

Cytotoxic effects of individual and binary combinations of zearalenone and ochratoxin a on liver

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ABSTRACT

Co-contamination with mycotoxin represents a serious concern for human and animal health. In this *in vitro* study, we investigated the combined effects of ZEA and OTA, mycotoxins which frequently contaminate cereals, in binary mixtures on the viability of human liver cancer cell line (HepG2). Cell viability was assessed after 24 h using a neutral red assay. An antagonistic effect was observed for binary toxins combinations affecting 25% of cell viability (CI=4.18), which turn into a synergistic effect as followed: slight at IL₅₀ (CI=1.51), moderate at IL₇₅ (CI=0.554) and strong at IL₉₀ (CI=0.203). In conclusion, our results show an important additive and even synergistic cytotoxic effect of two commonly occurred mycotoxins: zearalenone and ochratoxin when they are present simultaneously in food or feed. The co-exposure to mycotoxins lead to a higher toxicity than the exposure to single toxin. Our study provides important data for mycotoxins risk assessment. In this context, a re-evaluation of the guidance levels for mycotoxins will be required in the future, in order to reduce the health risk associated with the possible consumption of mycotoxin co-contaminated food or feed.

Keywords: zearalenone, ochratoxin, liver, co-contamination, HepG2

INTRODUCTION

Mycotoxins are toxic secondary metabolites synthesized by mold, which can seriously affect human and animal health. The most common mycotoxins known for the severe poisoning they produce in humans and animals are mainly synthesized by five major types of fungi: *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* and *Claviceps* (Liew & Mohd-Redzwan, 2018, Alshannaq & Yu, 2017).

Ochratoxin A or OTA is produced by species of *Aspergillus* (*A. ochraceus*) and *Penicillium* (*P. verrucosum*, *P. viridicatum*) being found in cereals, mainly wheat, corn, rye, barley, but also rice, soy, coffee, cocoa, beans, peas, peanuts and dehydrated fruits (e.g. figs, grapes). It is also present in cereal products

such as flour, bread, pasta (Torovic et al., 2017), beer and wine (Bellver Soto et al., 2014). The principal toxic effect of OTA is mainly nephrotoxic, but it can induce other toxic effects as mutagenicity, teratogenicity, hepatotoxicity, neurotoxicity, immunotoxicity etc (Kuiper-Goodman & Scott, 1989, Zhu et al., 2017)

Zearalenone (ZEA, ZEN) or F2 toxin is part of an oestrogenic mycotoxin family produced by *Fusarium* species, mainly *F.graminearum*, *F. semitectum*, *F. equiseti*, *F. crookwellense*, *F. culmorum*, but and other species such as *F. tricinctum*, *F. moniliforme*, *F. oxysporum*, *F. sporotrichoides* and *F. laterinum*. ZEN has mainly estrogenic and anabolic activities (Pazaiti et al., 2012, Kuiper-Goodman et al., 1987) causing serious alterations in the reproductive tract of laboratory and domestic animals (Kuiper-Goodman et al., 1987, Diekman & Green, 1992).

Frequently cereals are contaminated with more than one mycotoxin, and the co-occurrence of mycotoxins in food and animal feed has been found around the world (Streit et al., 2012). Monbaliu et al., (Monbaliu et al., 2010) have shown that among wheat or maize samples obtained from Europe, 75% were contaminated with more than one mycotoxin. In particular ochratoxin and zearalenone were found to co-occur frequently in cereal samples from Europe (Streit et al., 2012).

However, many studies have investigated the toxic effect of single toxins, ignoring the interactions among mycotoxins, i.e., additive, synergistic, or antagonistic toxic effects. Liver represent the main detoxification organ and an important target for the mycotoxins. For this reason, in this study, we investigated the hepato-cytotoxic effect of OTA, ZEA and their mixture using HepG2 cell as a cellular model.

MATERIALS AND METHODS

Cell culture and reagents. Cytotoxicity tests were performed on HepG2 cells. The cells were grown in DMEM supplemented with 2 mM glutamine (Gibco BRL), 1% penicillin/streptomycin (10,000 units of penicillin/mL and 10 mg streptomycin/mL), and 10% foetal bovine serum at 37 °C in a 5% CO₂ humidified atmosphere. Purified OTA and ZEA (Sigma) were dissolved in DMSO/culture media (1:4, v:v), aliquoted and stored at -20°C before dilution in cell culture medium.

Measurement of cell viability. Cell viability in response to OTA and ZEA was assessed through neutral red (NR) assays. Briefly, 2x10⁵ HepG2 cells were cultured in culture media, in 96 well plates for 24h and then treated for another 24h with different concentrations of individual toxins (0-100 μM) and their combinations in a ratio OTA: ZEA of 1:4. After 24h, the media was

removed and replaced with a solution of NR (50µg/mL) for two hours. Then, the cell was fixed with a solution of formaldehyde 1% and the dye was solubilised using a mixture of ethanol: acetic acid: water (50:1:49). The absorbance was read at 540nm using a spectrophotometer (Tecan Infinite M200 Pro).

Analysis of the interaction between mycotoxins

When cells are exposed to the mixtures of mycotoxins, the resulting interaction type can be analyzed using the Chou and Talalay method based on the median-effect equation, coupled with isobologram method described by (Chou, 2006, Marin et al., 2019). The CompuSyn software version 1.0 was used to generate the isobolograms and to calculate the combination index (CI) values for binary combinations of OTA and ZEA as well as the dose reduction index (DRI) values, which indicated the folds of dose reduction for OTA and ZEA.

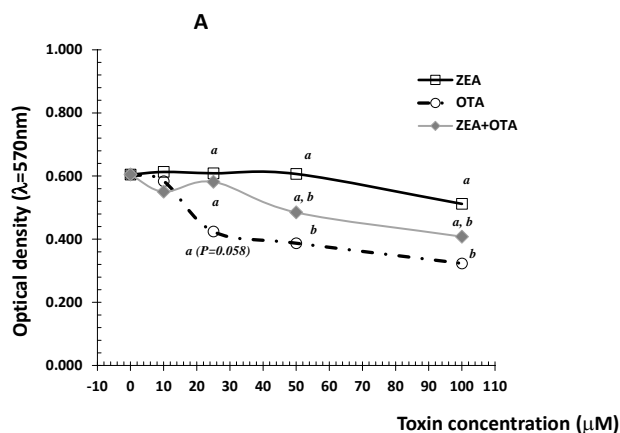
Statistical analysis

The cytotoxicity tests were performed in two independent experiments and three triplicate treatments was realized for each experiment. The cytotoxicity data were presented as mean \pm standard error (SE). IC₅₀ was calculated using GraphPad Prism statistics package, version 5.02 (GraphPad Software, USA). StatView software 6.0 (SAS Institute, Cary, NC) with one-way ANOVA followed by Fisher PSLD test for multiple comparisons was used for analyses of differences between groups. P values lower than 0 .05 were considered significant.

RESULTS AND DISCUSSION

Cytotoxic effect of individual toxin and their combination on hepatic cells

OTA and the combination of OTA+ZEA induced a dose-dependent reduction of viability of cells treated with mycotoxins (Fig.1). No significant effect was observed in ZEA case when compared to control. The cytotoxic effect induced by OTA was significantly higher than that induced by ZEA and OTA+ZEA combination at least for high toxin concentrations (25µM, 50µM and 100µM). In other studies ZEA induced a dose dependent decrease of cell viability in cell types from human and animal origin (Li et al. 2018; Marin et al. 2019).



B

Toxin IC50 (microM)	Mean \pm SD
OTA	4.1 \pm 0.6
ZEA	92.9 \pm 10.9
OTA + ZEA	10.0 \pm 1.2

Figure 1. The effect of contamination with ZEA, OTA and their binary combinations on HepG2 cells viability (A) and the values of cytotoxicity index (IC50) for ZEA, OTA and their combinations (B). ^{a,b,c} indicates significant differences between treatments ($P < 0.05$).

Other experiments on HepG2 cells following treatment with OTA at concentrations even lower than that used in our experiment (0.005- 0.5 μM), have shown that cell viability decreased also in a dose-dependent manner (Shin et al. 2019). The values of the cytotoxic effect for toxins combination at the concentration used in our experiment were always between the values for independent toxin. Based on the cytotoxicity data, the two mycotoxins and their combination were ranked for their cytotoxicity against HepG2 cells as following: OTA > OTA+ZEA > ZEA (Figure 1).

Assessment of interactive effects induced by mycotoxins on cell viability

The values of the combination index (CI) reflecting the inhibition level (IL) induced by the combination of the two toxins by 25, 50, 75 and 90% and the doses of toxins required for cell viability reduction with 25, 50, 75 and 90% (dose reduction index, DRI) are presented in Table 1. An antagonistic effect was observed for binary toxins combinations affecting 25% of cell

viability (CI=4.18), which turn into slight synergistic effect at IL50 (CI=1.51) moderate synergistic at IL75 (CI=0.554) and strong synergistic effect at IL90 (CI=0.203).

Table 1. Combination and dose reduction index values for the combination between ZEA and OTA for cell viability

Cell Viability	CI value*	Toxin concentration (μM)		DRI	
		ZEA	OTA	ZEA	OTA
fa = 0.25 (IL ₂₅)	4.18	1326.40	337.098	0.474	0.482
fa = 0.5 (IL ₅₀)	1.51	505.61	112.57	1.397	1.244
fa = 0.75 (IL ₇₅)	0.554	192.73	37.59	4.11	3.21
fa = 0.9 (IL ₉₀)	0.203	73.47	12.55	12.13	8.29

*Combination index (CI) values for binary toxins combinations affecting 25, 50, 75% and 90% of cell viability. CI values were calculated using CompuSyn software (Chou and Martin, 2005). CI values indicate synergism (CI<1.1), additive effect (0.9<CI<1.1) and antagonism (CI>1.1). Dose reduction index (DRI) indicates fold of dose reduction for different percentages of the decrease of cell viability (25, 50, 75 and 90%) in binary toxins combinations as compared to each mycotoxin alone. fa= Fraction affected

The DRI values indicating the factor by which the dose of each mycotoxin in the combination is reduced due to the synergy, ranged from 0.474 to 12.13 for ZEA and from 0.482 to 8.29 for OTA. This means that for a 90% decrease of cell viability by the combination OTA+ZEA, 12.13 less ZEA and respectively 8.29 less OTA are needed to produce the same effect as the independent toxin. In a similar way, other authors have shown that the exposure of HepG2 cell to a mixture of two mycotoxins: aflatoxin B1 and ZEA induced similar effect as observed by us for the combination ZEA + OTA; low *fa* level was associated with a slight antagonistic effect, while a synergism was obtained at increased *fa* level (Li et al. 2018).

Interrelation between combination index (Figure 2A), fraction affected (Figure 2B), toxin concentration (Figure 2C) and dose reduction index (Figure 2D) for the combination between ZEA and OTA for cell viability are presented in the Figure 2. The isobolograms corresponding to different percentage of cell viability inhibition (50, 75 and 90 %) are presented in the Figure 2 for the OTA+ZEA mixture. The points positioned below the diagonal lines indicate the synergy between the binary mixture of the toxins at 50, 75 and 90% of affected fraction. The dose-effect curve parameters (Dm and m) for cell

viability is presented in Table 2. Excepting low concentrations of mycotoxins, the synergistic effect of ZEA-OTA mixture was 1.2–12 time more potent than OTA or ZEA alone in term of cell cytotoxicity.

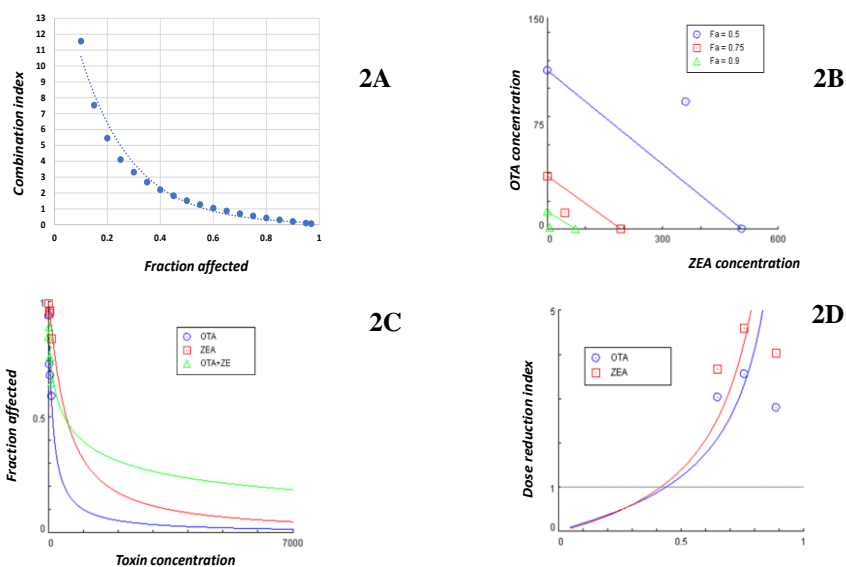


Figure 2. Interrelation between combination index, fraction affected, toxin concentration and dose reduction index for the combination between ZEA and OTA for cell viability

Table 2. Dose effect relationship parameters for ZEA, OTA and their binary combinations on the cell viability

Drug/Combo	Dm	m	r
OTA	112.5	-1.001	-0.933
ZEA	505.6	-1.139	-0.931
OTA+ZEA	452.33	-0.537	-0.815

Dm is the median-effect dose which is required to produce 50% inhibition of the assessed parameters.

m is the slope of the dose-effect relationship, where $m > 1$, $m = 1$ and $m < 1$ mean hyperbolic, sigmoidal and negative sigmoidal dose-effect curve.

Other recent study that investigated individual and combined cytotoxicity effect of zearalenone and ochratoxin A on HepG2 but using a full factorial design as mathematical model found an antagonism for the combination of ZEA (30 and 60 μM) and OTA (6 and 12 μM) (Zheng et al. 2018). However, these results were based on the assumption that mycotoxin dose-effect curves are linear (simple addition of effects, factorial analysis of variance) which is not useful for modelling nonlinear dose-effect curves (Kifer et al. 2020). Our study has used the Chou-Talalay model combined with an isobologram that

has been applied in the majority of the recently published studies concerning the mycotoxin combinations; use of this model allow the estimation of confidence intervals for the combination index which enables the application of statistics (Kifer et al. 2020).

CONCLUSION

Our results show an important additive and even synergistic cytotoxic effect of two important mycotoxins: zearalenone and ochratoxin when they are present simultaneously. The co-exposure to mycotoxins can lead to a higher toxicity than the exposure to single toxin. Our study provides important data for mycotoxins risk assessment. In this context, a re-evaluation of the guidance levels for mycotoxins will be required in the future, in order to reduce the health risk associated with the possible consumption of mycotoxin co-contaminated food or feed.

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