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High content analysis: A sensitive tool to detect and quantify the cytotoxic, synergistic and antagonistic effects of chemical contaminants in foods

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HIGHLIGHTS

- High content analysis (HCA) is a useful tool to determine cytotoxicity of complex mixtures.
- Conventional endpoints MTT and NR were compared to HCA endpoints.
- Tertiary mycotoxin mixture (OTA/FB1/AFB1) revealed more cytotoxicity using HCA endpoints over conventional endpoints.
- Binary combination (OTA/FB1) revealed synergistic interactions using HCA previously undetected by conventional endpoints.

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ABSTRACT

Aflatoxin B1 (AFB1), ochratoxin A (OTA) and fumonisin B1 (FB1) are important mycotoxins in terms of human exposure *via* food, their toxicity and regulatory limits that exist worldwide. Mixtures of toxins can frequently be present in foods, however due to the complications of determining their combined toxicity, legal limits of exposure are determined for single compounds, based on long standing toxicological techniques. High content analysis (HCA) may be a useful tool to determine total toxicity of complex mixtures of mycotoxins. Endpoints including cell number (CN), nuclear intensity (NI), nuclear area (NA), plasma membrane permeability (PMP), mitochondrial membrane potential (MMP) and mitochondrial mass (MM) were compared to the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) and neutral red (NR) endpoints in MDBK cells. Individual concentrations of each mycotoxin (OTA 3 μg/ml, FB1 8 μg/ml and AFB1 1.28 μg/ml) revealed no cytotoxicity with MTT or NR but HCA showed significant cytotoxic effects up to 41.6% ($p \leq 0.001$) and 10.1% ($p \leq 0.05$) for OTA and AFB1, respectively. The tertiary mixture (OTA 3 μg/ml, FB1 8 μg/ml and AFB1 1.28 μg/ml) detected up to 37.3% and 49.8% more cytotoxicity using HCA over MTT and NR, respectively. Whilst binary combinations of OTA (3 μg/ml) and FB1 (8 μg/ml) revealed synergistic interactions using HCA (MMP, MM, NI endpoints) not detected using MTT or NR. HCA is a highly novel and sensitive tool that could substantially help determine future regulatory limits, for single and combined toxins present in food, ensuring legislation is based on true risks to human health exposure.

1. Introduction

High content analysis (HCA) is a novel technology that quantitatively examines chemical induced toxicity at sub-cellular microscopic resolution. HCA has the potential to identify new drug targets, predict *in vivo* toxicity, suggest molecular targets; measure the effects of compounds and biological molecules; and measure cellular and intercellular movement (Buchser et al., 2004–2012). HCA has been shown to be highly sensitive and specific using pre-lethal toxicity assays to establish subtle changes in cell health rather than overt, gross cytotoxicity detected by conventional 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) and neutral red (NR) assays (O'Brien et al., 2006; O'Brien and Haskins, 2007; O'Brien, 2008 O'Brien, 2008; Taylor, 2007; Xu et al., 2004). It is capable of combining automated fluorescence microscopy and advanced image analysis software, to measure multiple cellular events in single cells (Walsh et al., 2011).

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Cytotoxicity parameters such as cell number (CN), nuclear intensity (NI), nuclear area (NA), mitochondrial membrane potential (MMP), mitochondrial mass (MM) and plasma membrane permeability (PMP) measure selected cellular metabolic events and were optimised for HCA as they are hallmarks of cell health (O'Brien and Haskins, 2007; O'Brien, 2008; Walsh et al., 2011). Mitochondria are essential for the life eukaryotic cells and their responses are compound, concentration specific as well as the specific mitochondrial function affected. Enhanced biogenesis of mitochondria can increase MM due to increased mitochondrial respiration and this corresponds with reduced MMP (O'Brien and Haskins, 2007). Cell membrane permeability is a common indicator of cell viability but occurs in the late stages of cytotoxicity when the membrane barrier can no longer be maintained, whilst swelling of nuclei is linked to compound induced necrosis. NI correlates to nuclear size with large nuclei showing lower intensities (Mirochnitchenko et al., 1999). The assay used in the present study was based on the multi-parameter cytotoxicity assay developed by O'Brien et al. (2006). The simultaneous analysis of six parameters of cell cytotoxicity (CN, NA, NI, PMP, MMP and MM) gives substantially more information than conventional approaches by analysing a large proportion of cells in each well.

Morphological, functional and biochemical parameters to elucidate pathophysiology are capable of being measured with higher sensitivity, which has been reflected through lower IC50 values being measured by HCA compared to MTT assay (Rawlinson et al., 2010). HCA has also been validated against human *in vivo* toxicity data for numerous marketed drugs (Abraham et al., 2008; O'Brien et al., 2006; O'Brien and Canelas-Domingos, 2009; Xu et al., 2008). To date HCA has not been applied to determine the toxicity of single or complex mixtures of chemicals in foods.

The cytotoxic effects for Madin-Darby bovine kidney (MDBK) cells have been described for the binary and tertiary combination of ochratoxin A (OTA), fumonisin B1 (FB1) and aflatoxin B1 (AFB1) using conventional cytotoxicity assays MTT and neutral red (NR) (Clarke et al., 2014). The current study was designed to assess cytotoxicity using a multiple parameter approach which would directly compare the sensitivity of this technology to conventional methods and determine its applicability to become one of the tools in determining legislative limits for chemical food contaminants alone and in complex mixtures. The tertiary mycotoxin mixture (OTA, FB1 and AFB1) and binary mixture (OTA and FB1) was the model selected for evaluation, as a previous study has revealed cytotoxicity and synergy with these particular combinations (Clarke et al., 2014). This mixture of toxins occurs in foods at the concentrations selected for the study, and incorporates concentrations corresponding to biologically relevant concentrations naturally contaminating foodstuff (OTA 3 μg/ml, FB1 8 μg/ml and AFB1 1.28 μg/ml) (Bhat et al., 1997; Puntaric et al., 2001; Warth et al., 2012). Co-occurrence of the three mycotoxins has also been reported in a range of commodities including barley, feed, peanuts and infant food (Park et al., 2002; Sangare-Tigori et al., 2005; Warth et al., 2012); with low dose exposure over a long time period resulting in cancers and other irreversible effects (Rodncks et al., 1977; Smith and Moss, 1985).

This is the first study reported which has evaluated HCA as a means of quantifying the toxicity of single and complex mixtures of chemical food contaminants.

2. Methods

2.1. Chemicals

OTA and FB1 were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). AFB1, phosphate buffered saline (PBS) and formalin solution were obtained from Sigma–Aldrich (Dorset, England). TrypLE™ Express, minimum essential media (MEM), MEM non-essential amino acids (NEAA), foetal bovine serum, trypan blue and Countess™ cell counting chamber slides where obtained from Life Technologies (Paisley, Scotland). Mitochondrial membrane potential dye, permeability dye and Hoechst nuclear stain were purchased from Perbio (Northumberland, England). All other reagents were standard laboratory grade.

2.2. Cell culture and treatment

Madin-Darby bovine kidney (MDBK) (Veterinary Sciences Division (VSD), Stormont) were routinely cultured in a humidified atmosphere of 5% CO_2 at 37 °C. MDBK cells were grown in 75 cm^2 flasks in MEM media supplemented with 10% foetal bovine serum and 1% NEAA. TrypLE™ Express trypsin was used to disperse the cells from the flasks, while cell counting and viability checks prior to seeding plates were achieved by trypan blue staining and using a Countess® automated cell counter.

Cells were seeded into BD Falcon 96 well microtitre plates (BD Biosciences, Bedford, MA, US) for assays at a density of 2×10^4 cells/ml. Cells were allowed to attach for 24 h before mycotoxin treatment. Stock concentrations of mycotoxins were prepared in methanol. Final concentrations of test mycotoxins were achieved by adding culture media, giving a final methanol concentration of 0.5% (v/v). Cells were treated with individual mycotoxins AFB1 (0.1 ng/ml–1.28 μg/ml), OTA (0.5 ng/ml–3 μg/ml) and FB1 (0.25–8 μg/ml) for 48 h. Binary combinations (FB1/OTA) and tertiary combinations were also tested using the same concentrations and exposure time. A total of seven combinatory concentrations were tested individually and in binary and tertiary mixtures with controls 0.5% (v/v) methanol in media.

2.3. HCA cytotoxicity assay

Cellomics® HCS reagent series multiparameter cytotoxicity instructions and dyes were utilised. Mitochondrial membrane potential dye was prepared by adding 117 μl of anhydrous DMSO to make a 1 mM stock. Permeability dye was used as provided in kit. The live cell stain was prepared by adding 2.1 μl permeability dye to 6 ml of complete media that had been preheated to 37 °C, and then 21 μl of mitochondrial membrane potential (final concentration 3.5 μM). After the 48 h incubation, 50 μl of live cell stain was added to each well for 30 min at 37 °C and protected from light. Cells were fixed with 10% formalin solution for 20 min at room temperature protected from light and washed with PBS. Hoechst 33342 dye at a final concentration of 1.6 μM was added to each well and incubated for 10 min at room temperature protected from light, after which cells were washed with PBS and evaluated on CellInsight™ NXT High Content Screening (HCS) Platform (Thermo Fisher Scientific, UK). This instrument analyses epifluorescence of individual cell events using an automated micro-plate reader analyser interfaced with a PC (Dell precision T5600 workstation). Hoechst dye was used to measure nuclear morphology (CN, NI and NA), Permeability dye was used to measure PMP and mitochondria membrane potential dye used to measure mitochondrial function (MMP and MM). Data was captured for each plate at 10× objective magnification in the selected excitation and emission wavelengths for Hoechst dye (Ex/Em 350/461 nm), permeability dye (Ex/Em 491/509 nm) and mitochondrial membrane potential dye (Ex/Em 554/576 nm). Nine field view images were acquired in each well to examine each parameter.