>5 µg OTA/mL</td>
<td>17 ± 3.1</td>
<td>4.8 ± 0.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15 µg OTA/mL</td>
<td>1 ± 0.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>25 µg OTA/mL</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LLC-PK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>336 ± 21</td>
<td>0.7 ± 0.1</td>
<td>6.3 ± 0.9</td>
<td>19 ± 2.7</td>
</tr>
<tr>
<td>2.5 µg OTA/mL</td>
<td>116 ± 18</td>
<td>13.1 ± 1.3</td>
<td>0.2 ± 0.1</td>
<td>1.7 ± 0.9</td>
</tr>
<tr>
<td>5 µg OTA/mL</td>
<td>26 ± 3</td>
<td>5.3 ± 0.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15 µg OTA/mL</td>
<td>2 ± 0.1</td>
<td>2 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>25 µg OTA/mL</td>
<td>1 ± 0.1</td>
<td>1 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RK 13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>385 ± 24</td>
<td>5.7 ± 1.1</td>
<td>0.7 ± 0.1</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>2.5 µg OTA/mL</td>
<td>267 ± 19</td>
<td>21.9 ± 3.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5 µg OTA/mL</td>
<td>190 ± 12</td>
<td>25.2 ± 2.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15 µg OTA/mL</td>
<td>153 ± 13</td>
<td>66.2 ± 5.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>25 µg OTA/mL</td>
<td>119 ± 8</td>
<td>9.4 ± 1.3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^{(a)}\)Values represent the means ± SD of total, apoptotic and mitotic cells calculated in 15 optical fields (magnification 200×).

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![Figure 1](https://example.com/figure1.png)

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, \(^*P<0.05\).
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 LD₅₀ 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-
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 \( \times \) 10\(^{-9}\) g 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.\(^3\)\(^6\) Apoptosis (measured by caspase-3 activity and DNA fragmentation), but not necrosis (measured by LDH leakage), was observed in MDCK-C7 cells treated with 2.5 \( \mu \)g 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.

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,\(^1\) activation of caspases\(^4\) and/or activation of calpain.\(^4\) 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.\(^4\) The loss of cell-cell or cell-matrix connections as well as shrinkage of cells and apoptosis could also be observed.\(^4\)\(^5\) 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

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**Figure 4.** OTA induces structural changes of actin cytoskeleton. LLC-PK1 cells were treated with 2.5, 5, 15 and 25 \( \mu \)g 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 \( \mu \)g 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 process ends in induction of necrotic cell death. Our results show that 2.5 to 25 \(\mu\)g 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 \(\mu\)g OTA/mL for MDCK and LLC-PK1 cells or with 15 \(\mu\)g 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 \(\mu\)g/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.

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OCHRATOXIN A INDUCES CELL DEATH

József Petrik, Ana Malić, Karmela Barišić, Lada Rumora, Tamás Kőszegi, Stjepan Pepeljnjak, Tihana Žanić-Grubišić i Ivana Čepelak

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 uticaj 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.

SAŽETAK

Okratoksin A inducira apoptotičko i nekrotičko umiranje bubrežnih stanica