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Species- and sex-specific renal cytotoxicity of Ochratoxin A and B *in vitro*

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With 4 figures and 8 tables

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Summary

Four different cell models were chosen for comparison of OTA and OTB toxicity: primary porcine (PKC), rat (RPTC) and human renal proximal epithelial cells (HKC) from both sexes and a porcine renal cell line: LLC-PK1. Culture conditions were tested and optimized for each respective cell type (species/sex and origin). All cell types were characterized for epithelial origin and growth patterns and following optimization of dosing strategies and assay procedures, a strict study design was implemented to avoid systemic variations. Due to possible sensitivity differences, three simple endpoints were chosen to provide basic data for interspecies comparison: neutral red uptake, MTT reduction and cell number. Of the endpoints tested neutral red appeared the most sensitive, although all three parameters yielded comparable EC_{50}'s. Sex-differences were observed between male and female HKC cells following 96 h exposure to OTA, with HKC(m) being more sensitive than HKC(f). No sex-difference was observed in PKC cells, however, the PKC were approximately 3 and 10 times more sensitive than HKC(m) and HKC(f), respectively, to OTA and OTB. Interestingly, the CI_{95} of the EC_{50} values obtained for OTA (15.5–16.5 µM) and OTB (17.0–21.0 µM) were comparable in the PKC cells. In contrast, OTB had lower cytotoxicity than OTA in HKC and LLC-PK1 (approx. 2-fold) and no effects in RPTC. Overall, HKC(m) were nearly as sensitive as PKC towards OTA, followed by RPTC, LLC-PK1 and HKC(f), thus suggesting a sex specific sensitivity in humans towards OTA induced cytotoxicity.

Introduction

The mycotoxin ochratoxin A (OTA) is a secondary metabolite of certain ubiquitous *Aspergillus* and *Penicillium* species. Ochratoxins are known contaminants of human foodstuffs e.g. cereals, beer, wine and coffee and animal feeds arising due to sub-optimal storage conditions (SPEIJERS and VAN EGMOND 1993; STUDER-ROHR et al. 1995). Oral exposure to moderately high concentrations of OTA induces nephrotoxicity in domestic and laboratory animals (STOEV et al. 1998). Furthermore, chronic exposure to OTA via foodstuffs has been associated with the human renal disease Balkan endemic nephropathy (BEN) and with increased incidences of urothelial tumors (UT). Although patients presenting with BEN often are also afflicted with UT, neither the etiology of BEN or UT is known, nor can it *a priori* be assumed that the two diseases have a common causative agent(s) or etiology(ies). Epidemiologic studies of the regions endemic for BEN and UT suggest that women are slightly more affected than men (BOBINAC 1996; TATU et al. 1998). These sex differences are also apparent in other species. Dosing of F344 rats with low (21–210 µg OTA/ kg bw) for 2 years produced a ten-fold higher incidence of renal cortical tumors in male than in female rats (BOORMAN 1989). A similar carcinogenicity study conducted with $B6C3F_1$ mice, however using up to 4 mg OTA/ kg bw, produced a 62% incidence of renal

cortical tumors in male mice while female mice were completely refractive (BENDELE et al. 1985). To summarize, the available human and animal data show large species- and sex-differences in susceptibility towards OTA-induced nephrotoxicity and carcinogenicity (BENDELE et al. 1985; BOORMAN et al. 1992), however the mechanism(s) resulting in these differences as yet, remain to be elucidated. Slight modifications in the *in vitro* techniques, namely cell culture in the presence or absence of serum, OTA concentrations employed and assay procedures can severely alter the experimental outcome (BONDY and ARMSTRONG 1998) and thus, result in different toxicity rankings.

A study with IHKE-1 cells of human origin reported a significant decrease in cell number and an increase in LDH release within 24 hours following exposure to 50 nM OTA in the absence of serum (BENESIC et al. 1999). A further study using LLC-PK1 cells, also carried out in the absence of serum, reported an EC_{50} value of approximately 15 µM for MTT reduction following 24 hour OTA exposure (BONDY and ARMSTRONG 1998). Other authors have reported EC_{50} values of approximately 1 µM OTA and 5 µM OTA for unscheduled DNA synthesis and inhibition of thymidine incorporation, in primary porcine urinary bladder epithelial cells (DÖRRENHAUS and FÖLLMANN, 1997) and rat proximal tubular cells (GEKLE et al. 1995a) respectively, using defined serum-free media supplemented with specific growth factors and hormones and a ≤ 24 hour exposure duration.

In contrast, at least ten-fold higher OTA concentrations were required to induce significant morphological nuclear changes and DNA degradation after 24 hours in hamster kidney and HeLa cells in the presence of serum (SEEGERS 1994).

These drastic discrepancies in the findings reported for OTA *in vitro* cytotoxicity with regard to the concentrations required to produce comparable effects (ranging from nM – µM), lead to difficulties in the elucidation of the underlying mechanisms of ochratoxin toxicity. Therefore, it was deemed necessary to investigate the acute OTA- and B-induced cytotoxicity in a single, stringently designed study. To this end four different cell models were chosen for testing: primary porcine, rat and human renal proximal epithelial cells from both sexes and a porcine renal cell line: LLC-PK1. Culture conditions were tested and optimized for each respective cell type (species and origin, e.g. primary or cell line). All primary cell types were characterized for epithelial origin and growth patterns and following preliminary testing of dosing strategies and assay procedures, three simple endpoints were chosen to provide basic data for interspecies comparison: neutral red uptake (BORENFREUND and PUERNER 1985), MTT reduction (ISLET et al. 1989) and cell number. Ochratoxin B (OTB), a natural analog of OTA, which differs from OTA only by the substitution of a hydrogen group for the chloride group at the isocoumarin moiety, has been shown to be much less toxic *in vivo* (DOSTER et al. 1972; PECKHAM et al. 1971). Therefore, OTA and OTB were used for comparison in all tests.

Material and methods

Materials: Highly purified ochratoxin A and B (>98% purity, benzene free) were kindly provided by Dr. M. E. STACK, FDA, Washington, D.C. Media and other cell culture chemicals were purchased from Gibco Life Technologies, Karlsruhe, Germany and Fetal calf serum (FCS) from Biochrom, Berlin, Germany. Unless otherwise stated, all other chemicals were purchased from Sigma, Taufkirchen, Germany.

Animals and tissues: Female and male Fischer rats (CDF®(F344)/Crl BR) (150–200 g) were purchased from Charles River, Sulzfeld, Germany and maintained by veterinarians and specifically skilled animal personnel in the animal research center (TFA) of the University of Konstanz. Whole kidneys from freshly-killed improved German hybrid pigs of both sexes were obtained from a local slaughterhouse.

Human tissue samples: Human renal biopsy material (male and female patients) was obtained from the local hospital (Klinikum Konstanz) in collaboration with the Department of Urology, Prof. Dr. HOCHBERG, subsequent to patient information and receipt of the signed patient's agreement form. Tissue samples as well as patient data were handled in compliance with the stipulations put forth by the ethics committee of the University of Konstanz and in fulfillment of German law and the Declaration of Helsinki (1964) pertaining to personal data protection and handling of human biopsy material.

Cell preparation and culture: Isolation and culture of primary rat proximal kidney cells (RPTC). Briefly, rats were anesthetized with sodium pentobarbital, kidneys were removed and placed in ice-cold HBSS-EGTA-buffer (5.36 mM KCl, 0.34 mM NaH_2PO_4, 0.44 mM KH_2PO_4, 145 mM NaCl, 10 mM Hepes, pH 7.4, 300 mosM, supplemented with 0.2 mM EGTA, 2000 U/l penicillin and 2 mg/l streptomycin sulfate). After decapsulation and removal of the papillary and medullary part, the remaining cortex was minced with scissors, washed twice in HBSS-EGTA and twice in HBSS (centrifugation at 1,000 rpm (174 × g), room temperature, 2 min). The resultant pellet was resuspended in 30 ml of HBSS at 37 °C, containing 1 mg/ml collagenase (Type I from *Clostridium histolyticum*), 4 mM $CaCl_2$, 1 mM $MgCl_2$, and incubated on a magnetic stirrer at 37 °C for 20 min. The suspension was filtered through a 200 µm gauze and incubated for 10 min. Following filtration through a 40 µm gauze, the cell suspension was washed twice with HBSS as described above. The pellet was resuspended in 20 ml HBSS, mixed with 128 ml 45% isosmotic Percoll (LASH 1992) and transferred into four polycarbonate Oak Ridge centrifugation tubes (Sorvall, Bad Homburg, Germany). Cells were centrifuged at 12,000 rpm (20,000 × g) at 4 °C for 30 min using a Sorvall RC-5 superspeed centrifuge equipped with an SS34 rotor. Thereafter, the upper 24 ml of all tubes, containing predominantly proximal tubular cells,

and the remaining 13 ml of all tubes, containing predominantly distal tubular cells, respectively, were carefully collected, pooled and washed several times with HBSS. The resulting pellet contained single cells, cell aggregations and small tubular fragments. This was resuspended in DMEM/F12 medium, containing 10% FCS and antibiotics (1000 U/l penicillin, 1 mg/l streptomycin sulfate) and seeded into Primaria™ plasticware (Becton Dickinson, Heidelberg, Germany) at a density of 2 mg tissue/cm^2. Cultures were maintained in a standard humidified atmosphere (95% O_2/ 5% CO_2 at 37 °C). After 96 hours without disturbance the medium was replaced by serum-free medium (DMEM/F12, 5 mg/l transferrin, 5 mg/l insulin, 50 nM hydrocortisone, 10 µg/l epidermal growth factor, 50 µg/l prostaglandin E1, 4 µg/l thyroxin, 5 µg/l selenious acid, 500 U penicillin and 500 µg streptomycin sulfate) and changed every 48 hours. Cell cultures reached confluency within 192 hours of seeding. Only cultures of passage 0 were used for experiments.

Primary human kidney cells (HKC) and primary porcine kidney cells (PKC) were prepared by collagenase digestion as described above and seeded, at a density of 1×10^4 cells/cm^2 in Primaria™ plasticware in MEM/Dval medium (to suppress fibroblast growth), supplemented with 10% FCS and antibiotics and maintained in a standard humidified atmosphere (95% O_2/ 5% CO_2 at 37 °C). Medium was changed every 48h, after one week, medium was replaced by DMEM/F12 medium, supplemented with 10% FCS and antibiotics. Cells were passaged using trypsin/EDTA and seeded at 1×10^4 cells/ cm^2 on normal tissue culture plastics for experiments. Passages 1–2 were used for experiments.

LLC-PK1 cells were obtained from the European Collection of Cell Cultures, Salisbury, UK (ECACC No. 86121112) and were cultured in DMEM supplemented with 10% FCS and antibiotics under standard conditions. Passages 1–20 were used for experiments.

Characterization of cell cultures: Initial cell viability following cell preparation was tested using trypan blue exclusion (POLLOCK and FIELD 1993). Only cell preparations with >90% viability were used for subsequent cultures.

Cell origin was examined by enzyme distribution: γ-glutamyl-transferase activity was used as a marker for proximal tubular epithelial origin (LASH 1992) and determined spectrophotometrically using the Sigma GGTP kit (Sigma procedure no. 545). Hexokinase activity was used as a marker for distal tubular origin and determined spectrophotometrically according to a standard method (BERGMEYER 1974). Only preparations of predominantly proximal tubular origin (>85%) were used for culture (LASH 1992).

General morphology was examined via light microscopy and transmission electron microscopy.

Expression of cytokeratins was examined as a marker for epithelial character using immunofluorescent methodology. Briefly, cells were seeded in 8-chamber culture slides (Becton Dickinson, Heidelberg, Germany). Subconfluent cultures were fixed (PBS, containing 2% (w/v) para-formaldehyde and 0.1% (v/v) Triton X-100), incubated with polyclonal anti-keratin AE1/AE3 (Roche Diagnostics, Mannheim, Germany) and with anti-mouse-Ig-FITC (Roche Diagnostics, Mannheim, Germany) co-stained with DAPI and examined under a fluorescence microscope. In addition, cytokeratins were detected by western blot. Cells of various passages were harvested and intermediate filaments were isolated according to standard procedure (ECKERT and KARTENBECK 1997) in order to ensure that cells maintain their epithelial characteristics during passaging of the cultures. Proteins were separated on a 12% polyacrylamide gel (LAEMMLI 1970) and electroblotted onto a nitrocellulose membrane (Schleicher und Schuell, Dassel, Germany). Cytokeratins were detected using polyclonal anti-keratin AE1/AE3 (Roche Diagnostics, Mannheim, Germany), anti-mouse-Ig, conjugated with alkaline phosphatase (Roche Diagnostics, Mannheim, Germany), and detected with Sigma Fast™ Red (Sigma, Taufkirchen, Germany).

Growth characteristics were studied by establishment of growth curves for all cell types using a Coulter Counter following trypsinisation.

Incubations: Sterile ochratoxin stock solutions were prepared with 0.1 M $NaHCO_3$ and diluted in culture medium. Nominal concentrations of 0.001 to 100 µM were used. RTPC cells were incubated after the first medium change, all other cells after attachment. Ochratoxins (OTA and OTB) were replenished with every medium change.

Cytotoxicity assays: MTT reduction was assayed spectrophotometrically (ISLET et al. 1989) using a microtiter plate reader (SLT, Crailsheim, Germany). Briefly, following exposure, LLC-PK1 cells and primary cells were incubated with 454,5 µg MTT/ml medium at 37 °C for 1 hour and 2.5 hours, respectively, the supernatants were then discarded and the intracellular dye was solubilized with 95% isopropanol/ 5% formic acid. Absorbances were then read at 550 nm.

Neutral red uptake was measured in a similar manner (BORENFREUND and PUERNER 1985). Following exposure, and subsequent incubation with 83 µg/ml neutral red for 90 mins at 37 °C, cells were gently washed with PBS (136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, ≤ 300 mOsM) the intracellular dye was solubilized with 0.05 M NaH_2PO_4 in 50% (v/v) ethanol. Absorbances were read at 550 nm.

Cell number was determined using a Coulter Counter after trypsination.

Statistical analysis: Results from cytotoxicity experiments were expressed as percentage of control and analyzed with a one-tailed Dunnett's test. The effective concentrations causing 50% effect (EC_{50}) of each assay endpoint were calculated from concentration-response curves fitted by GraphPad Prism version 3.00 for Windows and Excel software (Microsoft Excel 2000). Unless otherwise stated, data are presented as arithmetic means with corresponding 95% confidence intervals from at least three independent preparations carried out in duplicate.

Results

Characterization of cell cultures

In order to design the study preliminary examinations of all cell cultures were necessary. The aim was to use cultures with optimized growth conditions, of epithelial character, with known origin and stable morphological features. Therefore, cells were inspected microscopical-

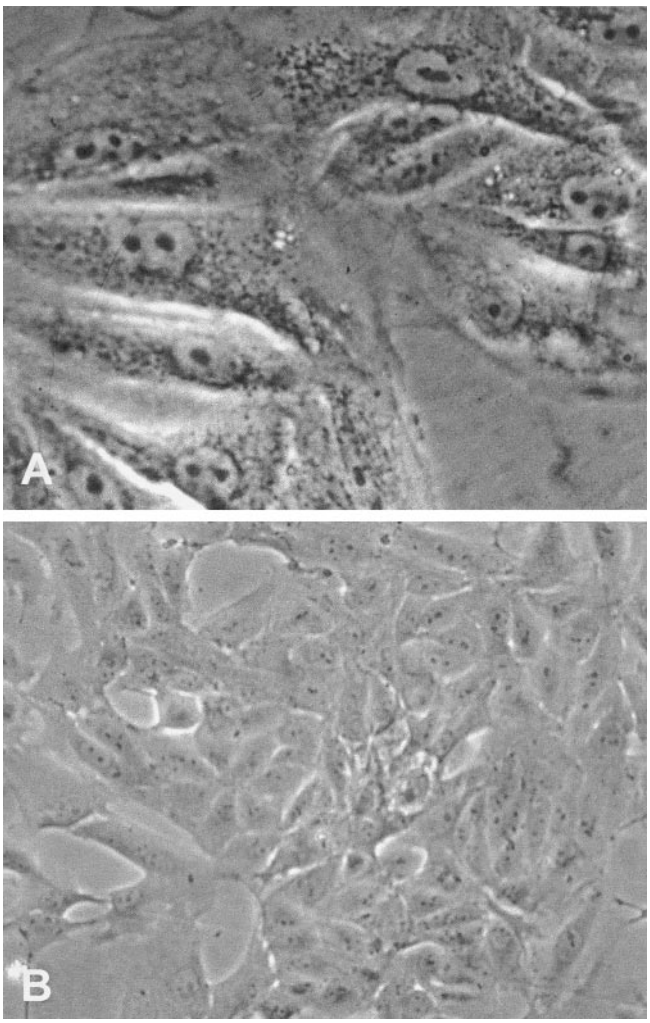


Fig. 1. Representative photographs of primary cell cultures: **A:** male human kidney cells (magnification ×640), **B:** female porcine kidney cells (magnification ×320).

ly via routine inverse light microscopy and transmission electron microscopy (TEM). All examinations revealed the typical features of proximal tubular epithelial cells (fig. 1): All cells displayed a cuboidal shape and monolayer growth with the exception of LLC-PK1, which tended to overgrow each other when postconfluent. No fibroblast growth was observed. The monolayers displayed typical cobblestone-like morphology and dome formation was seen at or near confluency. All cell types expressed a brush border on the apical side. Cytokeratin expression patterns, as determined by fluorescence microscopy and western blotting (fig. 2) remained stable throughout at least two passages following initial preparation. In order to compare the growth behavior of different passages, growth curves were generated of all cultures. All cell types showed comparable proliferation rates and characteristics. The growth curve of male PKC, as illustrated in figure 3, is therefore representative for the growth curves of the other cell types.

Effect of serum on cell proliferation

In order to test the effects of serum deprivation on cell proliferation characteristics, parallel growth curves in the presence and absence of FCS, were generated for two different cell types. LLC-PK1 and male PKC served as representatives for a cell line and for primary cells, respectively (fig. 4). Both cultures showed similar growth rates and reached confluency within four days in the presence of 10% FCS. If, however, 10 hours after seeding medium with FCS was substituted for medium without FCS, LLC-PK1 cells showed a significant suppression (30–40%) of cell proliferation (determined at 24 hours under FCS free conditions). This level of suppression remained stable throughout the duration of the experiment, i.e LLC-PK1 cells maintained under FCS-free conditions did not attain confluency within 96 h.

The effect of serum deprivation was more pronounced in primary PKC cells. Immediately following serum (FCS) deprivation, PKC cells growth-arrested and began to detach from the substrate. Following 48 h culture under serum-free conditions, the cells began to die. After 96 h, only 50% of the initially seeded cells remained (Fig. 4).

Light microscopic observations revealed that both cell-types attached only loosely to the substrate in the absence of FCS and tended to detach easily. This effect was more obvious in the primary cells, however, the general epithelial morphology of both cultures remained unchanged.

Effect of serum on OTA cytotoxicity

OTA concentration-response curves were generated and EC_{50} values calculated in the presence and absence of FCS (tables 1 and 2). The standard endpoints for assessing cytotoxicity were neutral red uptake (NR) and MTT reduction. The endpoints displayed comparable sensitivities in detecting OTA cytotoxicity, although NR appeared to detect cytotoxicity at an earlier time point than MTT. EC_{50} values were always 1–2 orders of magnitude lower in the FCS-free experiments. However, cells maintained under FCS-free conditions appeared significantly more susceptible to smallest disturbances, be this of a chemical or physical nature and resulted in the detachment of the cells from the substrate.

Comparison of OTA and OTB cytotoxicity using neutral red uptake as endpoint

OTA and OTB concentration-response curves were assayed at 24, 48, 72 and 96 hours exposure to OTA or OTB (0.001-100 mM) and EC_{50} values calculated (tables 3 and 4).

After short-term (24–48 h) exposure to OTA HKC and RPTC showed distinct sensitivity differences between cells from males and females, with cells from

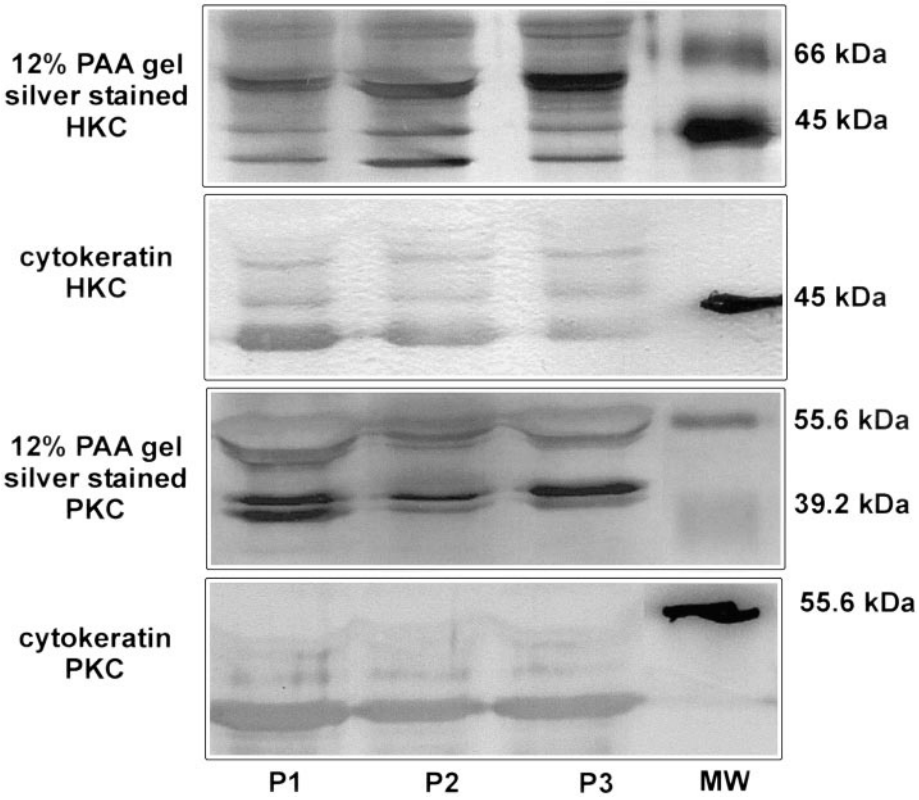


Fig. 2. Expression of cytokeratins in primary cells. Representative polyacrylamide gel and western blot sections stained as described in the Methods section. HKC, m, and PKC, m, respectively. P1, passage 1; P2, passage 2; P3, passage 3, MW, molecular weight marker proteins.

Table 1. Effect of serum on OTA cytotoxicity of LLC-PK1 cells.

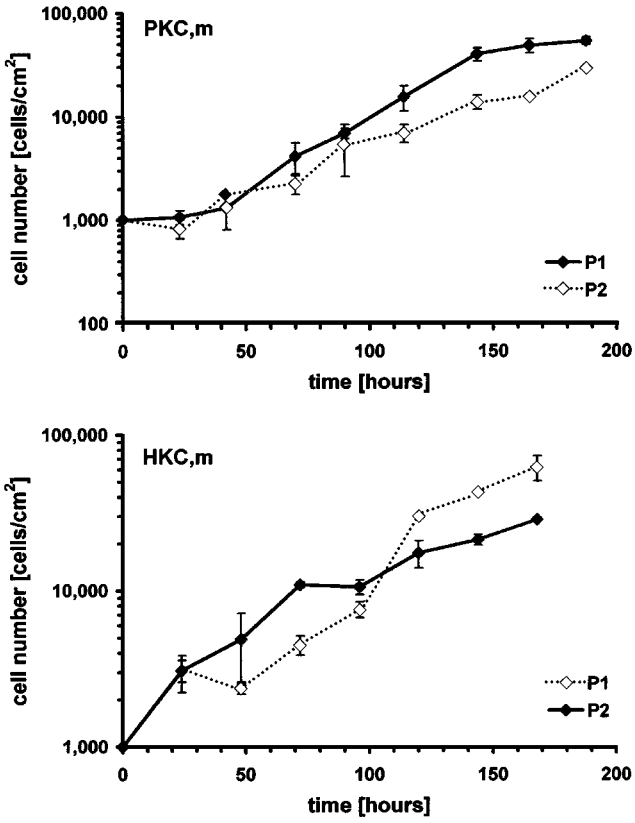
Assay	FCS	24 hours EC_{50} [µM]	48 hours EC_{50} [µM]	72 hours EC_{50} [µM]	96 hours EC_{50} [µM]
NR	+	>100 [a)]	19.0 [16.0–22.0]	19.0 [17.0–21.0]	20.0 [18.0–22.0]
NR	–	9.0 [8.9–9.1]	0.32 [0.31–0.33]	0.78 [0.68–0.88]	0.66 [0.60–0.72]
MTT	+	>100 [a)]	>100 [a)]	25.0 [24.0–26.0]	25.0 [24.0–26.0]
MTT	–	75.0 [65.0–85.0]	0.55 [0.50–0.60]	0.34 [0.32–0.36]	0.36 [0.34–0.38]

Values are expressed as arithmetic means of the effective concentrations [µM] causing 50% effect (EC_{50}) with corresponding 95% confidence intervals from at least three independent preparations carried out in duplicate as described in the Methods section. NR, neutral red uptake assay, MTT, MTT reduction assay, FCS, fetal calf serum, a) no effects within the testing concentration range.

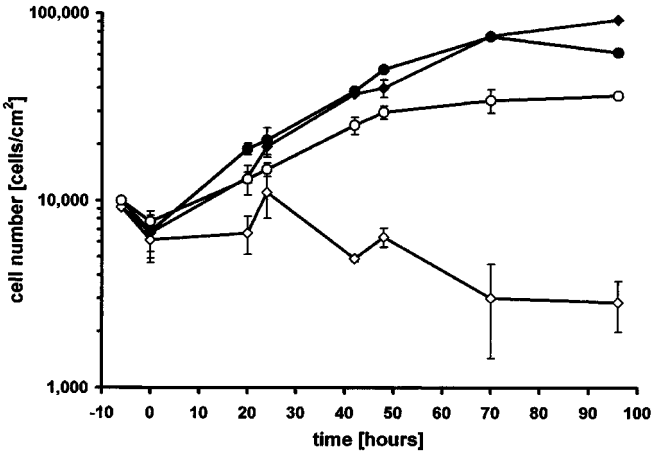
Table 2. Effect of serum on OTA cytotoxicity of primary male porcine kidney cells (PKC, m).

Assay	FCS	24 hours EC_{50} [µM]	48 hours EC_{50} [µM]	72 hours EC_{50} [µM]	96 hours EC_{50} [µM]
NR	+	40.0 [39.5–40.5]	17.0 [16.0–18.0]	17.0 [16.8–17.2]	16.0 [15.5–16.5]
NR	–	6.0 [5.8–6.2]	0.4 [0.37–0.43]	0.46 [0.45–0.47]	0.85 [0.71–0.99]
MTT	+	>100 [a)]	12.0 [11.0–13.0]	13.0 [12.5–13.5]	8.0 [7.6–8.4]
MTT	–	35 [28.5–41.5]	14.0 [11.5–16.5]	0.36 [0.33–0.39]	0.7 [0.62–0.78]

Values are expressed as arithmetic means of the effective concentrations [µM] causing 50% effect (EC_{50}) with corresponding 95% confidence intervals from at least three independent preparations carried out in duplicate as described in the Methods section. NR, neutral red uptake assay, MTT, MTT reduction assay, FCS, fetal calf serum, a) no effects within the testing concentration range.



◀ **Fig. 3.** Growth pattern of male PKC and HKC. P1, passage 1; P2, passage 2. Values represent mean ± SD.



▲ **Fig. 4.** Effect of serum on cell growth . Growth curves represent means of cell numbers per square centimeter of growth area ± SD from three independent preparations carried out in duplicate as described in the Methods section. LLC-PK1 cells with FCS (●) and without FCS (○), male primary porcine kidney cells with FCS (◆) and without FCS (◇).

Table 3. Comparison of OTA cytotoxicity in cells from various species using neutral red uptake as endpoint.

Tissue	48 hours EC_{50} [µM]	72 hours EC_{50} [µM]	96 hours EC_{50} [µM]
HKC, f	76.0 [72.0–80.0]	24.5 [23.0–26.0]	20.0 [19.5–20.5]
HKC, m	25.0 [22.0–28.0]	24.0 [21.0–27.0]	13.0 [11.0–15.0]
PKC, f	18.0 [17.0–19.0]	15.5 [15.0–16.0]	16.0 [15.8–16.2]
PKC, m	17.0 [16.0–18.0]	17.0 [16.8–17.2]	16.0 [15.5–16.5]
RPTC, f	48.0 [46.0–50.0]	30.0 [24.0–36.0]	22.0 [19.0–25.0]
RPTC, m	17.0 [15.5–18.5]	20.0 [15.0–25.0]	28.0 [24.0–32.0]
LLC–PK1, (m)	19.0 [16.0–22.0]	19.0 [17.0–21.0]	20.0 [18.0–22.0]

Values are expressed as arithmetic means of the effective concentrations [µM] causing 50% effect (EC_{50}) with corresponding 95% confidence intervals from at least three independent preparations carried out in duplicate as described in the Methods section. HKC, primary human kidney cells, PKC, primary porcine kidney cells, RPTC, primary rat proximal tubular cells, m, male, f, female, (m), originally male.

Table 4. Comparison of OTB cytotoxicity in cells from various species using neutral red uptake as endpoint.

Tissue	48 hours EC_{50} [µM]	72 hours EC_{50} [µM]	96 hours EC_{50} [µM]
HKC, f	>100 [a)]	66.0 [63.0–69.0]	84.0 [79.0–89.0]
HKC, m	56.0 [51.0–61.0]	48.0 [42.0–54.0]	12.0 [11.0–13.0]
PKC, f	23.0 [21.5–24.5]	19.5 [18.5–20.5]	20.0 [19.5–20.5]
PKC, m	19.0 [18.0–20.0]	19.0 [18.8–19.2]	19.0 [17.0–21.0]
RPTC, f	>100 [a)]	>100 [a)]	>100 [a)]
RPTC, m	>100 [a)]	>100 [a)]	>100 [a)]
LLC–PK1, (m)	52.0 [46.0–58.0]	41.0 [36.0–46.0]	46.0 [42.5–49.5]

Values are expressed as arithmetic means of the effective concentrations [µM] causing 50% effect (EC_{50}) with corresponding 95% confidence intervals from at least three independent preparations carried out in duplicate as described in the Methods section. HKC, primary human kidney cells, PKC, primary porcine kidney cells, RPTC, primary rat proximal tubular cells, m, male, f, female, (m), originally male, a) no effects within the testing concentration range.

males being more sensitive than their female counterparts. At the 48 hour time point this difference represented a factor of 3 (table 3) which was already apparent after 24 hours (data not shown). Following longer exposure times (72–96 h), this sex-specific difference in sensitivity was no-longer evident.

No cytotoxic effects were detectable after 24 hours of exposure to OTB in any cell type (data not shown). Following 48, 72 and 96 h exposure to OTB, HKC(m) were observed to be a factor of 1.4–7 more sensitive than HKC(f). The effects were observed to be time-dependent in HKC(m) whereas for all other cell types the EC_{50} values remained constant throughout the 96 h assay period. PKC cells, whether male or female, were more sensitive than the HKC(m) at the 48 and 72 h time-point and less sensitive at the 96 h time-point. Interestingly, the EC_{50} values obtained for OTA and OTB were comparable in the PKC cells, suggesting comparable susceptibility of these cells to OTA and OTB (tables 3 and 4). In contrast, OTB had no cytotoxic effects in RPTCs over either the concentration range or time points tested. Lower susceptibility (approx. 2-fold) to OTB than to OTA was also demonstrated in LLC-PK1 cells.

Comparison of OTA and OTB cytotoxicity using MTT reduction as endpoint

OTA-and OTB-mediated cytotoxicity was assessed over the same concentration range and duration as for neutral red assays and EC_{50} values were calculated (tables 5 and 6).

In general, MTT reduction was not as sensitive as neutral red uptake for detection of OTA-and OTB-mediated cytotoxicity. PKC were most sensitive towards OTA in this system, followed by RPTC, LLC-PK1 and HKC. No significant effects were seen with any cell type after 24 hours of exposure to OTA or OTB (data not shown). However, effects became apparent after 48 hours and later. Sex-differences were observed between male and female HKC cells following 96 h exposure to OTA, with HKC(m) being more sensitive than HKC(f). No sex-difference was observed in PKC cells, however, the PKC cells were approximately 3 and 10 times more sensitive than HKC(m) and HKC(f), respectively, to OTA and OTB (tables 5 and 6). Indeed, PKC showed comparable sensitivity to OTA and OTB. The EC_{50}'s determined with MTT (tables 5 and 6) were similar to those determined with

Table 5. Comparison of OTA cytotoxicity in cells from various species using MTT reduction as endpoint.

Tissue	48 hours EC_{50} [µM]	72 hours EC_{50} [µM]	96 hours EC_{50} [µM]
HKC, f	>100 a)	43.0 [33.0–53.0]	55.0 [50.0–60.0]
HKC, m	>100 a)	47.0 [44.0–50.0]	28.8 [28.6–29.0]
PKC, f	13.0 [12.0–14.0]	7.0 [6.5–7.5]	5.5 [5.0–6.0]
PKC, m	12.0 [11.0–13.0]	13.0 [12.5–13.5]	8.0 [7.6–8.4]
RPTC, f	12.0 b)	12.0 b)	4.1 b)
RPTC, m	70.0 b)	5.2 b)	24.0 b)
LLC–PK1, (m)	>100 a)	25.0 [24.0–26.0]	25.0 [24.0–26.0]

Values are expressed as arithmetic means of the effective concentrations [µM] causing 50% effect (EC_{50}) with corresponding 95% confidence intervals from at least three independent preparations carried out in duplicate as described in the Methods section. HKC, primary human kidney cells, PKC, primary porcine kidney cells, RPTC, primary rat proximal tubular cells, m, male, f, female, (m), originally male, a) no effects within the testing concentration range, b) means of two independent preparations carried out in duplicate.

Table 6. Comparison of OTB cytotoxicity in cells from various species using MTT reduction as endpoint.

Tissue	48 hours EC_{50} [µM]	72 hours EC_{50} [µM]	96 hours EC_{50} [µM]
HKC, f	>100 a)	>100 a)	>100 a)
HKC, m	>100 a)	>100 a)	29.0 [28.0–30.0]
PKC, f	15.0 [14.5–15.5]	12.0 [11.8–12.2]	10.0 [9.8–10.2]
PKC, m	10.0 [9.5–10.5]	12.0 [11.8–12.2]	10.0 [9.0–11.0]
RPTC, f	>100 a)	>100 a)	85.0 b)
RPTC, m	>100 a)	>100 a)	>100 a)
LLC–PK1, (m)	>100 a)	60.0 [55.0–65.0]	84.0 [78.0–90.0]

Values are expressed as arithmetic means of the effective concentrations [µM] causing 50% effect (EC_{50}) with corresponding 95% confidence intervals from at least three independent preparations carried out in duplicate as described in the Methods section. HKC, primary human kidney cells, PKC, primary porcine kidney cells, RPTC, primary rat proximal tubular cells, m, male, f, female, (m), originally male, a) no effects within the testing concentration range, b) means of two independent preparations carried out in duplicate.

NR (tables 3 and 4). For all other cell types the MTT reduction assay was less sensitive than neutral red uptake for detection of OTB-mediated cytotoxicity. HKC(m) cells were more affected than female HKC (factor ≥ 3.4) after 96 hours of exposure to OTB. With shorter exposure times, no significant effect was detectable. The effects of OTB on HKC(m) cells was also observed to be time-dependent, whereas for all other cell types the EC_{50} values remained constant throughout the duration of the assays.

Comparison of OTA and OTB using cell number as endpoint

OTA and OTB concentration-response curves were generated as described for neutral red assays above and EC_{50} values were calculated (tables 7 and 8). PKC(m) showed effects as early as 24 hours following exposure to OTA or OTB, all other cell types were not affected within the tested concentration range at this time point (data not shown). Using this endpoint, human cells showed the greatest difference between the sexes, with HKC(m) cells being approximately a factor of 4 more sensitive than HKC(f) cells to OTA following 48 and 72 h exposure and approximately a factor 3 following 96 h exposure. Similar differences between HKC(f) and HKC(m) cells were noted following OTB exposure, with HKC(m) cells being more sensitive than HKC(f) cells (at least a factor 14 at 96 h exposure). Furthermore, the cytostatic effect of both OTA and OTB in human cells appeared to be time-dependent. In contrast, no sex-difference was detectable in PKC following either OTA or OTB exposure and the effects remained constant over the assay period. Overall, using cell number as endpoint for OTA and OTB cytotoxicity, porcine primary cells (PKC) were the most sensitive cell type followed by LLC-PK1 and HKC. Due to the extremely low cell numbers remaining for seeding in the six-well plates, cell numbers were not sufficiently high to be used as a method for detecting cytotoxicity in rat primary cells. The comparison of all three detection systems (NR, MTT and cell number) demonstrated high congruence of the EC_{50}'s obtained with OTA and OTB in PKC, LLC-PK1 and HKC at the 96 hour time points. A higher variability is observed at the earlier time-points.

Table 7. Comparison of OTA cytotoxicity in cells from various species using cell number as endpoint.

Tissue	48 hours EC_{50} [µM]	72 hours EC_{50} [µM]	96 hours EC_{50} [µM]
HKC, f	>100 [a)]	>100 [a)]	25.0 [24.0–26.0]
HKC, m	27.0 [15.0–39.5]	24.5 [22.5–26.5]	7.9 [7.3–8.5]
PKC, f	10.1 [9.9–10.3]	3.8 [3.6–4.0]	5.2 [4.7–5.7]
PKC, m	13.0 [12.0–14.0]	10.0 [8.5–11.5]	6.5 [6.2–6.8]
RPTC, f	nd	nd	nd
RPTC, m	nd	nd	nd
LLC–PK1, (m)	20.0 [18.0–22.0]	18.0 [15.0–21.0]	17.0 [15.0–19.0]

Values are expressed as arithmetic means of the effective concentrations [µM] causing 50% effect (EC_{50}) with corresponding 95% confidence intervals from at least three independent preparations carried out in duplicate as described in the Methods section. HKC, primary human kidney cells, PKC, primary porcine kidney cells, RPTC, primary rat proximal tubular cells, m, male, f, female, (m), originally male, nd, not determined, a) no effects within the testing concentration range.

Table 8. Comparison of OTB cytotoxicity in cells from various species using cell number as endpoint.

Tissue	48 hours EC_{50} [µM]	72 hours EC_{50} [µM]	96 hours EC_{50} [µM]
HKC, f	>100 [a)]	>100 [a)]	>100 [a)]
HKC, m	>100 [a)]	>100 [a)]	7.0 [6.5–7.5]
PKC, f	4.0 [3.8–4.2]	4.4 [4.3–4.5]	5.5 [5.3–5.7]
PKC, m	11.0 [10.0–12.0]	11.5 [11.0–12.0]	9.0 [8.6–9.4]
RPTC, f	nd	nd	nd
RPTC, m	nd	nd	nd
LLC–PK1, (m)	26.0 [23.0–29.0]	17.0 [14.5–19.5]	17.0 [15.0–19.0]

Values are expressed as arithmetic means of the effective concentrations [µM] causing 50% effect (EC_{50}) with corresponding 95% confidence intervals from at least three independent preparations carried out in duplicate as described in the Methods section. HKC, primary human kidney cells, PKC, primary porcine kidney cells, RPTC, primary rat proximal tubular cells, m, male, f, female, (m), originally male, nd, not determined, a) no effects within the testing concentration range.

Discussion

The detection of cytotoxicity of a compound *in vitro* is primarily dependent on the availability of the compound to the cells. In this regard confounding factors, e.g. the addition or absence of serum in the culture medium may alter the toxicity of a specific compound by several orders of magnitude. For example the cytotoxicity of compounds e.g. OTA with an inherently high affinity to plasma proteins (HAGELBERG et al. 1989) may be severely overestimated when assayed under serum free conditions (BONDY and ARMSTRONG 1998; GEKLE et al. 1995b). Indeed, the cytotoxicity of OTA in LLC-PK1 and PKC cells was more than 30-fold and 7-fold greater (table 1 and 2), respectively, when assayed in medium without serum than with serum. The visual assessment of LLC-PK1 and PKC cells, maintained under serum-free conditions, presented with major morphological alterations already 24hr after initial seeding. The question thus arises whether the observed higher cytotoxicity of LLC-PK1 cells maintained under serum free conditions resulted from the higher bioavailability of OTA to the cells or, more likely, rendered the cultured cells generally more susceptible to any kind of cytotoxic agent. The latter hypothesis is supported by observations of BONDY and ARMSTRONG (BONDY and ARMSTRONG 1998), who found greater cytotoxicity of OTA when added to the LLC-PK1 culture 24 h after seeding than when added directly at seeding in the absence of serum. In addition, under *in vivo* conditions, OTA would never occur without the presence of plasma proteins. Thus using serum free conditions without growth stimulating factors (EGF, insulin, prostglandin etc.) may produce questionable results with regard to the potential prevailing mechanism(s) (GEKLE et al. 1995b). as well as to interpretation and extrapolation of the potential risk associated with OTA exposure *in vivo*.

The comparison between the three different cytotoxicity endpoints used in this study demonstrated that generally all endpoints provide similar results. Neutral red appeared slightly more sensitive an endpoint than MTT or cell numbers in this study. The latter observation stands in contrast to findings by BONDY and ARMSTRONG (BONDY and ARMSTRONG 1998), who reported that MTT was more sensitive than NR. This discrepancy may be explained by the observation that in morphologically changed LLC-PK1 cells maintained under serum free and therefore stressful (suboptimal) conditions for 24 hr (BONDY and ARMSTRONG 1998), mitochondrial activity (MTT) may have already been compromised (reduced) while lysosomal integrity (capability for NR retention) remained intact.

With the exception of primary HKC(f) cells primary cell types were significantly more susceptible to OTA mediated toxicity than the LLC-PK1 cell line. Using the 72 or 96 h time points for a preliminary ranking, male and female PKC and HKC (m) were the most sensitive, followed by RPTC and LLC-PK1 cells (tables 3, 5 and 7). These data would suggest a higher susceptibility of pigs and men to OTA induced renal toxicity. This suggestion is supported by the fact that pigs develop nephropathies within 10 months of exposure to moderate levels of OTA (KROGH 1987, 1992; KROGH et al. 1976, 1988; STOEV 1998). In contrast, no human male specificity for the increased incidences in BEN or UT was observed in the endemic areas (NIKOLOV and CHERNOZEMSKY 1990; NIKOLOV et al. 1996). However, the concentrations employed in this study were much higher than those observed to be present *in vivo*. The most likely mechanism associated with the observed effects of OTA is overt cell necrosis, although apoptosis was also observed at similarly high concentrations in hamster kidney cell lines (SEEGERS 1994). It is thus suggested that OTA-induced overt cell necrosis would not occur in the *in vivo* situation, i.e. under the nutritional conditions which are assumed to be responsible for BEN and UT. If indeed OTA is causal in the etiology of BEN and UT, other mechanisms e.g. OTA induced cytostasis or enhanced apoptosis, cell dedifferentiation may be of greater importance (O'BRIEN et al. 2001). The fact that the elimination half-life of OTA in humans, with approximately 36 days (STUDER-ROHR et al. 2000), is considerably longer than noted in other mammalian species (mice (40 h) (FUKUI et al. 1987; HAGELBERG et al. 1989), rats (55–120 h) (BALLINGER et al. 1986; GALTIER et al. 1979; HAGELBERG et al. 1989), pigs (72–120h) (GALTIER et al. 1981; MORTENSEN et al. 1983) or monkeys (820 h) (KUIPER-GOODMAN and SCOTT 1989), that tissue homogenates from the human renal cortex display a higher binding affinity and capacity for [^{3}H]-OTA than the respective tissue homogenates from rat, mouse or pig [abstract, DIETRICH et al., Toxicological Sciences 54: 170, 2000], as well as the data presented here, suggest that OTA may constitute a higher risk in humans than previously considered.

In comparison to OTA an approximately 2- fold lower cytotoxicity was observed in LLC-PK1 cells exposed to OTB (tables 3–8), corroborating earlier findings by Heussner et al. [abstract; HEUSSNER et al., Toxicological Sciences 42: 288, 1998] and Bondy and Armstrong (BONDY and ARMSTRONG 1998). In contrast, primary RPTC appeared refractive to OTB, while being just as susceptible to OTA as other primary cell types (tables 3–6). These findings support observations by O'BRIEN et al. (O'BRIEN et al. 2001), which suggest that OTB has a completely different mode of action than does the structurally practically identical OTA.

In conclusion, when investigating the toxicity of substances to cells in culture with a view to extrapolation to, or comparison with the *in vivo* situation, care should be taken with the study design and the endpoints chosen so that risks are neither over- nor underestimated. The cell models described here appear to represent the *in vivo* situation and therefore provide a suitable basis for the further investigation into the mechanism(s) of action of OTA.

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