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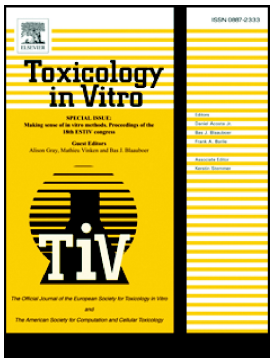

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M. Pinhão, A.M. Tavares, S. Loureiro, H. Louro, P. Alvito, M.J. Silva

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Combined cytotoxic and genotoxic effects of ochratoxin A and fumonisin B_1 in human kidney and liver cell models

Pinhão M., MSc[a], Tavares A.M., MSc[a], Loureiro S., PhD[b], Louro H., PhD[a,c], Alvito P., PhD[b,d], Silva M. J., PhD[a,c] *

[a] Department of Human Genetics, National Institute of Health Doutor Ricardo Jorge, I.P. (INSA), Av. Padre Cruz, 1649-016, Lisbon, Portugal. marianaa.pinhao@gmail.com; anar..p'avares@gmail.com; henriqueta.louro@insa.min-saude.pt; m.joao.silva@insa.min-saude.pt

[b] CESAM - Centre for Environmental and Marine Studies, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal. sloureiro@ua.pt

[c] ToxOmics - Centre for Toxicogenomics and Human Health, NOVA Medical School/FCM, Universidade NOVA de Lisboa, Portugal.

[d] Food and Nutrition Department, INSA, Av. Padre Cruz, 1649-016 Lisboa, Portugal paula.alvito@insa.min-saude.pt

*Corresponding Author:

Maria João Silva

Department of Human Genetics

National Institute of Health Doutor Ricardo Jorge, I.P. (INSA)

Av. Padre Cruz, 1649-016, Lisbon, Portugal

m.joao.silva@insa.min-saude.pt; Tel.: +351 217 519 234

Abstract:

Food products can be contaminated by several fungi species and each species may produce different mycotoxins, leading to human combined exposure. Although predictions about the joint toxic effects of mycotoxins can be made from their individual toxicities, experimental data is still limited to allow a reliable hazard assessment. Thus, this study aimed to characterize the combined cytotoxic and genotoxic effects of ochratoxin A (OTA) and fumonisin B1 (FB_1) in cell lines representative of their target organs, kidney and liver. Interactions were ascertained using mathematical extensions to the reference models of concentration addition and independent action. Cytotoxicity (MTT assay) data modeling revealed a synergistic pattern for low doses of both FB_1 and OTA shifting to antagonism at higher concentration levels, irrespectively of the reference model applied. Concerning genotoxicity assessment, neither OTA nor FB_1, individually or in combination, induced a prominent increase in DNA damage (comet assay) or oxidative DNA damage (FPG-comet assay). In conclusion, this study revealed a synergistic cytotoxic effect for OTA and FB_1 at low concentration levels. Given that human co-exposure to these two mycotoxins is probable to occur at low doses, these results raise concerns regarding their potential health outcomes that seem to differ from those predicted by an additive model.

Keywords: ochratoxin A; fumonisin B_1; liver and kidney toxicity; genotoxicity; interactive effects

Abbreviations:

OTA, Ochratoxin A; MoA, Mode of Action; IARC, International Agency for Research on Cancer; FB_1, Fumonisin B1, CA, Concentration Addition; IA, Independent Action; FPG, Formamidopyrimidine DNA glycosylase; DMSO, Dimethyl sulfoxide; DPBS, Dulbecco's Phosphate-Buffered Saline; DMEM, Dulbecco's Modified Eagle Medium; FBSi, heat-inactivated fetal bovine serum; HEPES, hydroxyethyl-piperazineethane-sulfonic acid buffer; MTT, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; BSA, bovine serum albumin; PBS, Phosphate-Buffered Saline; EMS, ethyl methanesulfonate; ANOVA, Analysis of Variance; SS, sum of squares; DR, Dose-ratio dependent; DL, Dose-level dependent, ROS, Reactive Oxygen Species

1. Introduction

Mycotoxins are secondary metabolites produced by some genera of fungi that contaminate agricultural cereal crops in the field or during harvest and storage (Agriopoulou et al., 2020). Human exposure to mycotoxins occurs mainly through the ingestion of contaminated food products or the inhalation of airborne fungi spores and can represent a hazard to human health (Marin et al., 2013). Recent food occurrence and human biomonitoring studies have indicated that humans are frequently exposed to multiple mycotoxins, e.g., fumonisins, ochratoxin, zearalenone and deoxynivalenol. Moreover, *in vivo* studies have evidenced that co-exposure may cause adverse health effects even when individual concentrations of mycotoxins do not exceed legal guidance values (Ráduly et al., 2020). This finding has raised a significant concern over potential combined effects of mycotoxins that may differ from the simple addition of their single effects, possibly impacting on their health risk (Clarke et al., 2014; Corcuera et al., 2011; Domijan et al., 2015).

Ochratoxin A (OTA) is produced by *Aspergillus* and *Penicillium* genera (Ráduly et al., 2020) and is one of the mycotoxins most commonly found in food products, e.g., cereal and cereal-based food, dried fruits, coffee, alcoholic beverages, and spices (Do et al., 2015). OTA primarily targets the kidney and human exposure has been associated to nephropathies development and cancer of the upper urinary tract (Agriopoulou et al., 2020). It is presently classified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic to humans (group 2B, IARC, 1993), mainly based on animal studies data. In addition, genotoxicity, teratogenicity, neurotoxicity and immunotoxicity have been reported in man and other animal species (Agriopoulou et al., 2020; Heussner and Bingle, 2015; Ráduly et al., 2020). Its Mode of Action (MoA) includes the inhibition of protein synthesis,

epigenetic effects (Hadjeba-Medjdoub et al., 2012; Pfohl-Leszkowicz and Manderville, 2012; Vettorazzi et al., 2013), mitochondrial damage (Kamp et al., 2005; Liu et al., 2012) and lipid peroxidation (Kumar et al., 2014).

Fumonisins are produced mainly by fungi of the *Fusarium* genera (Agriopoulou et al., 2020; Kamle et al., 2019), being fumonisin B_1 (FB_1) the most abundant toxic form (Creppy et al., 2004) found in maize and related products, e.g., bread or pastry (Agriopoulou et al., 2020; EFSA, 2014). Human exposure has been associated to immunosuppression (Domijan et al., 2015; Kamle et al., 2019), and neurotoxicity (Doi and Uetsuka, 2011). Rodent studies have shown that kidney and liver are the target organs for FB_1-mediated toxicity and developmental toxicity has also been reported in several animal species (IARC, 2002). FB_1 exhibits tumor promoter properties and is classified by IARC as possibly carcinogenic to humans (group 2B, IARC, 2002). *In vitro* studies have revealed that although it is not mutagenic, FB_1 induces oxidative damage and is clastogenic in mammalian cells (Knutsen et al., 2018) besides causing apoptosis, necrosis, cell regeneration and proliferation (EFSA, 2014). FB_1 adverse effects are mainly mediated by the inhibition of ceramide synthases, which are key enzymes in the sphingolipid metabolism (EFSA, 2014).

Previous studies have suggested that interactions between OTA and FB_1 occur in renal cell lines, pointing to synergism, although they did not present mathematical modelling data, which is crucial to confirm the observed effects (Creppy et al., 2004; Klarić et al., 2008). Currently, two reference mathematical models are broadly accepted to explain the interactive effects of chemical mixtures: the concentration addition (CA) and the independent action (IA) models (Jonker et al., 2005; Loureiro et al., 2010; Tavares et al., 2013). The first assumes that chemicals have the same MoA, while the latter does not (Loureiro et al., 2010). Both models assume that the chemicals do not interact and, for that reason, deviations from the predictions indicate interaction (Syberg et al., 2008). It is then possible to conclude if the

chemicals have stronger (synergistic) or weaker (antagonistic) effects than expected from an additive effect, or if they have a relationship dependent on dose level or dose ratio (Loureiro et al., 2010; Tavares et al., 2013).

Given the human co-exposure to OTA and FB_1 and the possibility of interactive effects at hepatic or renal levels, this study intended to characterize the combined cytotoxicity and genotoxicity of those mycotoxins in two cell lines representative of their target organs, using the reference mathematical models of CA and IA.

2. Materials and Methods

Mycotoxins

A stock solution of OTA (9910 µM) was prepared in dimethylsulfoxide (DMSO; Sigma-Aldrich) and successive dilutions (working solutions) of the stock solution were done with Dulbecco's phosphate buffered saline (DPBS; Gibco, Life Technologies Limited, Paisley, UK) plus DMSO (10%). The exposure medium consisted of the culture medium with 2% FBSi to which the appropriate volume of the selected working solution was added, to try that the final concentration of DMSO in the medium was below 1%. FB1 was reconstituted in DPBS with DMSO (50%) to obtain a stock solution (30 mM). Similarly, successive working solutions were prepared with DPBS and the appropriate volume of the selected working solution was added to the exposure medium to try that the final concentration of DMSO in the medium was below 1%.

Cell lines and treatments

Both cell lines were obtained from the American Type Culture Collection: the human hepatocellular carcinoma cell line HepG2 (ATCC HB-8065) and the human proximal renal tubular epithelial cells HK-2 (ATCC CRL-2190). All solutions for cell culture were obtained from Life Technologies Limited (Paisley, UK). Cells were maintained in DMEM-F12

medium containing L-Glutamax, and supplemented with 10% or 15% heat-inactivated fetal bovine serum (FBSi; HK-2 and HepG2 cells lines, respectively), 2.5% HEPES buffer (25 mM), 1% penicillin/streptomycin (10000 units/mL of penicillin and 10000 µg/mL of streptomycin) and 1% amphotericin B (0.25 mg/mL), at 37°C, in 5% CO2, humidified atmosphere.

For the MTT and the Comet assays, the cells were plated in 96 or 24-well plates, (respectively) at the density of 10^5 cells/mL, and maintained at 37°C, in 5% CO_2. HepG2 cells were allowed to attach and grow before exposure to the toxins for 24 hours, and HK-2 cells for 48 hours. All treatment solutions were prepared in DMEM-F12 culture medium supplemented with 2% FBSi in order to keep the cell growth conditions, while minimizing the possibility of OTA affinity to serum proteins, that could decrease its availability in medium leading to underestimation of effects (EFSA, 2006; van der Valk et al., 2018).

Experimental design

Firstly, the cytotoxicity of each individual mycotoxin was assessed by establishing a concentration-response curve and the concentration that inhibited 50% cell viability (IC_{50}) was determined. The concentrations of OTA used for this purpose in HK-2 cells ranged from 2.5 to 160 µM, and in HepG2 cells from 5 to 60 µM. The concentrations of FB_1 tested in kidney cells ranged from 2.5 to 1080 µM, and in the liver cells from 40 to 320 µM. Afterwards, the combined cytotoxic effect of these toxins was evaluated by the determination of cell viability after exposure to binary mixtures of OTA and FB_1 in the same cell lines. A full factorial design was used for the cytotoxicity assessment and is depicted in Figure 1. All exposures were carried out for 24 hours

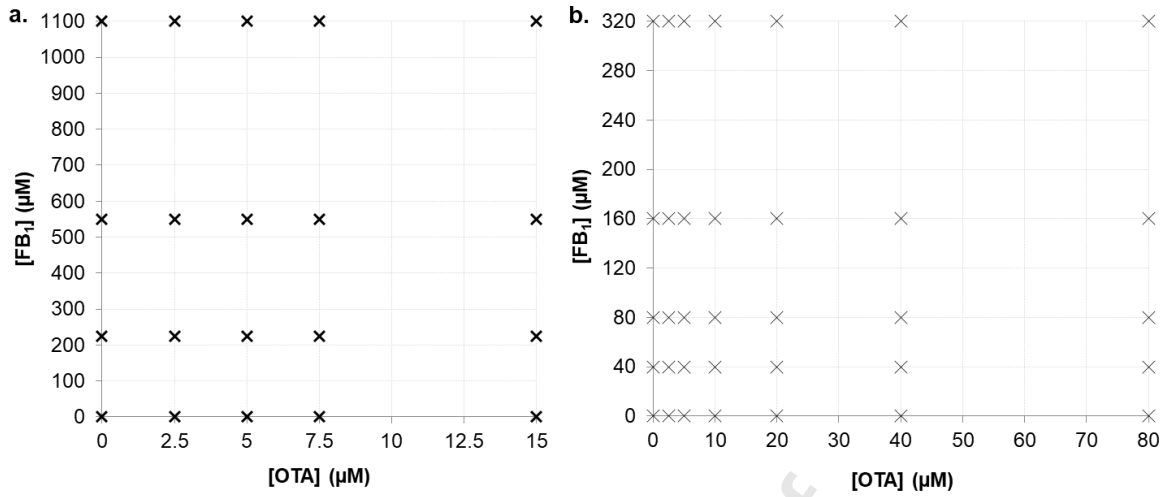

Figure 1 - Experimental design used for assessing the combined cytotoxicity of OTA and FB_1 by the MTT assay in (a) HK-2 cells and (b) HepG2 cells.

The genotoxicity of each individual mycotoxin was also assessed. The concentrations of OTA used in HK-2 cells ranged from 1.25 to 7.5 µM, and in HepG2 cells from 10 to 40 µM. The concentrations of FB_1 tested in HK-2 cells ranged from 225 to1100 µM, and in the HepG2 cells from 40 to 160 µM. The experimental design used for the combined genotoxicity assessment is depicted in Figure 2.

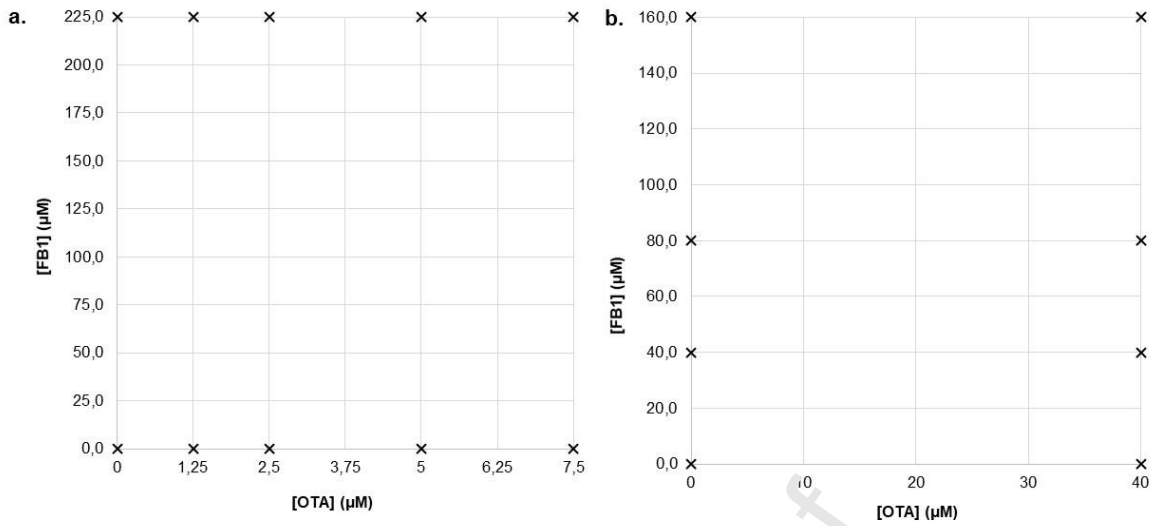

Figure 2 - Experimental design used for assessing the combined genotoxicity of OTA and FB_1 by the Comet assay in (a) HK-2 cells and (b) HepG2 cells.

MTT assay

The MTT assay was carried out according to Mosmann (1983) (Mosmann, 1983) with slight modifications. HK-2 and HepG2 cells were exposed to a concentration-range of each mycotoxin during 24 h. Given that preliminary experiments suggested that HepG2 cells were very tolerant to FB_1 and no IC_{50} was likely to be achieved, the top concentration tested was set at 320 µM. After cells exposure, the treatment medium was removed, and the cells were washed twice with pre-heated DPBS. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Calbiochem; Darmstadt, Germany) was reconstituted in DPBS (5 mg/mL) and then diluted in DMEM-F12 culture medium (0.5 mg/mL); 100 µL of this solution were added to the cells. The plates were incubated for 2-4 hours at 37°C, in 5% CO_2, to allow MTT incorporation. After this incubation, the MTT solution was removed and 100 µL of DMSO was added to dissolve the formazan crystals formed inside the viable cells after MTT incorporation. The plates were shaken for 15-30 minutes at room temperature and the results were measured

spectrophotometrically at 570 nm with a filter of reference at 690 nm, using a Multiskan Ascent spectrophotometer (Thermo Labsystems).

Controls were included in all experiments: negative controls consisting of DMEM-F12 culture medium supplemented with 2% FBSi, positive controls consisting of SDS 1%. Despite the attempt to maintain the DMSO concentration below 1% in all experiments, there were rare exceptions where this limit was exceeded. The highest concentration in the culture medium (1.98%) was reached when HK-2 cells were exposed to concentrations of 1100 µM of FB1 combined with 15 µM of OTA. When final DMSO concentrations ≥ 1% were reached in the exposure medium, a vehicle control consisting of DMSO at the concentration reached was included to correct for a possible DMSO toxicity. For each experiment, 3 replicate wells were used for each treatment condition.

The relative cell viability of treated cells was calculated in comparison to the mean absorbance of the vehicle control (which is assumed to correspond to 100% cell viability). The results were expressed as the mean value ± standard error of the mean (M ± SEM) of 3 independent experiments per treatment condition. The toxic effect of binary mixtures in cells was expressed as the percentage of cytotoxicity relative to control, calculated as the difference between the measured viability and 100% viability for each mixture tested.

Alkaline Comet Assay

The comet assay was carried out according to Collins et al. with modifications (Collins, 2014). After exposure, cells were embedded in 1% low melting point agarose and placed onto microscope slides previously coated with a 1% normal melting point agarose. The microscope slides were then immersed in lysis solution [with 89% Lysis Buffer (NaCl 2.5 M, Na2EDTA.2H2O 100 mM, Tris-HCl 10 mM; NaOH until pH=10), 10% DMSO and 1% Triton-X100] for at least 1h, at 4°C. After lysis, slides were washed in enzyme buffer (HEPES 40 mM, KCl 100 mM, acid EDTA 0.5 mM, BSA 0.2 mg/mL; KOH until pH=8). All

reagents used in comet assay were obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO). The nucleoids were exposed to Formamidopyrimidine DNA glycosylase (FPG) enzyme (kindly provided by Dr. A. R. Collins, University of Oslo, Norway) − which converts oxidized pyrimidines into DNA breaks allowing detection of oxidative DNA damage (Collins, 2009) −, or buffer only, and incubated at 37°C for 30 minutes. The slides were then covered by electrophoresis buffer (NaOH 0.3 M, Na2EDTA.2H2O 1mM; pH=13) for 30 minutes, allowing the DNA to unwind; electrophoresis was performed for 25 minutes at 28 V and 300 mA. The slides were immersed in DPBS for 10 minutes to neutralize the pH,and left to dry at room temperature overnight. The slides were stained with ethidium bromide (0.125 µg/mL), and observed in a fluorescence microscope (Axioplan2 Imaging, Zeiss) with the assistance of specific image-analysis software (Comet Imager 2.2, from MetaSystems, GmbH).

A positive control was included in all experiments consisting of ethyl methanesulfonate (EMS) at 10 mM for 60 minutes or hydrogen peroxide (H_2O_2) at 200 µM for 30 minutes, as well as a negative control, consisting of DMEM-F12 culture medium supplemented with 2% FBSi. When final DMSO concentrations above 1% were reached in the exposure medium, a vehicle control consisting of DMSO at the highest concentration reached was included to correct for a possible toxicity caused by DMSO. The maximum DMSO percentage (1.98%) was reached at the highest FB1+OTA concentrations, i.e. 1100 µM of FB1 combined with 15 µM of OTA. Two slides were prepared for each treatment condition, and two or three replicate experiments were performed in each case. 50 nucleoids were analyzed per gel, therefore, 100 were analyzed per slide, and 200 per treatment.

The amount of DNA damage was assumed to be accurately expressed by the percentage of the DNA in the tail of the nucleoids, as this parameter is linearly related to the DNA damage caused to the cells (Collins, 2014). The median of the percentage of DNA in the tail of the nucleoids was calculated for each slide, and the results were expressed as the mean

value (± SEM) of medians (100 nucleoids per slide, 2 slides per replicate experiment, two or three independent experiments).

Statistical Analysis and Data Modeling

Statistical analysis was performed using SPSS Statistics 20 (IBM, New York, USA). The results of both MTT and Comet assays were analyzed using a parametric One-Way ANOVA test (followed by the Tukey post-hoc) or Student's *t*-test. The normality of the data was evaluated with Shapiro-Wilk's test, and the homogeneity of variances assumption was verified with Levene's test. Whenever possible, the existence of a dose-response curve was determined by regression analysis, and in the cytotoxicity assays for the individual toxins, the IC_{50} values were calculated from the curve equation that best fitted the experimental data. In all cases, statistical significance was assumed for $p \leq 0.05$.

Data obtained from the mixture exposures was analyzed using the MIXTOX tool (Jonker et al., 2005) by comparing the observed data with the predicted effects, based on the individual exposure toxicities, and using the reference conceptual models (CA and IA). Both CA and IA models were then mathematically extended to derive deviations for synergism/antagonism, dose ratio and dose level dependency, by forming a nested framework. From this mathematical modeling, there were two output parameters (a and b) that enable the prediction of a biological effect. More details on the mathematical derivation of these functions can be obtained in Jonker et al. (2005) (Jonker et al., 2005) and in Table 1 for the biological significance of these parameters. To attain the best model fit and the best model descriptors, data fit was evaluated by the method of maximum likelihood and statistically compared through likelihood testing. The significant level of 0.05 using the Chi-square test was used based on the decrease in the residuals of the sum of squares (SS) and an increase in the description of the variation of the data (R^2). When a significant deviation

from CA and IA model was identified, the effect pattern was deduced directly from the parameter values (Table 1).

Table 1 - Analysis of mixture toxicity data and interpretation of additional parameters (a and b) that define the functional form of deviation pattern from the reference models of concentration addition (CA) and independent action (IA).

Deviation Pattern	Parameter a (CA and IA)	Parameter b (CA)	Parameter b (IA)
synergism/ antagonism (S/A)	a>0: antagonism a<0: synergism		
Dose-ratio dependent (DR)	a>0: antagonism except for those mixture ratios where negative b value indicate synergism	b_i>0: antagonism where the toxicity of the mixture is caused mainly by toxicant i	
	a<0: synergism except for those mixture ratios where positive b value indicate antagonism	b_i<0: synergism where the toxicity of the mixture is caused mainly by toxicant i	
Dose-level dependent (DL)	a>0: antagonism low dose level and synergism high dose level	b_{DL}>1: change at lower IC_{50} level	b_{DL}>2: change at lower IC_{50} level
		b_{DL}=1: change at IC_{50} level	b_{DL}=2: change at IC_{50} level
	a<0: synergism low dose level and antagonism high dose level	0<b_{DL}<1: change at higher IC_{50} level	1<b_{DL}<2: change at higher IC_{50} level
		b_{DL}<0: No change but the magnitude of S/A is DL dependent	b_{DL}<1: No change but the magnitude of S/A is effect level dependent

a and b are parameters of the deviation function, which are then transposed to the biological interpretation of the mixture; bDR- parameter b referring to Dose Ratio deviation; bDL- parameter b referring to Dose Level deviation; SS- the objective function used, as the sum of squares; df – degree of freedom; p refers to the significance of the fit to the CA or IA models (χ^2) or for the likelihood test (conceptual model vs deviation).

3. Results

3.1. Cytotoxicity assessment

3.1.1. Single mycotoxins cytotoxicity

The cytotoxic effects of single OTA and FB_1 were assessed on HK-2 and HepG2 cells after 24 hours exposure, and a dose-response curve was derived (Figure 3). The IC_{50} values were calculated for each mycotoxin, whenever possible.

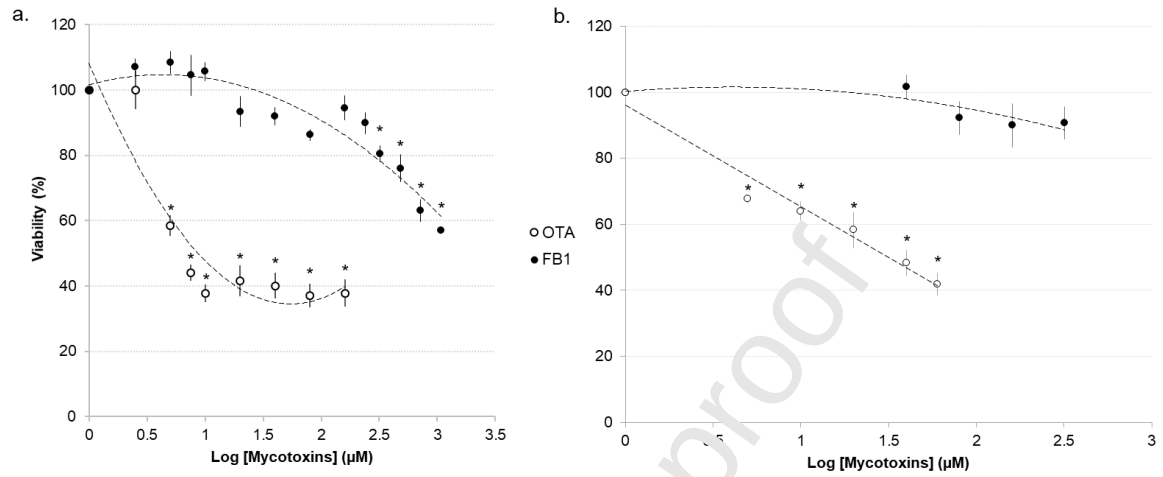

Figure 3 - Viability of (a) HK-2 and (b) HepG2 cells exposed to OTA and FB_1. Results are expressed as percentage of viability *vs.* logarithm of the toxins concentration. 100% of viability corresponds to the viability of unexposed cells. * - significantly different compared to the negative control ($p<0.01$).

In HK-2 cells, both mycotoxins showed accentuated cytotoxic effects in the tested concentration range (Figure 3a). OTA led to a significant loss of cell viability in all concentrations tested except for the lowest one, in comparison to the negative control ($p<0.001$; One-Way ANOVA followed by Tukey post-hoc). The dose-response curve that best fit this data is a polynomial model ($y=24.46x^2 – 84.92x + 108.21$; $R^2=0.86$), from which an IC_{50} of 8.71 µM was derived. The reduction of cell viability by 50% with FB_1 was not reached experimentally, and therefore, an IC_{50} of 2118.29 µM was estimated from the dose-response polynomial curve equation suggested by regression analysis ($y=-7.58x^2 + 9.70x + 101.62$; $R^2=0.89$).

In HepG2 cells (Figure 3b), OTA caused a marked loss of viability, significantly different from the negative control for all tested concentrations ($p<0.01$). The mathematical model that best fitted the data was a linear function ($y=-30.71x + 96.02$; $R^2=0.97$). From this dose-response association, it was then possible to calculate the IC_{50} of this toxin of 31.50 µM.

FB_1, on the other hand, did not cause a significant reduction in viability (p=0.295; One-Way ANOVA), not being possible to derive a realistic IC_{50} value for this cell line from the dose-response curve that best adjusted to the experimental data ($y=-3.62x^2 + 4.40x + 100.26$; $R^2=0.71$).

3.1.2. Combined mycotoxins cytotoxicity

Both cell lines were exposed to several concentrations of OTA and FB_1, as well as to their combinations; the results were then modeled in order to reveal possible interactions between these two toxins, using the CA and the IA mathematical models. The derived parameters a and b, and their significance, were then checked against information in Table 1 to infer on the biological interpretation.

The results of the combined cytotoxicity of the two mycotoxins are represented in Figure 4. In HK-2 cells, the mixtures cytotoxic effects increased with the OTA concentration; the co-exposure to FB_1 increased slightly the cytotoxicity of OTA, in particular at the two lowest concentrations tested (Figure 4a). In HepG2 cells, the cytotoxic effects of the mixtures were mainly driven by OTA toxicity (Figure 4b).

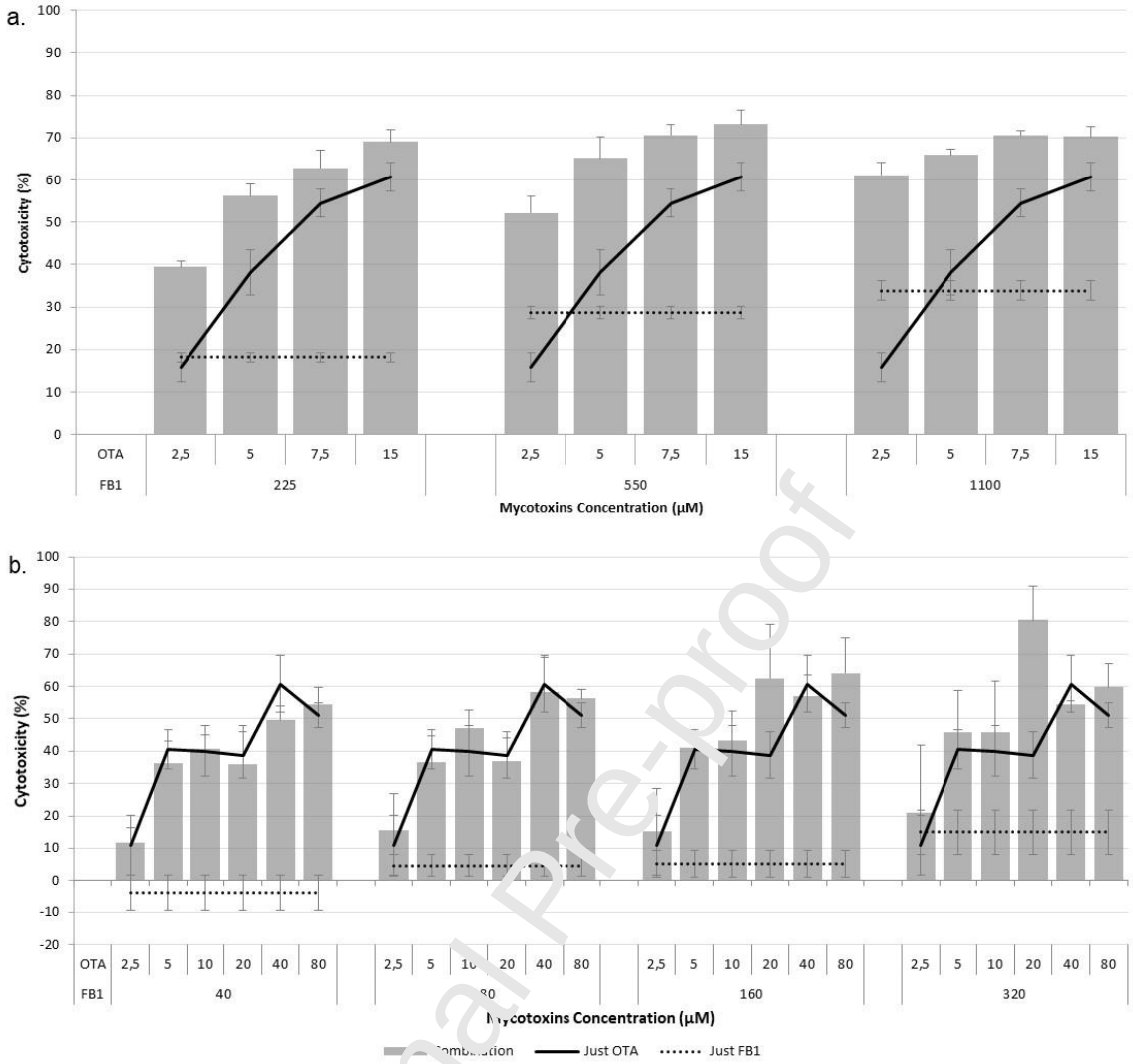

Figure 4 - Viability of (a) HK-2 and (b) HepG2 cells exposed to OTA, FB_1 and their mixtures. Results are expressed as percentage of cytotoxicity relatively to the negative control.

Tables 2 and 3 summarize (and Figure S1 graphically represents) the cytotoxic responses of HK-2 and HepG2 cells after modeling, by using the two conceptual models, CA and IA.

Table 2 - Summary of the analysis of the combined toxicity of OTA and FB_1 in HK-2 cells

Parameter	CA model	Deviation from CA model	IA model	Deviation from IA model
		DL[a]		**DL[a]**
Max	0.98	**0.98**	0.98	**0.98**
slope (OTA)	1.08	**1.22**	0.87	**1.06**
slope (FB_1)	1.05	**0.66**	0.58	**0.68**
IC_{50} (OTA)	6.66	**7.96**	7.81	**8.45**
IC_{50} (FB_1)	1145	**2498**	2316	**2316**
a [b]	-	**-4.63**	-	**-2.67**

b_{DR} [b]	-	**-**	-	**-**
b_{DL} [b]	-	**0.33**	-	**1.27**
SS [c]	133	**57**	83	**64**
r^2 [d]	0.86	**0.94**	0.91	**0.93**
df	4	**2**	4	**2**
χ^2	797		847	
Likelihood test		**75**		**19**
p [e]	$3x10^{-171}$	**$4x10^{-17}$**	$6x10^{-182}$	**$7x10^{-5}$**

[a] Dose Level deviation; [b] parameters of the deviation function, which are then transposed to the biological interpretation of the mixture. [c] objective function used, as the sum of squares; [d] coefficient of determination; [e] significance of the fit to the CA model (χ^2) or for the likelihood test (conceptual model vs deviation); Data in bold depicts the best fit.

Table 3 - Summary of the analysis of the combined toxicity of OTA and FB_1 in HepG2 cells

Parameter	CA model	Deviation from CA model	IA model	Deviation from IA model
		DL[a]		**DL[a]**
max	0.98	**0.93**	0.98	**0.95**
slope (OTA)	0.5	**0.63**	0.5	**0.54**
slope (FB_1)	2	**1115**	2	**1.61**
IC_{50} (OTA)	60	**53**	60	**51**
IC_{50} (FB_1)	1000	**519**	1000	**1644**
a [b]	-	**3.67**	-	**-0.007**
b_{DR} [b]	-	**-**	-	**-**
b_{DL} [b]	-	**0.68**	-	**-6.33**
SS [c]	1124	**775**	1147	**792**
r^2 [d]	0.53	**0.67**	0.52	**0.67**
df	4	**2**	4	**2**
χ^2	1244		1221	
Likelihood test		**349**		**354**
p [e]	$4x10^{-268}$	**$2x10^{-76}$**	$4x10^{-263}$	**$1.36x10^{-77}$**

[a] Dose Level deviation; [b] parameters of the deviation function, which are then transposed to the biological interpretation of the mixture. [c] objective function used, as the sum of squares; [d] coefficient of determination; [e] significance of the fit to the CA model (χ^2) or for the likelihood test (conceptual model vs deviation); Data in bold depicts the best fit.

Data modeling pointed to a synergistic pattern of cytotoxicity in mixtures where both mycotoxins presented low doses in the exposure to HK-2 cells (a<0), shifting to antagonism at higher doses, irrespectively of the starting model applied (CA or IA). The shift from synergism to antagonism took place at a dose level higher than the IC_{50} of the mixture

(b_{DL}=0.33, when the CA model was used as the starting point, and b_{DL}=1.27, after fitting to the IA model) (Table 2).

Regarding the results from the exposure of HepG2, data modeling led to similar results for a dose level deviation, with synergism at lower doses of OTA and FB_1 (a<0). Changes to antagonism were predicted to occur at a dose level higher than the IC_{50} (b_{DL}=0.68) when the CA was the starting model, or at higher levels than the ones used in the experiment (**(**b_{DL}=-6.33), when the IA model was used (Table 3).

3.2. Genotoxicity assessment

The genotoxic potential of OTA and FB_1 was assessed individually and in combination, using the comet assay. Figure 5 presents the results of the conventional and the FPG-modified comet assay in both cells lines after exposure to OTA or FB_1 individually. The results of the conventional comet assay after exposure to the mixtures were all negative (Figure S2). Figure 6 shows the results of the FPG-modified comet assay after cells exposure to mixtures of both toxins. Likewise, with the addition of the FPG enzyme neither OTA nor FB_1, individually or in combination, caused any significant effect in the percentage of DNA in tail, irrespectively of the cell line (Figures 5 and 6). In addition, hedgehogs were rarely observed suggesting that there were no signs of early apoptotic cells at the concentration range tested.

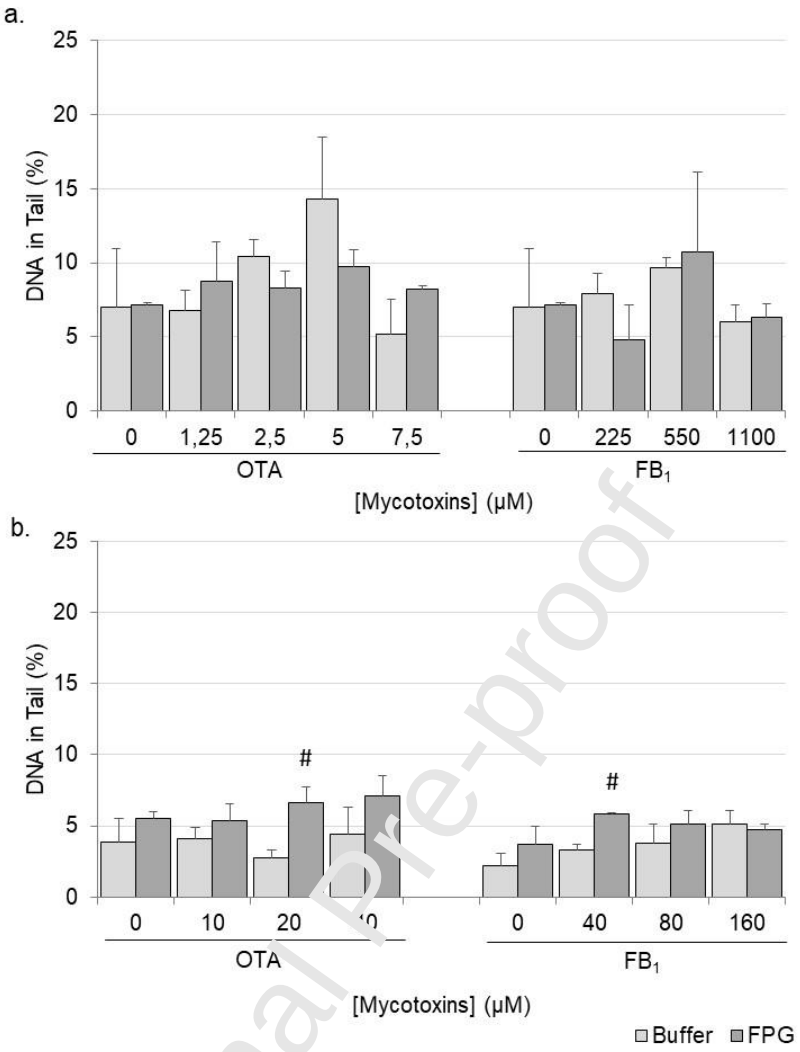

Figure 5 - Comet and FPG-comet assays results following cells exposure to OTA and FB_1: (a) HK-2 and (b) HepG2 cells. Results are expressed as mean percentage (± SEM) of DNA in the tail. * -significant increase of DNA damage over the negative control; # - significant increase of oxidative DNA damage compared to the respective conventional comet outcomes.

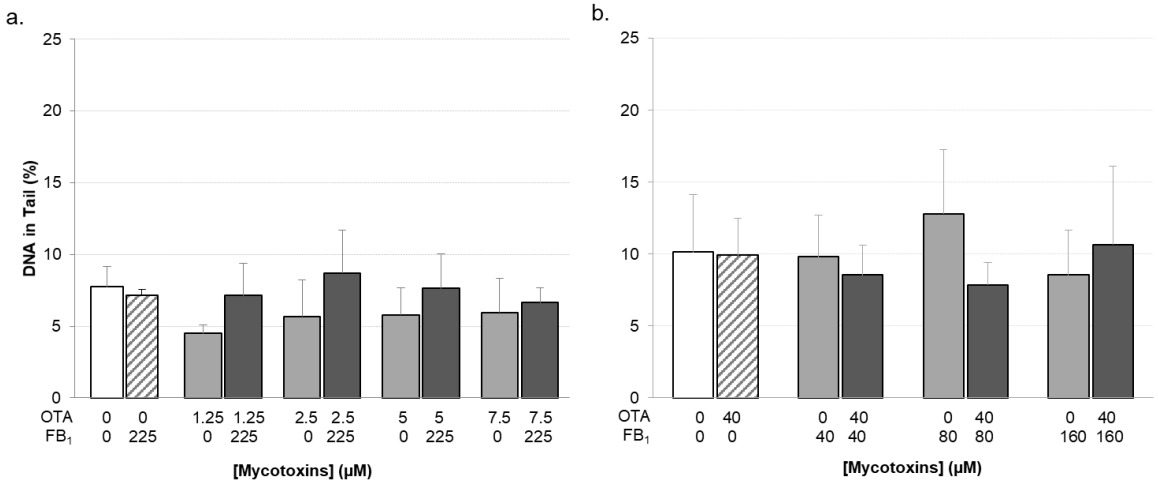

Figure 6 - FPG-modified comet assay results following (a) HK-2 cells exposure to FB_1 (stripes), OTA (light grey) and its mixtures (dark grey); (b) HepG2 cells exposure to OTA (stripes), FB_1 (light grey), and its mixtures (dark grey). Results are expressed as the mean percentage of DNA in the tails of the comets.

Lastly, the comparison of the conventional comet assay results with those of the modified version (i.e. DNA strand breaks vs. total damage, including oxidative lesions), showed that in liver cells, individual OTA and FB_1 treatments caused a significant increase in the level of oxidative DNA damage, measured as FPG sensitive sites: 20 µM of OTA (p = 0.03; Student's t-test) and 40 µM of FB_1 (p = 0.005) (Figure 5b). In the HK-2 cell line, on the other hand, the individual toxins did not significantly affect the level of oxidative lesions occurring in the DNA (Figure 5a).

4. Discussion

The presence of OTA and FB_1, among other mycotoxins, has been reported in food surveys (Assunção et al., 2016; García-Moraleja et al., 2015; Martins et al., 2018) and in human biomonitoring studies involving the general population (Al-Jaal et al., 2019; Martins et al., 2019), and thereby human co-exposure is real and constitutes a current public health concern. Moreover, both mycotoxins are nephrotoxic and hepatotoxic and may be carcinogenic to man, although through dissimilar mechanisms of action. This study focused on the cytotoxic and the genotoxic potential of OTA, FB_1 and their mixture in human liver and kidney cell lines, in order to disclose potential interactive effects.

The results revealed a significant dose-related cytotoxic effect of OTA in both cell lines, after 24 hours of exposure. By comparing the IC_{50} values it became apparent that OTA was more toxic for HK-2 cells than for HepG2 cells (8.71 µM vs. 31.50 µM, respectively). The IC_{50} value obtained in HK-2 cells was within the concentration range previously described for mammalian kidney cell lines (9.7 to 37 mM) (Bouslimi et al., 2008; Costa et al., 2016;

Creppy et al., 2004), and the sensitivity of this cell line also agrees with OTA nephrotoxicity (Klarić et al., 2010). Likewise, the IC_{50} value obtained in HepG2 cells was within the wide range reported in the literature, from 8.89 µM to 360 µM (Bouaziz et al., 2008; Corcuera et al., 2011; Guerra et al., 2005; Sobral et al., 2018; Wang et al., 2014). In another cell line representative of the intestine (Caco-2 cell line), OTA was also considered significantly cytotoxic with IC_{50} values ranging from 7.4 µM (Creppy et al., 2004) to 17 µM (Tavares et al., 2013). Even though the reported values clearly fluctuate between studies due to differences associated with methodologies, exposure periods, or cell lines sensitivity, it is generally agreed that OTA represents a health hazard and these findings reinforce the need of a close monitoring of its content in food products.

The analysis of FB_1 toxicity revealed a low potential to cause cell death in the concentration-range tested, either in HK-2 or in HepG2 cells. This observation is consistent with those of other works that also reported a low or inexistent toxicity in porcine kidney (Lei et al., 2013), jejunal epithelial cells (Wan et al., 2013), murine macrophages, Caco-2, HepG2 and bovine kidney cells (Clarke et al., 2014; Sobral et al., 2018). However, positive cytotoxicity results have also been reported, with values of IC_{50} in several *in vitro* models ranging from 31.2 µM to 64.3 µM (Creppy et al., 2004). Similarly to OTA, such contrasting findings are possibly related to variations in techniques and cells or tissues. Nevertheless, and considering the inconsistencies reported in the literature, FB_1 must remain conservatively considered as a reasonable hazard to public health.

To ascertain the combined cytotoxicity of OTA and FB_1, mixtures of these toxins were tested in several concentrations according to a factorial design and two reference mathematical models, the CA and IA models, were applied to explore interactive effects. EFSA has advocated that the CA model can generally be used as the most conservative approach to analyze interactions between chemicals in a mixture, as long as the single components cause a similar adverse outcome (EFSA, 2013), e.g., hepatic or renal toxicity.

Nevertheless, it is useful to consider the MoA of the single chemicals to allow an informed choice of the model and to better interpret the output generated. While both mycotoxins' MoA are complex, it was likely that some interactive effects occurred in co-exposed cells, possibly mediated by the induction of ROS or the depletion of antioxidant defenses. This led to the assumption that the most reasonable mathematical model would be the CA model. However, since each mycotoxin also acts by mechanisms other than oxidative stress, this assumption could be reductionist. Following these mechanistic-driven considerations, instead of making a theoretical choice, both models were applied to better explore the interactions between OTA and FB_1. Modelling of cytotoxicity results led to the same conclusion for the two cell lines: the combinations between the lowest concentrations of mycotoxins caused higher cytotoxic effects than expected from the sum of individual effects, representing a synergistic behavior, shifting to antagonism at higher concentrations. Since the exposure to mycotoxins in real-life scenarios occurs mostly at low doses, these results point to a greater health hazard than expected from the individual effects. Although the actual mechanisms that mediate OTA or FB_1 toxicities are complex and not fully understood, it is known that both can induce oxidative stress and apoptosis. The OTA MoA involves inhibition of protein synthesis (Arbillaga et al., 2007; Kamp et al., 2005), reactive oxygen species production (Arbillaga et al., 2007; Cui et al., 2010; Hadjeba-Medjdoub et al., 2012) and, consequently, lipid peroxidation (Kumar et al., 2014) and oxidative DNA damage (Cui et al., 2010; Hadjeba-Medjdoub et al., 2012; Liu et al., 2012) that may ultimately lead to apoptosis (Cui et al., 2010; Kamp et al., 2005). FB_1 is also able to induce oxidative stress, increasing lipid peroxidation (Kouadio et al., 2005) and decreasing the levels of glutathione in plasma and blood cells (Fodor et al., 2008), although its main mechanism of action relies on the inhibition of the ceramide synthase activity, leading to the depletion of ceramide (which is involved in stress response pathways, such as apoptosis, necrosis or inflammation) and accumulation of sphingosine and sphinganine, which are cytotoxic and inductors of

apoptosis (Bouhet and Oswald, 2007; Lallès et al., 2009; Lessard et al., 2009; Luongo et al., 2008). The disruption of the lipidic metabolism creates an imbalance between the rates of apoptosis and proliferation that is suggested to be a critical determinant in the process of hepato- and nephrotoxicity and tumorigenesis in animal models (IARC, 2002). Thus, it can be hypothesized that the synergistic toxicity at low concentrations may be associated with depletion of the cell antioxidant defenses committing cells to apoptosis. Indeed, OTA activates p53-dependent apoptotic processes, leading to cell cycle disruption and ultimately cell death (Bouaziz et al., 2008), while FB_1 is thought to alter the entire stress response pathway resulting in the activation of programmed cell death too (Bouhet and Oswald, 2007). Therefore, a synergistic interaction at this level is likely to occur. The apparent shift to antagonism at higher concentrations may be due to inherent limitations of the cytotoxicity assay used, since the reduction of MTT is dependent on the mitochondria metabolic activity, and both mycotoxins have been documented to interfere with mitochondrial normal function (Bouaziz et al., 2008; Domijan et al., 2015; Kouadio et al., 2005). Other works addressing OTA and/or FB_1 mixtures produced different conclusions regarding the interactive cytotoxic effects of these two toxins: in MDBK cells, these toxins appear to induce additive effects (Clarke et al., 2014), while in rat brain glioma, Caco-2 and Vero cells, OTA and FB_1 were synergistic (Creppy et al., 2004). Similar shifts from synergism to antagonism have been reported in previous studies involving different combinations of mycotoxins and different cell lines. In a recent study, an interactive cytotoxic effect between FB_1 and patulin was described in Caco-2 cells, changing from synergism to antagonism according to the dose-ratio of the mycotoxins in the mixture (Assunção et al., 2019). In a human hepatocellular cancer cell line (Hep3B), OTA, citrinin and sterigmatocystin were tested simultaneously and the cytotoxic response of the mixture corresponded to synergism at low toxin doses, changing to antagonism at higher concentrations (Anninou et al., 2014). Overall, the wide variety of findings observed among studies regarding mixtures of mycotoxins,

highlights the relevance of more research focused on their MoA, in order to make a more informed choice on the reference model for analysis of interactions and, consequently, allowing to draw firm conclusions regarding interactions between mycotoxins.

The genotoxic potential of single and combined OTA and FB_1 was also analyzed in this study through the alkaline comet assay. The addition of an enzymatic treatment step (FPG-comet assay) allowed to increase the sensitivity of the technique and to measure oxidative lesions in the DNA by transforming oxidative lesions into detectable DNA strand breaks. Those differences, despite reaching statistical significance, were not considered entirely relevant in terms of their biological meaning, given the low level of induced DNA damage. These results agree with those described by Qi et al. (2014) and Assunção et al. (2019) but differ from others showing that OTA is capable of inducing oxidative lesions (Ali et al., 2011; Arbillaga et al., 2007; Bouaziz et al., 2008; Costa et al., 2016). It is possible that the potential to induce these DNA lesions is, once again, dependent on the experimental system used (tissues, culture conditions, and concentration ranges) or on the type of oxidative lesions induced that might not be detectable by the FPG-comet assay. The results also pointed to the fact that FB_1 does not induce DNA damage under the conditions tested, although there are studies showing that FB_1 can cause ROS-induced DNA damage in whole blood (Domijan et al., 2015), Wistar rats (Domijan et al., 2007) and intestinal cells (Kouadio et al., 2005).

Furthermore, none of the combinations of OTA and FB_1 were able to increase the level of DNA damage in comparison with the control. These findings suggested that OTA and FB_1 do not interact at a genotoxic level, under the tested conditions. In the literature, the number of reports discussing genotoxic interactive effects between OTA and FB_1 are scarce, but the existing evidence also describes no interaction between them (Klarić et al., 2010). This absence of interactive effects is plausible given that OTA is genotoxic mainly through

pre-mutagenic guanine specific DNA adducts formation (Pfohl-Leszkowicz and Manderville, 2012), while FB_1 acts through a non-genotoxic mechanism being considered as a tumor promoter (IARC, 2002, 1993). This effect is not ascertained by the common genotoxicity assays, e.g., the comet assay. Nevertheless, a joint effect of OTA and FB_1 on different phases of the cell transformation process towards a malignant phenotype should not be neglected and other approaches should be used to further explore this possibility. In particular, modern high throughput methodologies, e.g., transcriptomics, might provide new mechanistic insights, for instance, into the patterns of differentially expressed genes, pathways and biological functions affected in exposed cells.

5. Conclusions

This study aimed to disclose possible interactive cytotoxic and genotoxic effects of OTA and FB_1 in liver and kidney cells, two cell lines representative of the target organs of these toxins. Regarding cytotoxicity, the results revealed that both toxins individually are cytotoxic and, more importantly, that their combined effect is synergistic in conditions relevant for human exposure. The observed synergism may raise concerns about potential health outcomes from exposure to this mixture, given that they are higher than those predicted from an additive effect. Although neither OTA nor FB_1, individually or in combination, induced genotoxic effects in this study, a possible joint effect at cell transformation level should not be disregarded.

Author Contributions:

Conceptualization, Maria João Silva; Data curation, Maria João Silva; Funding acquisition, Paula Alvito; Investigation, Mariana Pinhão and Ana Tavares; Methodology, Henriqueta

Louro; Project administration, Paula Alvito; Experimental design and mixture toxicity data treatment, Susana Loureiro; Supervision, Maria João Silva; Writing – original draft, Mariana Pinhão, Susana Loureiro and Maria João Silva; Writing – review & editing, Mariana Pinhão, Susana Loureiro, Ana Tavares, Henriqueta Louro and Maria João Silva.

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Highlights:

- Ochratoxin A significantly induced cytotoxicity in HK-2 and HepG2 cells.
- Fumonisin B_1 did not induce cytotoxicity in HK-2 nor HepG2 cells.
- Joint effects of both toxins were assessed through the Concentration Addition and Independent Action mathematical models.
- Synergistic cytotoxic effects were observed when combining low ochratoxin A and fumonisin B_1 concentrations in both cell models.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: