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Individual and combined cytotoxic effects of aflatoxin B_1, zearalenone, deoxynivalenol and fumonisin B_1 on BRL 3A rat liver cells

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ABSTRACT

This study was performed to determine the individual and combined cytotoxic effects of Aflatoxin B_1 (AFB_1), zearalenone (ZEA), deoxynivalenol (DON) and fumonisin B_1 (FB_1) on BRL 3A rat liver cells. After the mycotoxins treated the BRL 3A cells for 12, 24 and 48 h, cell viability was determined using the MTT assay. The cytotoxicity of individual mycotoxins on BRL 3A cell viability in decreasing order were DON > AFB_1 > ZEA > FB_1. The central composite design (CCD) was used to assess the toxicity of binary and ternary mixtures of these mycotoxins. The mixtures of AFB_1 + ZEA and AFB_1 + DON showed the synergetic toxic effects on BRL 3A cells. These toxins decreased the viability of cells by inducing intracellular reactive oxygen species (ROS) production and promoting apoptosis in the BRL 3A cells. This effect was mediated by an upregulation of the stress and apoptotic genes Hsp70, p53, Bax, Caspase-3 and Caspase-8, along with a downregulation of the antiapoptotic gene Bcl-2. In conclusion, our results suggested that the coexistence of AFB_1 and ZEA or DON in agricultural products could be more hepatotoxic than individually, suggests that the toxicological interactions of these toxins need to be better understood to assess health risks.

1. Introduction

Mycotoxins are secondary metabolites of fungi, which are toxic to both humans and animals and are primarily produced by five genera: *Aspergillus*, *Fusarium*, *Penicillum*, *Alternaria* and *Claviceps* (Steyn, 1995). These toxins can contaminate various types of crops like maize, wheat, barley, millet, peanuts, peas and oily feedstuffs, which have been shown to affect approximately 25% of the world's agricultural commodities every year (Kanora and Maes, 2009). Livestock ingestion of feed contaminated by mycotoxins can pose serious problems in the health and productivity, and thus cause significant economic losses (Zain, 2011).

Approximately 400 mycotoxins have been currently identified in more than 100 fungi strains (Jard et al., 2011). Aflatoxins B_1 (AFB_1), zearalenone (ZEA), deoxynivalenol (DON) and fumonisin B_1 (FB_1) are recognized as the principal mycotoxins observed in agricultural products, usually co-occurring in food and animal feed (Monbaliu et al., 2010; Streit et al., 2012; Serrano et al., 2012; Solfrizzo et al., 2014). Produced by the fungi *Aspergillus*, AFB_1 is the most lethal mycotoxin, exhibiting harmful hepatotoxic, mutagenic, carcinogenic and teratogenic effects on many species of livestock. It is also classified as a Group one carcinogen (IARC, 1987; Rawal et al., 2010). ZEA is an estrogenic mycotoxin produced by Fusarium, exhibiting a similar structure to estrogen and therefore competing with 17β-estradiol for binding to the estrogen receptor, leading to problems in fertility and reproduction ability (Takemura et al., 2007). DON is a member of the trichothecenes family and mainly produced by *Fusarium*. Ingestion of DON-contaminated feed can induce anorexia, vomiting, and impair immune function in various livestock species. DON can also induce apoptosis in haemopoietic progenitor cells (Parent-Massin, 2004) as well as inhibit protein, DNA and RNA synthesis (Audenaert et al., 2013). FB_1 was originally isolated from *Fusarium moniliforme* and has been associated with equine leukoencephalomalacia (Marasas et al., 1988), porcine pulmonary edema, hydrothorax syndrome (Harrison et al., 1990) and chicken spiking disease (Ledoux et al., 1992). Many studies have showed that FB_1 can induce apoptosis in the kidneys

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and the liver through the inhibition of sphinganine N-acetyl transferase, an enzyme involved in the *de novo* biosynthesis of sphingosine (Merrill et al., 2001).

The symptoms of mycotoxicosis not only depend on the type of mycotoxin, but also relate to the exposure amount and duration, species, age, sex, health, environment, nutritional state and particularly for the interactions with other toxic insults (Bennett and Klich, 2003; Zain, 2011). Furthermore, because of the rather complex process and simultaneous contamination of several mycotoxins in various crops utilized for feed production (Murphy et al., 2006; Streit et al., 2012), extremely toxic interactions can potentially occur as a result. However, the toxic effects of the combination of different mycotoxins on animal health and productive remain limited. As AFB_1 is the most prevalent toxin and is often found with other mycotoxins (ZEA, DON and FB_1) in food and animal feed. Therefore, it is extremely important to evaluate the combined cytotoxic effects among these mycotoxins. Because liver is the primary organ for toxin metabolism, we selected BRL 3A rat liver cells to investigate the individual and combined cytotoxic effects of AFB_1 as well as the other selected mycotoxins (ZEA, DON and FB_1).

2. Materials and methods

2.1. Chemicals and reagents

AFB_1, ZEA, DON and FB_1 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ham's F12K and fetal bovine serum (FBS) was obtained from Hangzhou Sijiqing Biological Engineering Material Co., Ltd. (Zhejiang, China). Antibiotics and trypsin/EDTA solution were purchased from the Beyotime Institute (Shanghai, China) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (Solon, USA). All the other chemicals and reagents used in the experiment are analytically pure and were purchased from Sinopharm Chemical Reagent Co., Ltd (Beijing, China).

2.2. Cell culture, treatments and viability assay

The rat liver cell line BRL 3A was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. This cell line has been well characterized for its relevance to toxicity models (Boess et al., 2003). Before the various mycotoxins treatments, cells were grown in F12K medium supplemented with 100 IU penicillin, 100 mg streptomycin/mL, and 10% FBS. All cell culture plates were maintained in a humidified incubator containing 5% CO_2 and 95% air at 37 °C. Cell counting was conducted using a hemacytometer. Suspension of cells in exponential growth period was prepared and was seeded at 30,000 or 10^6 cells/well in 96 or 6-well plates, respectively, cells were allowed to attach for 8 h before treatment with the mycotoxins. The MTT and lactate dehydrogenase (LDH) were assayed in the 96-well plates, the cells collected for assays of apoptosis, reactive oxygen species (ROS) production, RNA and protein quantifications were cultured in the 6 well plates.

Stock solutions of individual mycotoxins were prepared as described before (Lei et al., 2013). Cells were treated with various dilutions of each individual mycotoxin, AFB_1 (from 0 to 60 μM), ZEA (from 0 to 140 μM), DON (from 0 to 60 μM) and FB_1 (from 0 to 160 μM). Solutions of AFB_1 combination with the other mycotoxins (ZEA and DON), the treatment concentrations followed central composite design (see below), were prepared by mixing the stock solutions of the individual mycotoxins and diluted by F12K.

After BRL 3A cells were exposed to the individual or combined mycotoxins for 24 h, 48 h and 72 h, cell viability was determined using the MTT assay (Mosmann, 1983). Briefly, the cells were treated with toxins for the indicated times and then incubated at 37 °C for 4 h in MTT at a final concentration of 1000 μg/mL. After discarding the supernatants, the intracellular formazan product was dissolved in DMSO and the absorbance of each sample was spectrophotometrically measured at 570 nm and 630 nm (reference wave) using a microplate reader. Meanwhile, after the cells were exposed to mycotoxins for 24 h, the apoptotic rate of treated cells was assayed using an FITC Annexin V Apoptosis Detection kit II (BD Bioscience) by a flow cytometer (BD FACSCalibur). The LDH activity (Babson and Babson, 1973) in the medium supernatant, along with ROS (Lei et al., 2013) as well as Caspase-3 and Caspase-8 activities in the cells were determined as previously described (Wu et al., 2013). Specifically, the LDH activity was determined using a colorimetric method and the results are expressed as relative LDH activity, which is the ratio between the LDH activity of the sample and that measured in control. Caspase-3 and Caspase-8 activities were quantified by spetrophotometric detection of chromophpre (pNA) its cleavage from labeled substrates IETD-pNA and DEVD-pNA, respectively. ROS were detected by using the membrane-permeable probe DCFH-DA method.

2.3. Central composite design for mixtures

The central composite design (CCD) can use to determine levels of various parameters and was performed to assess the interrelationships among all parameters simultaneously (Montgomery, 1991; Heussner et al., 2006). Thus, we used CCD design to evaluate the combined cytotoxic effects between mycotoxins.

In the binary and ternary mixtures experiments, the inhibition concentration of 30% (IC_{30}) of cell viability of each mycotoxin from MTT assay (48 h) was chosen as the center point. The coded and natural values of the factors are shown in Tables 1 and 2. The statistical designs of the binary and ternary mixtures experiments are shown in Tables 3 and 4. The cytotoxic effects of combined mycotoxins were assessed using the MTT assay. All runs were repeated at least three times with duplicate samples.

2.4. Real-time qPCR and western-blot analyses

The BRL 3A cells were collected after treatments with mycotoxins along with the respective control for 24 h. Total RNA was prepared from fresh cells by using Trizol (Invitrogen) and following the manufacturer's instructions. The mRNA levels of pertaining genes involved in apoptosis were determined by qPCR (7900 HT; Applied Biosystems). The genes assayed and primer sequences used for each gene are presented in Supplemental Table 1. The 2^{-ddCt} method was used for the quantification with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene, and the relative abundance was normalized to the control (as 1). Protein concentration determinations and western blot analyses of the pertaining samples were performed as previously described with minor revision (Liu et al., 2012). Briefly, the supernatants of the homogenates (20 μg protein/lane) were loaded onto an SDS-PAGE

Table 1
Nominal concentration range for binary mixture test.

Coded	AFB_1 (μM)	ZEA (μM)	DON (μM)
-r (−1.414)	22	78	0.2
−1	24	82	1
0	29[a]	92[a]	3[a]
1	34	102	5
r (1.414)	36	106	5.8

[a] IC_{30} of cell viability of each mycotoxin was chosen as the center point.