Abstract

The in vitro cytotoxicity of aflatoxin B\(_1\) (AFB\(_1\)), citrinin (CIT), ochratoxin A (OTA), patulin (PAT), and zearalenon (ZEA) was determined using the MTT bioassay. The study was performed on three cell lines originated from kidney fibroblasts of farm animals: SK-6, PO, and FLK. The dose response curves for each mycotoxin were generated and the concentrations causing 50% cell death (CD\(_{50}\)) were estimated. PAT and OTA were the most potent toxins for SK-6 cells, with CD\(_{50}\) of 2.01 \(\mu\)mol/l and 2.58 \(\mu\)mol/l, respectively; (0.31 mg/l and 1.04 mg/l, respectively). Based on molar concentration OTA was less toxic than PAT but five times more potent than CIT. The PO cell line was generally more resistant to the mycotoxins tested at the concentration between 0.1 and 10.0 \(\mu\)g/ml. This study shows that the cytotoxicity of Penicillium toxins to kidney cell lines depends on toxin concentration and the all tested cell lines were very resistant to ZEA produced by Fusarium species. The in vitro biological assay thus provides an excellent system for quantifying the low CD\(_{50}\) expressed by mycotoxins in feed.

Key words: kidney fibroblasts, mycotoxins, cytotoxicity, MTT assay.

Mycotoxins are natural products of filamentous fungi and they can be toxic when introduced even in a low concentration (ppb, ppm) to animals and man. The mycotoxins are mainly produced by five genera of filamentous fungi: Aspergillus (aflatoxins, ochratoxins, sterigmatocystin, and cyclopiazonic acid); Penicillium (patulin, ochratoxin, citrinin, and cyclopiazonic acid); Fusarium (deoxynivalenol, nivalenol, zearalenone, T-2 toxin, fumonisins, and moniliformin); and Alternaria (tenuazonic acid, alternariol methyl ester, Claviceps (ergot alkaloids) (19, 20). They are predominantly found in plant-based products and as residues and metabolites in food of animal origin.

There are many reviews (2, 15, 23) of the toxicity and/or genotoxicity exhibited by several mycotoxins. The following mycotoxins produced by Aspergillus, Penicillium, and Fusarium were selected for the present study: aflatoxin B\(_1\), ochratoxin A, citrinin, patulin, and zearalenon. All the mycotoxins have been found in feed and food.

Aflatoxin B\(_1\) (AFB\(_1\)) is a carcinogenic mycotoxin found in commercial feed, food, and airborne grain dust. Aflatoxin B\(_1\) requires biotransformation to the AFB\(_1\)-8,9-epoxide by the bioactivation system and subsequent covalent binding to DNA or proteins, to exert its carcinogenic potential (14). Patulin (PAT) is a secondary metabolite very toxic to some species of farm animals (13). The potency of PAT in inhibiting DNA synthesis and lymphocyte proliferation was found to be three times that of OTA, and to be similarly effective at a molecular level (4). It is considered to be a mutagen. Ochratoxin A (OTA) is linked with kidney diseases in many parts of the world and the evidence supporting its role in the aetiology of Balkan endemic nephropathy and associated urinary tract tumours has been gathered for several years (8, 10). Ingestion of citrinin (CIT) also causes nephrotoxicosis in domestic and laboratory animals and may be associated with human nephropathy (1). Zearalenone (ZEA) has been detected in feed and foodstuffs and has been implicated in several mycotoxicoses of farm animals. ZEA and its metabolites have estrogenic and anabolic activities in several species (rodents, pigs, and monkeys). However, a limited number of studies have been carried out to determine the genotoxicity of ZEA and the results are controversial (3).

The MTT assay has been used for numerous medical, microbiological, and toxicological tests. The main advantage of this functional colorimetric assay is its simplicity and independence of the radiolabelling. It has been reported on the suitability of the MTT assay for the cytotoxicological evaluation of several mycotoxins on various cell line cultures (9, 18). However, the origin of tissue source is the open way for potentially greater advantages in this area. One of them is the ability to render cells much more susceptible to the cytotoxic effect of the mycotoxins, which in turn, allows its easier detection even at extremely low concentrations. As in the majority of the MTT tests, the human cell lines are used to determine the cytotoxicity of mycotoxins, the present study was undertaken to find out the possibility
to use the animal cell lines from the kidneys of farm animals, which might be the target for toxicological effects of nephrotoxic mycotoxins.

Material and Methods

Chemicals. The mycotoxins, AFB1, OTA, ZEA, PAT, and CIT were purchased from SIGMA. They were reconstituted in dimethyl sulphoxide (DMSO) obtained from the same supplier. MTT [3(4,5-dimethylthiazol-2-y1) 2,5-diphenyltetrazolium bromide] for cytotoxic assay (Sigma) and sterile buffer saline (PBS) was used for the dilution of the mycotoxins according to the running experiment.

Cell cultures. The swine kidney cell line SK-6 (Department of Swine Diseases, National Veterinary Research Institute, Pulawy, Poland) was cultivated in Minimum Essential Medium with Earle’s balanced salts and L-glutamine supplemented with 10% foetal calf serum and antibiotics. When cell lines exhibited 80–90% confluence, they were scrapped without trypsinisation for cellular detachment and seeded into fresh, pre-warmed (37°C), complete media.

The foetal lamb kidney cells (FLK) permanently infected with bovine leukaemia virus (BLV) was obtained from Dr Van Der Maaten (National Animal Diseases Centre, Aimes, USA), and foetal lamb kidney cells non infected (PO) from Dr Ribe (Federal Research Centre for Viral Diseases, Riems, Germany). The cell lines were cultivated in RPMI 1640 (Gibco, USA), supplemented with 10% foetal calf serum (FCS) and antibiotics. The cells were detached by scraping (without trypsin), and for the test, their number was adjusted to 0.5 x 10⁶ cells/ml in complete medium with FCS. The cells were propagated at 37°C and in 5% CO₂ atmosphere.

Cell line preparation. The cell suspension was adjusted to 1 x 10⁶ cell/ml and 900 µl of each cell line was pipetted into 24-well plate, and 100 µl of each mycotoxin in sterile PBS, representing the first concentrations of 0.1 – 10.0 µg/ml, was added and then followed by gentle agitation on a rotary shaker for 5 min. Immediately after agitation, 150 µl of each sample was placed into wells of a sterile 96-well plate covered with a lid to prevent evaporation. The samples were incubated for 3 d at 37°C in an incubator with 5% CO₂ atmosphere.

MTT assay. After the incubation was completed, 50 µl of MTT in PBS (0.4 mg/ml) was added to each well and incubated again at 37°C in CO₂ for 6 h. The supernatant was aspirated and 150 µl of DMSO was added to dissolve any intracellular formazan crystals and agitated for 15 min on a rotary shaker. An ELISA Reader (Dynatech MR 5000) set at wavelength of 570 nm was used to determine a survival ability of the cell lines through MTT cleavage. The mean optical density (OD) reading was used to calculate the percentage of cell survival and standard error for each dilution of test sample. The percentage of cell death/survival was calculated using this formula:

\[
\text{% of cell survival} = \frac{\text{mean OD treated cells} - \text{mean OD negative control cells}}{\text{mean OD negative control cells}} \times 100
\]

The MTT assay provides a linear relationship between cell number and formazan production at low and high cell densities, making routine determination with standard deviation easily calculated (data not presented). The cytotoxicity of mycotoxins was expressed as 50% of dead cells (CD₅₀) in relation to control cultures.

Lamb kidney cells (PO) were found to be the most resistant, whereas swine kidney cells (SK-6) were the most sensitive to all mycotoxins tested by MTT assay at concentrations between 0.1 and 10.0 µg/ml. The lamb kidney cells infected with EBB virus (FLK) were very sensitive only to OTA and PAT. The CD₅₀ results obtained from the exposure of the SK-6, PO, and FLK cell lines to the mycotoxins, utilising the MTT assay, are shown in Table 1. The results vary significantly between the toxins examined. Statistical analysis indicated that the difference between CD₅₀ for SK-6 and FLK cells, in comparison to PO, were statistically significant (P<0.05) for AFB₁, CIT, OTA, and PAT.

The dose response curves showed a dose dependent toxicity (Fig. 1). The concentrations producing 50% of cell death were estimated from these curves. OTA and PAT were found to be the most potent inhibitors of cell survival. Based on toxins concentration, PAT showed the highest potency and was approximately 10, 20, and 30 times more potent as an inhibitor than OTA, AFB₁, CIT, and ZEA, respectively. In cultured SK-6, the PAT concentration of 0.31 ± 0.18 µg/ml gave 50% inhibition of cell survival, but OTA required concentration of 1.04 ± 0.13 µg/ml, CIT 3.25 ± 0.29 µg/ml, and ZEA 9.98 ± 0.95 µg/ml to obtain similar cytotoxicity.

Gradients/slopes of the dose response curves were quite similar for AFB₁ and PAT, the latter however, required almost 10 times lower concentration to reach the 50% inhibition of cell viability than the former one.
With the increasing concentration of PAT, CIT, and AFB1, there was demonstrated a dramatically decrease in cell viability in all kidney cell lines, and PAT caused almost zero survival at 0.5 µg/ml but AFB1 at 1.0 – 5.0 µg/ml, dependently on the cell type. While the OTA caused generally a marked decrease in cell viability at the concentration of 5 µg/mL, the cell viability in SK-6 cell cultures was completely stopped. The particular attention should be paid to the normal (PO) and permanently infected with EBB virus (FLK) lamb kidney cells. The mycotoxins AFB1, CIT, and OTA caused lower viability and cells survival of FLK than PO cultures. In case of AFB1, a dramatic decrease of curve slopes was at 2 µg/ml in FLK cells and 5 µg/ml in PO cultures, and the difference was statistically significant (P<0.05). The weakest slope was observed in the curves for ZEA, showing 50% inhibition of cell viability at approximately 10 µg/ml or even higher concentration of the mycotoxin in the cultures of SK-6, PO, and FLK cells. In addition, there were not any significant differences between all the cell lines treated with ZEA. The results clearly indicated that the highest cytotoxicity was revealed for PAT and the lowest one for ZEA.

Based on molecular concentrations of OTA and PAT, the similar potency was showed (Table 2). PAT was approximately 10 times, or even more potent than CIT. The gradient/slope of the dose response curves were quite similar for AFB1, PAT, and CIT. The CIT however, required 10 times higher concentration to reveal that slope. The mycotoxins concentration range between zero and maximal inhibition of cell survival was approximately 10x (Figs 1-A, 1-B, 1-C, and 1-D). The weakest slope was observed on the curves for ZEA (Fig. 1-E) showing the inhibition of cell survival at approximately 100 times the concentration having practically no effect.

The dose response for OTA showed approximately 50 times concentration range between zero and maximal inhibition (Fig. 1C).

### Table 1
Cytotoxicity of mycotoxins using the MTT assay on SK-6, PO, and FLK cells, mean ± SD

<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>Cytotoxicity (CD50) µg/mL</th>
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<tbody>
<tr>
<td></td>
<td>SK-6</td>
</tr>
<tr>
<td>Aflatoxin B1</td>
<td>1.84 ± 0.15</td>
</tr>
<tr>
<td>Citrinin</td>
<td>3.25 ± 0.29</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td>1.04 ± 0.13</td>
</tr>
<tr>
<td>Patulin</td>
<td>0.31 ± 0.18</td>
</tr>
<tr>
<td>Zearalenone</td>
<td>9.98 ± 0.95</td>
</tr>
</tbody>
</table>

### Table 2
Concentration of *Penicillium* mycotoxins causing 50% cytotoxicity (CD50) in swine kidney cells estimated from dose response curves. Results are presented as weight per volume and as molar concentrations. Cytotoxicity potencies relative to that of ochratoxin A are indicated

<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>CD50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/L</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td>1.04</td>
</tr>
<tr>
<td>Patulin</td>
<td>0.31</td>
</tr>
<tr>
<td>Citrinin</td>
<td>3.25</td>
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</tbody>
</table>
Fig. 1. The effect of aflatoxin B₁ (A), citrinin (B), ochratoxin A (C), patulin (D), and zearalenone (E), on three kidney cell lines: swine (▼), lamb (■), and lamb kidney cells permanently infected with EBB virus(●). The values are expressed as percentage of control response and are means of at least four independent experiments.
Discussion

The MTT assay has been frequently used to measure the cytotoxicity of various mycotoxins acting on different cell lines, both of animal and human origin (5, 9, 17). The assay measures the conversion of the tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to MTT formazan in living cells and may be used to determine cell viability and the ability to proliferate. The reaction is catalysed by mitochondrial succinyl dehydrogenase and requires NADH, which has to be supplied by the cells, thus providing an indication of mitochondrial or respiratory competence.

The present study showed that all mycotoxins depressed cell viability at certain concentration. The varying cytotoxic response of different cell types to a mycotoxin may also be due to the differing metabolic activity of the target cells. The most commonly encountered explanations for cell to cell variability in toxin sensitivity is in the amount of target enzymes, other cellular constituents, or functions that can interact with the toxin, the amount of metabolic enzymes, which activate a non-toxic precursor molecule into a metabolite with non-specific toxicity, and the efficiency of detoxification mechanisms that protect cells and tissues that are less susceptible to the toxic agent (19).

Aflatoxin B1 is both water and lipid soluble and these properties facilitate its penetration through cell membranes and into cellular organelles. This is a toxin frequently cited, particularly with respect to the formation of AFB1 adducts resulting in genotoxicity and frequently cited, particularly with respect to the membranes and into cellular organelles. This is a toxin acting on laboratory animals, and from a combination of both in vivo and in vitro investigations, it would appear that the very important is metabolism of AFB1 to its active form AFB1-8,9-epoxide and subsequently to AFB1-DNA adduct formation (12, 14).

According to the data presented in Table 1, AFB1 was not the most potent mycotoxin in our studies and the results are consistent with other reports in the literature (9). The low cytotoxicity of AFB1 was probably due to the fact that AFB1 was not metabolised to the epoxide form, which is more toxic than intact AFB1 form. In general, the metabolic activation of AFB1 to its active form was probably not satisfactory; therefore, cytotoxicity was lower than that of other tested mycotoxins.

The nephrotoxic fungal toxins: OTA, OTB, and CIT are natural contaminants of feeds and foods. OTA is consistently more toxic than OTB and CIT, regardless of cell line or assay endpoint. This is consistent with previous studies in which OTA was more toxic than CIT to murine and bovine embryonic cells (7, 22) by using viable cell enumeration endpoint, and to MDCK cells (canine kidney) and HeLa cells by using MTT assay (6). OTA was also more toxic than OTB to HeLa cells by using viable cell enumeration (24). Whole-animal studies support the following rankings: the CD50 for OTA in rats was 20-22 mg/kg body weight compared to an LD50 for the same dose for CIT (16). OTB is structurally related to OTA, differing only by the presence of hydrogen, instead of a chlorine atom on the isocoumarin portion of the molecule. Clinical evidence indicates that this structural difference renders OTB less toxic to rats than OTA when administrated at the same level (21).

With respect to OTA, it is clear from our studies that its toxicity to SK-6 cells was very similar to the reported study where 1 µg/ml of the toxin reduced cell viability by 50% (9). This means that metabolic activity of SK-6 swine cell towards OTA was very close to that of human epithelial cell line.

Although direct comparison between in vivo and in vitro data is at the best ambiguous due to difference in toxin delivery, uptake, metabolism, and transport in cultured cells compared to whole animals, the units used in the present study (µg/ml) are mathematically equivalent to ppm or mg/kg units used in vivo studies. By this criterion, when in vitro CD50 values are compared to in vivo LD50 values, tissue culture systems were more sensitive to OTA and CIT than those in whole animals. The lowest CD50 for OTA was 1.50 µg/ml in PO cultures (Table 1) compared to 20 mg/kg body weight in rats (16). For CIT the lowest CD50 was 4.64 µg/mL also in PO cultures compared to 67 mg/kg body weight of rats. These CD50 values indicated higher cytotoxicity than results of Kitanabe et al. (6), who generated CD50 values for OTA and CIT of 5 µg/ml and 19 µg/ml, respectively, in HeLa cells using MTT assay. The lack of consistency with our results is probably due to higher sensitivity of kidney cells, which origin from the main target organ of OTA, PAT, and CIT toxicity. The results may indicate that even apparently minor alteration in assay protocols can result in variable toxicity determination for individual toxins as well as variable ranking between cell lines.

The potency of PAT at a dose inhibiting the viability of cell cultures was found to be 3 to 10 times higher than that of OTA, depending on cell lines. The effects of PAT cytotoxicity compared to those OTA cytotoxicity were calculated as stronger at a molecular level. These findings were reported previously in another type of cell cultures (11). In the present study, cell proliferation was totally suppressed at 0.5 µg/ml in swine and sheep normal kidney cells. A similar response to PAT in vitro was also described by Escoula et al. (4). The authors in a comprehensive study on mice have concluded that the exposure to PAT at levels consistent with potential human exposure in foods would not be likely to alter cellular responses. However, sublethal doses of PAT inhibited cellular functions in mice and rabbits after intra-gastric or intra-peritoneal administration (13). Furthermore, the kidney cells from pigs, which are more sensitive to PAT than other animals, were found in our study to be also the most sensitive cell cultures exposed to this toxin.

ZEA also induced a statistically significant inhibition of cell survival at the tested cell lines as compared to control. The cytotoxic effect induced by ZEA was the dose-dependent; in fact, by the gradual increasing its doses, the little but not significant reduction was observed. The similar effects were also
reported in bovine lymphocyte cultures (10). However, ZEA was more toxic to bovine lymphocytes than to kidney cells of swine and sheep. The detailed examination of the gradient/slope of the dose response curves (Fig. 1), allows imagining how big differences in the cytotoxicity may exist between three kidney cell lines treated with the five mycotoxins produced by different species of the filamentous fungi. The presence of proviral DNA of EBB leukaemia virus in the genome of sheep kidney cells decrease the ability of host to develop the defensive leukaemia virus in the genome of sheep kidney cells. filamentous fungi. mycotoxins produced by different species of the between three kidney cell lines treated with the five mycotoxins reached up to 30% of overall toxicity. The authors concluded that SK-6, PO, and FLK cells may be compared favourably with the existing models in terms of sensitivity to nephrotoxic fungal toxins, PAT, CIT, and OTA. However, the data also highlight the care that must be taken when designing and implementing in vitro assays since even small changes in assay protocols can influence the outcomes. This is a problem that could complicate studies trying to predict relative toxicity within groups of toxins. This study also indicates that clarifying the responses of target cell lines to test chemicals under a range of assays and culture conditions is a critical aspect of providing that an in vitro model will be an effective predictor of toxicity when applied as a screen for fungal toxins.

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References