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Aflatoxin M1 cytotoxicity against human intestinal Caco-2 cells is enhanced in the presence of other mycotoxins

Y.N. Gao [a, b, 1], J.Q. Wang [a, b, 1], S.L. Li [a, b], Y.D. Zhang [a, b], N. Zheng [a, b, *]

[a] Ministry of Agriculture Laboratory of Quality & Safety Risk Assessment for Dairy Products (Beijing), Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, 100193, PR China
[b] State Key Laboratory of Animal Nutrition, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, 100193, PR China

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ABSTRACT

Aflatoxin M1 (AFM1), a class 2B human carcinogen, is the only mycotoxin with established maximum residue limits (MRLs) in milk. Toxicological data for other mycotoxins in baby food, containing cereals and milk, either in isolation or in combination with AFM1, are sparse. The aim of this study was to investigate the cytotoxicity of AFM1, ochratoxin A (OTA), zearalenone (ZEA), and α-zearalenol (α-ZOL), individually and in combinations, in human Caco-2 cells. The tetrazolium salt (MTT) assay demonstrated that (i) OTA and AFM1 had similar cytotoxicity, which was higher than that of ZEA and α-ZOL, after a 72 h exposure; and (ii) the quaternary combination had the highest cytotoxicity, followed by tertiary and binary combinations and individual mycotoxins. Isobologram analysis indicated that the presence of OTA, ZEA, and/or α-ZOL with AFM1 led to additive and synergistic cytotoxicity in most combinations. The cytotoxicity of OTA was similar to that of AFM1, suggesting that OTA in food poses a health risk to consumers. Furthermore, AFM1 cytotoxicity increased dramatically in the presence of OTA, ZEA, and/or α-ZOL ($p < 0.01$), indicating that the established MRLs for AFM1 should be re-evaluated considering its frequent co-occurrence with other mycotoxins in baby food which contains milk and cereals.

1. Introduction

Combinations of mycotoxins, such as aflatoxin B1 (AFB1), ochratoxin A (OTA), and zearalenone (ZEA), are naturally present in cereal grains and animal feeds around the world (Gonzalez et al., 1999; Monbaliu et al., 2010; Sangare-Tigori et al., 2006; Streit et al., 2012; Zinedine et al., 2006). When animals such as dairy cows ingest feed contaminated with mycotoxins, these mycotoxins can be metabolized to aflatoxin M1 (AFM1), OTA, ochratoxin α (OTα), ZEA, and α-zearalenol (α-ZOL) and transferred into raw milk. Further, these metabolites remain stable during the processing of dairy products (Fink-Gremmels, 2008; Prandini et al., 2009). AFM1 is classified by the International Agency for Research on Cancer (IARC) as a class 2B carcinogen (IARC, 1993; Sugiyama et al., 2008). OTA, which is responsible for human Balkan endemic nephropathy, is a group 2B potential carcinogen (IARC, 1993; Pfohl-Leszkowicz and Manderville, 2007). ZEA, known for its strong estrogenic activity that causes functional and morphological changes in reproductive organs leading to human pregnancy disorders, has been classified as a group 3 carcinogen by IARC (IARC, 1993; Prouillac et al., 2012). Besides their typical toxicity, AFM1, OTA, and ZEA can impact intestinal and immune functions, causing human chronic intestinal inflammatory diseases (Maresca and Fantini, 2010).

Seventy-six cereal and oil product samples collected from China were analyzed for the occurrence of aflatoxins (AFs), AFB1, OTA, DON and ZEA, ZEA was the most prevalent toxin, with an incidence of 27.6%, AFs and AFB1 were detected in 14.5%, OTA in 14.5% and DON in 7.9% of the samples. In the same set of samples, 14.3%, 7.1% and 9.1% of rice, maize and oat samples contained both OTA and ZEA (Li et al., 2014). The rate of occurrence of mycotoxins in 80 cereal samples collected from Malaysian markets was 50%, 30% and 19% for aflatoxins (total amount of AFB1, AFB2, AFG1 and AFG2), OTA and ZEA, respectively (Soleimany et al., 2012). In Argentina, the co-occurrence of AFM1 (0.059 ppb), deoxynivalenol (DON) (0.338 ppb), and ZEA (0.125 ppb) in dairy milk was estimated by a

* Corresponding author. Institute of Animal Science, Chinese Academy of Agricultural Sciences, No.2 Yuanmingyuan West Road, Haidian District, Beijing, 100193, PR China.
E-mail address: jiaqiwang@vip.163.com (N. Zheng).
[1] The authors contributed equally.

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stochastic simulation model (Signorini et al., 2012). Previous work in our lab showed that AFM1, OTA, ZEA, and α-ZOL are present in raw milk in China, 15%, 45%, and 22% of the samples tested contained two, three, and four mycotoxins, respectively (Huang et al., 2014). In Italy, the presence of AFM1 and OTA in infant formulas was conducted, and AFM1 was found in 2 of 185 samples at levels of 11.8 ng l^{-1} and 15.3 ng l^{-1}, while OTA was detected in 133 (72%) samples in the ranging from 35.1 ng l^{-1} to 689.5 ng l^{-1} (Meucci et al., 2010). The mycotoxin could be absorbed into blood through intestinal, however, the concentration of mycotoxins in plasma would be reduced by the elimination of intestinal. In the 233 serum samples of healthy adults from Turkey, the AF levels were 0.98 ± 0.10 ng/ml in females and 1.35 ± 0.17 ng/ml in males, respectively (Sabuncuoglu et al., 2015). And in plasma samples (without conjugate hydrolysis), the concentration OTA was 0.23 ± 0.03 ng/ml in women and 0.26 ± 0.10 ng/ml in men (Munoz et al., 2010).

Mycotoxin contamination has attracted food safety concerns worldwide (Jolly et al., 2007; Pattono et al., 2011; Williams et al., 2004) since most of the toxic effects in humans are serious (Bouaziz et al., 2008, 2013; Kouadio et al., 2007; Li et al., 2014; Prandini et al., 2009; Tatay et al., 2014). In particular, the diet of young children is heavily based on milk and cereals, both of which have a high likelihood of mycotoxin contamination (Sherif et al., 2009; Tavares et al., 2013). The toxicity caused by multiple-mycotoxins can be classified as acute or, in the case of low dose exposure over a long period of time, chronic, leading to cancers and other irreversible effects (Clarke et al., 2014; James, 2005), including impairment of intestinal immune function, kidney and liver damage, and pulmonary edema (Clarke et al., 2015a; Oswald and Comara, 1998). Furthermore, mycotoxins may interact *in vitro*. Tatay et al. (2014) demonstrated moderate synergism between ZEA and α-ZOL at high concentrations and additivity at low concentrations in ovarian CHO-K1 cells, at 24 h. At 48 h and 72 h, the authors showed, additivity at all concentrations. Bouaziz et al. (2013) reported that the combination of ZEA and T-2 toxin induced a more significant decrease in cell viability and increase in oxidative damage in Vero cells at 24 h compared to individual toxins. This result may be explained by the fact that oxidative damage plays an important role in cytotoxicity. Li et al. (2014) showed that, although OTA and ZEA act individually through distinct mechanisms, in combination they affect cell viability additively. Therefore, it is important for consumers, particularly for young children, that we understand whether mycotoxin interactions increase or decrease toxicity.

Currently, AFM1 is the only mycotoxin with maximum residue limits (MRLs) in milk products worldwide (Sugiyama et al., 2008; Zheng et al., 2013). The MRLs of AFM1 in milk are 0.5 μg kg^{-1} in China and the United States (U.S.) and 0.05 μg kg^{-1} in the European Union (EU); however, no study has investigated the cytotoxic effects of AFM1 in combination with other mycotoxins in milk. Multiple mycotoxins in combination may produce different toxic effects form individual mycotoxins (Signorini et al., 2012). Previous work in our laboratory showed an interaction between OTA, ZEA, and α-ZOL in the human hepatoma G2 (Hep G2) cell line *in vitro* (Wang et al., 2014); however, the study did not include AFM1.

AFM1 toxicity can be used as a benchmark against which to compare other mycotoxins or mycotoxin combinations, which may provide valuable risk information for the development of food safety standards and related policies. Hence, to get a more comprehensive understanding of the combined impact of the mycotoxins existing in baby food, AFM1, OTA, ZEA and α-ZOL were included in this study. Using AFM1 as a reference, the objective of this study was to investigate the individual and combined cytotoxicity of AFM1, OTA, ZEA, and α-ZOL. The hypothesis of our study was that the interaction of AFM1, OTA, ZEA, and α-ZOL, the co-occurrence of which is frequent in baby food might increase cytotoxicity. Since intestine represents the first barrier against contaminants present in food, it could exposure higher concentration of mycotoxins than other organs. Human colon adenocarcinoma (Caco-2) cells, which are from intestinal origin, were used in this study. Caco-2 cells are sensitive to mycotoxins and are widely used in toxicology studies (Alassane-Kpembi et al., 2015; Sambuy et al., 2005).

2. Materials and methods

2.1. Toxins

AFM1, OTA, and ZEA were purchased from Fermentek Ltd. (Jerusalem, Israel), and α-ZOL was purchased from Sigma-Aldrich (St. Louis, MO, USA). OTA, ZEA, and α-ZOL were dissolved in methanol to the concentration of 5000 μg ml^{-1}, and AFM1 was dissolved to the concentration of 400 μg ml^{-1}. Stock solutions of mycotoxins were stored at −20 °C.

2.2. Cell culture and treatment

Caco-2 cells (passage number = 18), a line derived from a human colon adenocarcinoma, were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and maintained as described previously (Boveri et al., 2004). In the present study, the passage number between 20 and 35 were used. Briefly, Caco-2 cells were grown in an atmosphere containing 5% CO_2 and 95% air at 37 °C in constant humidity. The medium was complete DMEM (Gibco, CA, USA) containing 4.5 g/l glucose with L-glutamine and supplemented with 10% fetal bovine serum (FBS), antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin), and 1% non-essential amino acids (NEAA) (Life Technologies, CA, USA). After 3 days, confluence reached 80%, and the cells were trypsinized and plated at a density of 10,000 cells per well in culture medium in 96-well cell culture plates (Costar, Cambridge, MA, USA) (Lu et al., 2013; Wang et al., 2014).

Stock solutions of individual mycotoxins were prepared in methanol and diluted in serum-free medium; the final methanol concentration was lower than 1% (v/v) (Wan et al., 2013; Wang et al., 2014; Zhang et al., 2015). The final mycotoxin concentrations tested were as follows: AFM1 (0.12, 1.2, 3.6, 7.2 and 12 μM), OTA (0.2, 2, 6, 12 and 20 μM), ZEA (1, 10, 30, 60 and 100 μM), and α-ZOL (0.5, 5, 15, 30 and 50 μM). The control was serum-free medium with methanol at the same concentration as the test article (Wang et al., 2014). Caco-2 cells were exposed to individual mycotoxins for 24, 48, and 72 h. Mycotoxin combinations were prepared by mixing the stock solutions of individual mycotoxins and diluting in serum-free medium. The ratios of mycotoxins in the combination samples were based on the ratios of the IC_{50} values of the individual mycotoxins (Tatay et al., 2014; Wang et al., 2014), 1:1.5 for AFM1-OTA, 1:3 for AFM1-ZEA, 1:4.5 for AFM1-α-ZOL, 1:2 for OTA-ZEA, 1:3 for OTA-α-ZOL, and 1:1.5 for ZEA-α-ZOL. The IC_{50} value of each individual mycotoxin was calculated automatically using the computer software CalcuSyn v 2.0. These ratios were intended to yield a roughly similar toxicity for each mycotoxin combination. Caco-2 cells were exposed to mycotoxin combinations for 24 h (Lu et al., 2013; Prosperini et al., 2014).

2.3. Cytotoxicity assay

The tetrazolium salt (MTT) assay, which is based on the cellular conversion of (3,4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (Solon, USA) into formazan, was performed as described