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PRELIMINARY RESULTS ON THE INTERACTIVE EFFECTS OF DEOXYNIVALENOL, ZEARALENONE AND FUMONISIN B_1 ON PORCINE LYMPHOCYTES

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Fusarium mycotoxins, such as fumonisin B_1 (FB_1), deoxynivalenol (DON) and zearalenone (ZEN), frequently co-occur in feed raw materials and their presence is ubiquitous. The aims of this study were to determine the concentration that inhibits cell viability by 50% (IC_{50} values) for each mycotoxin (after 24, 48 and 72 h) and to investigate their combined effects in binary (DON + ZEN: DZ, DON + FB_1: DF, FB_1 + ZEN: FZ) and ternary (DFZ) mixtures using cyto- and genotoxicity on porcine lymphocytes as endpoints. The potency of cytotoxicity of the three toxins in an increasing order was FB_1 < ZEN < DON. The range of IC values depending on the period of exposure was 0.31–0.42 μg/ml and 16.6–22.9 μg/ml for DON and ZEN, respectively, and 101.15 μg/ml for FB_1 (50% viability was reached only after 72 h). The main interaction observed was antagonism regarding cytotoxicity. Lower and higher sets of concentrations were used for the genotoxicity (comet assay) experiments. When lower concentrations were used, antagonism was again the main interaction observed. However, at higher concentrations an antagonism was confirmed only for DFZ, whereas for DZ and FZ a synergism was observed. Interactions of DF were inconsistent in different exposure periods in both series of experiments. Further studies with additional endpoints should be performed (e.g. DNA fragmentation, protein synthesis) in order to elucidate the mechanisms underlying the interactions observed.

Key words: Comet assay, CCK-8 test, peripheral mononuclear cells, *Fusarium mycotoxins*, interactions

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The health effects of mycotoxins have been thoroughly studied and legislation has been implemented on the basis of single mycotoxin exposures in many countries of the world. However, as shown in recent surveys, mycotoxin co-occurrence is very frequent (Monbaliu et al., 2010; Rodrigues and Naehrer, 2012). In the latest survey conducted by BIOMIN GmBH (a commercial feed supplier), over 50% of the samples (feed raw materials) were contaminated with more than one mycotoxin. Furthermore, deoxynivalenol (DON), zearalenone (ZEN) and fumonisins were present in more than 50% of the samples tested worldwide (BIOMIN, 2016). *Fusarium* toxins, and especially fumonisin B_1 (FB_1), DON and ZEN, are the most likely to co-occur in nature. More specifically, the combinations of these mycotoxins present in low concentrations – which is a realistic scenario – should be investigated.

FB_1 is mainly produced by *Fusarium verticillioides* while the commonest main producer of DON and ZEN is *F. graminearum* (Pestka, 2010). The main mechanism of action of fumonisins is the disruption of sphingolipid metabolism due to their structural similarity (Voss et al., 2007). Trichothecenes inhibit protein synthesis by binding to the 60s ribosomal subunit of the cell (Ueno, 1984). Despite its low acute toxicity in comparison with other trichothecenes (i.e. T-2 toxin), DON has been widely studied since it is the commonest contaminant of cereal grains worldwide (BIOMIN, 2016). ZEN has a chemical structure similar to oestrogens, and thus it binds antagonistically to oestrogen receptors. Through this antagonistic binding, ZEN causes reproductive disturbances in both male and female animals, with swine being the most sensitive species (Minervini and Dell'Aquila, 2008). Besides its oestrogenic effects, ZEN is known to exert cytotoxic and genotoxic effects and to induce an immune response (Abid-Essefi et al., 2004; Vlata et al., 2006; Marin et al., 2010; Gao et al., 2013).

There are only few studies on the interactions of mycotoxins in binary (Ficheux et al., 2012; Bensassi et al., 2014; Cortinovis et al., 2014) and especially in ternary mixtures (Kouadio et al., 2007; Wan et al., 2013*a,b*). Therefore, the aim of this study was to determine the concentration that inhibits cell viability by 50% (IC_{50} values) of FB_1, DON and ZEN and to investigate their combined cyto- and genotoxic effects exerted on porcine lymphocytes in low concentrations. To the best of our knowledge, this is the first study on the interactions of FB_1, DON and ZEN on porcine lymphocytes.

Materials and methods

Chemicals

FB_1, DON and ZEN were purchased from Sigma Aldrich (Schnelldorf, Germany) and Cayman Chemical (Ann Arbor, Michigan, USA). The toxins were dissolved in pure ethanol (stock solutions). CCK-8 solution was provided by Do-

jindo EU GmbH (Munich, Germany). All other chemicals used were supplied by Sigma Aldrich (Schnelldorf, Germany).

Isolation and enumeration of lymphocytes

Blood was taken from the *v. cava cranialis* of healthy adult pigs. Blood (3 ml) was mixed with an equal volume of culture medium (RPMI-1640) in a 12-ml centrifuge tube. The mixture was gently overlaid onto an equal quantity of Histopaque 1077 (6 ml) and centrifuged for 30 min at 3000 rpm. The interface layer (mononuclear cells) was carefully transferred with a sterile Pasteur pipette into a new centrifuge tube, suspended in 12 ml RPMI-1640 and centrifuged at 4000 rpm for 20 min. After removing the supernatant, the cells were resuspended in fresh medium (12 ml) and centrifuged at 4000 rpm for 15 min. Then the cells were gently resuspended in 10 ml of complete cell medium [CCM; RPMI supplemented with penicillin and streptomycin solution (1%) and fetal bovine serum (10%)].

The cell suspension (100 µl) was mixed with an equal volume of 0.4% trypan blue and allowed to stand at room temperature for 2 min. Using a sterile pipette, a small amount (10 µl) was then transferred to both chambers of a Neubauer haemocytometer, covered with a cover slip and counted under light microscope. The cell viability (%) was calculated as: total number of viable cells × 100/total number viable plus non-viable cells. The cell number was adjusted to 2×10^6 cells/ml and $4–5 \times 10^6$ (minimum 95% cell viability) for cytotoxicity and genotoxicity assays, respectively.

Determination of IC_{50}

Preliminary experiments (n = 3) were performed to establish the IC_{50} value for each mycotoxin. Five concentrations and three exposure periods (24, 48 and 72 h) were used. The concentration ranges were chosen on the basis of data reported in the literature (Minervini et al., 2004; Cetin and Bullerman, 2005; Vlata et al., 2006; Maenetje et al., 2008; Mwanza et al., 2009) as well as the results of preliminary experiments (data not shown). The concentration ranges were 50–150 µg/ml, 0.07–0.84 µg/ml and 1–50 µg/ml for FB_1, DON and ZEN, respectively.

Combination experiments

The mycotoxins were tested in binary and ternary mixtures (n = 3) in low concentrations (below the IC_{50}) to assess interactive effects, if any. Binary and ternary mixtures of the toxins were investigated at 5 µg/ml (FB_1 and ZEN) and 0.07 µg/ml (DON) for cytotoxicity and genotoxicity. Higher concentrations (below the IC_{50}) were used for a second series of genotoxicity experiments (25, 0.21 and 10 µg/ml for FB_1, DON and ZEN, respectively). The combinations tested were: DON + FB_1 (DF), DON + ZEN (DZ), FB_1 + ZEN (FZ), DON + FB_1 + ZEN (DFZ).

Cytotoxicity assay

The conversion of the tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to MTT formazan in living cells is used for the determination of cell viability and proliferation (Mossman, 1983). In our study a water soluble tetrazolium salt was used [WST-8; cell counting kit-8 (CCK-8)]. The use of a water-soluble tetrazolium salt eliminates the additional step of crystal solubilisation. In addition, the detection sensitivity of CCK-8 is higher and its toxicity is lower than that of MTT.

After isolation the cell culture was subdivided in a 96-well microtitre plate with each well containing 100 µl of medium (2×10^5 cells per well). After 24-h incubation, 20 µl of phytohaemagglutinin (PHA) and 100 µl of mycotoxin or solvent were added to the wells. Control cells were treated with mycotoxin-free vehicle (0.9% NaCl sterile distilled water). Each treatment had 5 replicates and the incubation times were 24, 48 and 72 h. After each incubation period, 22 µl of CCK-8 was added to the wells and after 4-h incubation the absorbance was read at 450 and 620 nm under a Microplate Reader (DIALAB GmbH, Wiener Neudorf, Austria).

Genotoxicity (comet) assay

After isolation the cell culture was subdivided (100 µl) into microcentrifuge tubes which were incubated overnight in a humidified incubator at 37 °C and 5% CO_2. Then toxin or vehicle/carrier solutions were added to the tubes and were incubated for 24 h, 48 h and 72 h. After each incubation period the procedure described by Horvatovich et al. (2013) was followed, with slight modifications. The slides were washed with phosphate-buffered saline (PBS) instead of neutralising buffer and ethidium bromide (EtBr) was used instead of DAPI for staining the slides.

Calculations and statistical analysis

Dose-response curves were fitted to the best linear or non-linear models and the concentration that induced 50% loss of viability (IC_{50}) was calculated using the software OriginPro version 9.0 (OriginLab, Northampton, MA, USA).

The values obtained in the combination experiments were compared with expected values to detect any significant differences. Expected values were calculated as a mean value obtained after exposure to one substance alone plus a mean value obtained after exposure to a second or a third substance (Klaric et al., 2007):

Mean % (expected for myc1 + myc2) = mean % (myc1) + mean % (myc2) – 100% control

Mean % (expected for myc1 + myc2 + myc3) = mean % (myc1) + mean % (myc2) + mean % (myc3) – 100% control

Calculation of expected SD/SEM:

SD (expected for myc1 + myc2) = $[(\text{SD for myc1})^2 + (\text{SD for myc2})^2]^{1/2}$

SD (expected for myc1 + myc2 + myc3) = $[(\text{SD for myc1})^2 + (\text{SD for myc2})^2 + (\text{SD for myc3})]^{1/2}$.

The results were interpreted according to Klaric et al. (2007) as follows: *(i)* an additive effect was recorded if the measured values were not significantly above or below the expected values; *(ii)* a synergistic effect was recorded if the measured values were significantly above (tail intensity) and below (cell viability) the expected values; *(iii*) an antagonistic effect was recorded if the measured values were significantly below (tail intensity) and above (cell viability) the expected values.

Analysis and scoring for comet slides were performed with Epifluorescent Microscope (B600 TiFL) and Comet IV (version 4.3.1) software (Perceptive Instruments Ltd, Bury St Edmunds, UK). One hundred cells/gels (i.e. 400 cells/ concentration) were investigated during the analysis. The cells were classified by software analysis according to tail intensity (% tail DNA).

The statistical difference between observed and expected values was calculated with unpaired *t*-test.

Results

Cytotoxicity experiments

Determination of IC_{50}. A time- and dose-dependent decrease in cell viability was observed for all three toxins (Fig. 1). For DON and ZEN IC_{50} values could be calculated for all incubation times (Table 1). In contrast, FB_1 decreased cell viability by 50% only after 72 h. The potency of cytotoxicity in an increasing order was FB_1 < ZEN < DON.

Combined effects of binary and ternary mixtures. The mixtures of FB_1, DON and ZEN provided antagonism which was more evident after 72 h of exposure (Fig. 2). After 24 h of incubation the binary mixtures did not show interactions, whereas the ternary (DFZ) mixture exhibited a significant antagonism which was expressed for all incubation times. After 48 h an antagonistic effect was revealed for the binary mixtures of DON (DF and DZ), which was more expressed after 72 h. After 72 h an antagonistic effect was evident for mixtures of FZ as well. The potency of the mixtures after 24 and 48 h of incubation was DF < DZ < DFZ < FZ, whereas after 72 h the order was reversed as DFZ < FZ < DF < DZ.

Genotoxicity (comet assay) experiments

When using mixtures with lower concentrations of mycotoxins, an antagonism was observed for most of the mixtures in all exposure periods (Fig. 3a).

After 24 h an antagonism was observed for all mixtures but it was statistically significant only in the case of the ternary mixture. After 48 h, DZ and DFZ exerted an antagonistic effect but a statistically significant antagonism was found only for DFZ; DF displayed a synergistic effect, while FZ showed no interaction. After 72 h, all four mixtures showed an antagonism but it was statistically significant only for DF.

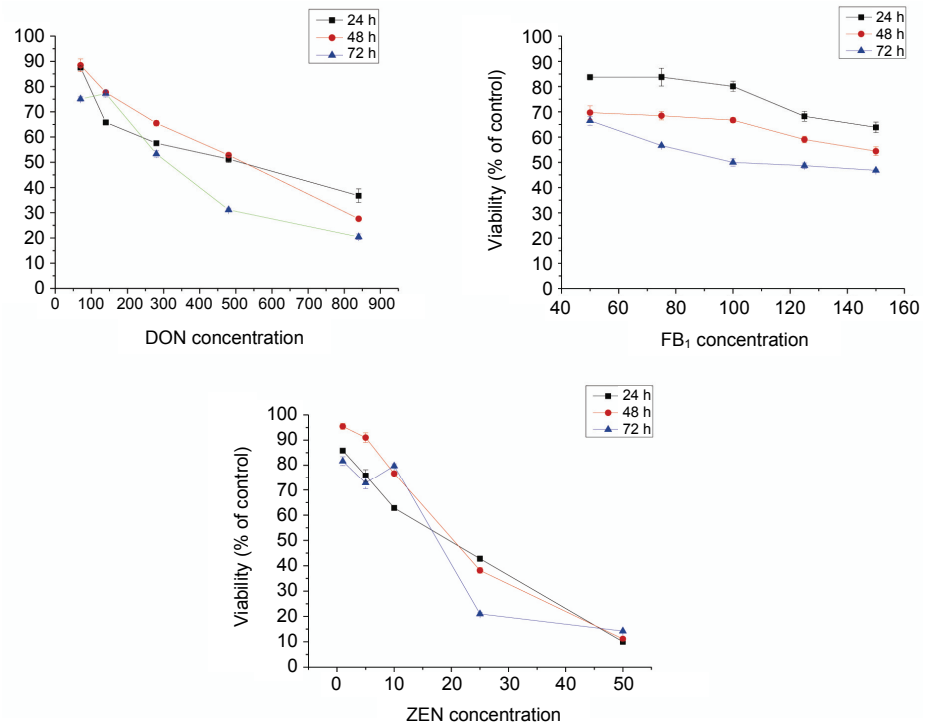

Fig. 1. Decrease of cell viability of porcine lymphocytes, determined by the CCK8 test, after 24, 48 and 72 h of incubation with FB_1 (μg/ml), DON (ng/ml) or ZEN (μg/ml)

Table 1

IC_{50} (mean ± SD) values calculated on porcine lymphocytes after 24 h, 48 h and 72 h of exposure to FB_1 (5 μg/ml), DON (0.07 μg/ml) or ZEN (5 μg/ml)

	IC_{50} (μg/ml)		
	24 h	48 h	72 h
FB_1	NA	NA	101.15 ± 7.80
DON	0.43 ± 0.02	0.41 ± 0.02	0.31 ± 0.01
ZEN	19.55 ± 1.20	20.60 ± 1.07	16.60 ± 2.05

NA: not applicable

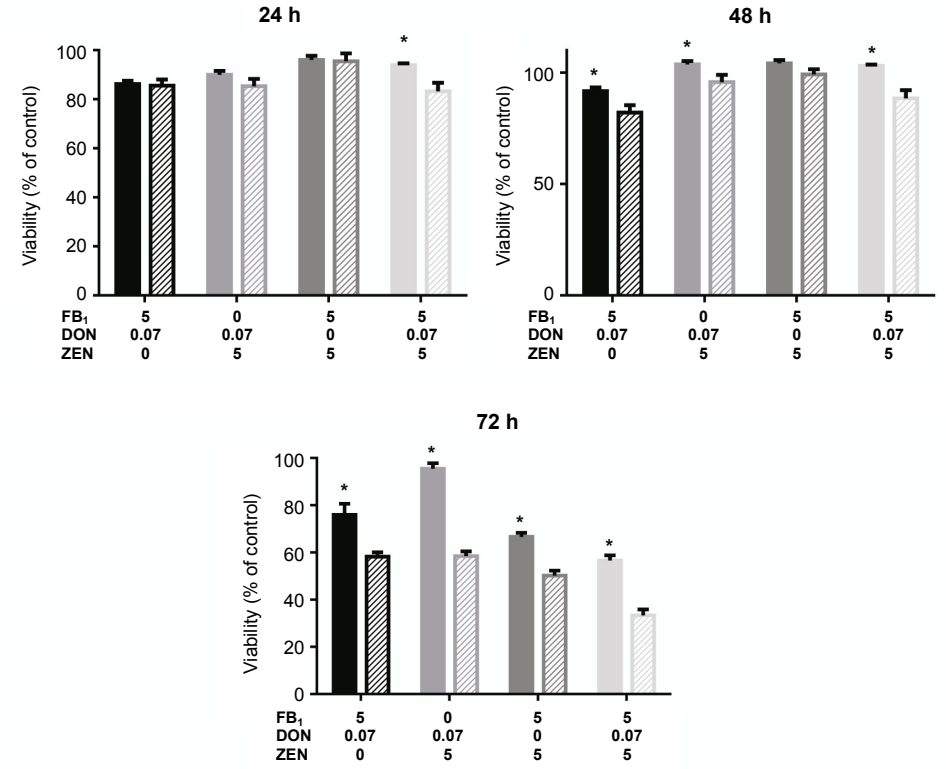

Fig. 2. Cytotoxic effects of binary and ternary mixtures of FB_1, DON and ZEN (μg/ml) on porcine lymphocytes after 24 h, 48 h and 72 h of incubation, as determined by the CCK8 test (sketched bars represent the calculated expected values; viability is expressed as percent of the control); $^{*}P < 0.05$

In order to obtain more pronounced effects, increased concentrations of mycotoxins (Table 2) were used for the further investigation of interactions. The concentrations used were 25, 0.21 and 10 μg/ml for FB_1, DON and ZEN, respectively. After 24 h, a significant antagonism was revealed by the mixtures of DF and DFZ, while DZ demonstrated a non-significant synergism and FZ showed no interaction (Fig. 3b). After 48 h the trend was the same for the mixtures of DZ and DFZ, while DF showed no interaction and FZ exerted an antagonistic effect which was not statistically significant. After 72 h an antagonism was expressed from the mixtures of DF and DFZ (non-significant and significant, respectively). On the other hand, the mixtures of DZ and FZ showed significant synergism.

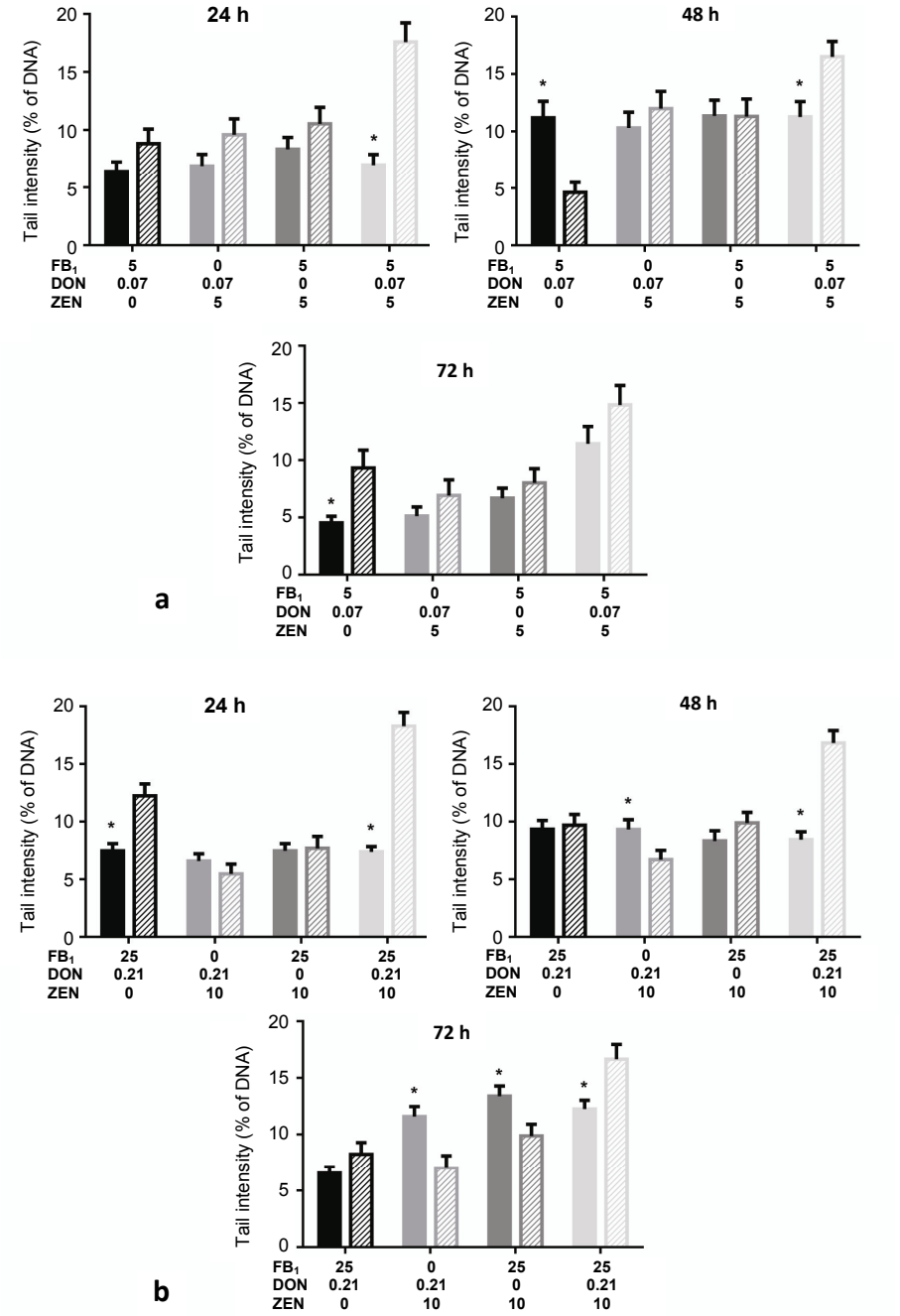

Fig. 3. Genotoxic effects of binary and ternary mixtures of DON, FB_1 and ZEN in lower (3a) and higher (3b) concentrations (μg/ml) on porcine lymphocytes after 24 h, 48 h and 72 h of incubation (sketched bars represent the calculated expected values; viability is expressed as percent of the control); $^{*}P < 0.05$

Table 2

IC_{50} (mean ± SD) values calculated on porcine lymphocytes after 24 h, 48 h and 72 h of exposure to FB_1 (5 μg/ml), DON (0.07 μg/ml) or ZEN (5 μg/ml)

Cell treatment	Tail intensity		
	24 h	48 h	72 h
Concentrations	5, 0.07, 5 μg/ml (FB_1, DON, ZEN)		
Control	6.21 ± 0.73	5.11 ± 0.53	5.39 ± 0.65
FB_1	7.98 ± 0.93	4.52 ± 0.66	7.91 ± 1.00
DON	7.05 ± 0.85	5.21 ± 0.60	6.81 ± 1.17
ZEN	8.76 ± 1.08	11.89 ± 1.37	5.50 ± 0.72
FB_1 + DON	6.41 ± 0.83	11.20 ± 1.42	4.53 ± 0.58
DON + ZEN	6.89 ± 1.01	10.31 ± 1.36	5.12 ± 0.80
FB_1 + ZEN	8.34 ± 1.03	11.36 ± 1.37	6.70 ± 0.86
FB_1 + DON + ZEN	6.97 ± 0.91	11.27 ± 1.33	11.45 ± 1.50
Concentrations	25, 0.21, 10 μg/ml (FB_1, DON, ZEN)		
Control	11.17 ± 0.68	7.36 ± 0.99	8.20 ± 0.82
FB_1	12.81 ± 0.65	10.12 ± 0.74	9.65 ± 0.68
DON	10.60 ± 0.85	6.94 ± 0.57	6.79 ± 0.77
ZEN	6.05 ± 0.55	7.13 ± 0.55	8.42 ± 0.79
FB_1 + DON	7.47 ± 0.62	9.34 ± 0.76	6.54 ± 0.57
DON + ZEN	6.59 ± 0.88	9.33 ± 0.84	11.50 ± 0.88
FB_1 + ZEN	7.74 ± 0.90	8.32 ± 0.89	13.40 ± 0.89
FB_1 + DON + ZEN	7.39 ± 0.94	8.44 ± 0.68	12.26 ± 0.76

Discussion

Although the number of studies with mycotoxin combinations tends to increase, there are still only few studies with the mixtures (and especially the ternary combinations) of FB_1, DON and ZEN.

In the present study DON was the most potent among the three mycotoxins investigated, with an increasing potency order of FB_1 < ZEN < DON, which is consistent with the results of Wan et al. (2013*a*) on swine jejunal epithelial cells. Kouadio et al. (2007) previously reported that the potency of FB_1, DON and ZEN on Caco-2 cells was ZEN > DON > FB_1 in decreasing order, confirming that FB_1 has low toxicity towards several cell lines.

From our experiments the calculated IC_{50} value was 101.15 μg/ml for FB_1 after 72-h exposure; on the other hand, Kouadio et al. (2007) could not calculate an IC_{50} value for FB_1 (the highest concentration used was 150 μM which is approximately 108 μg/ml) even after 72 h. In the study of McKean et al. (2006) only concentrations higher than 100 μM (72 μg/ml) exerted a cytotoxic effect (24-h exposure) to HepG2 and BEAS-2B cells, and the IC_{50} values were 399.2 μM

(288 μg/ml) and 355.1 μM (256 μg/ml), respectively. These IC_{50} values are 2.5-fold higher than those found in our study, which could be attributed to the higher sensitivity of porcine lymphocytes to FB_1. The lowest IC_{50} value for DON was 0.31 μg/ml after 72 h in our experiments, which is in total agreement with the findings of Goyarts et al. (2006*a,b*) who investigated the effect of DON on the proliferation of porcine blood lymphocytes using MTT assay. In the study of Meky et al. (2001) the IC_{50} value of DON on human lymphocytes was 0.216 μg/ml, which is significantly lower than the IC_{50} calculated in our study. This difference could be attributed to the longer exposure period (5 days). In our study the IC_{50} value of ZEN was 19.55 μg/ml after 24 h and decreased to 16.6 μg/ml after 72 h of exposure. According to the literature it appears that lymphocytes are less sensitive to ZEN than other cell lines, such as Vero kidney or Caco-2 cells. In the study of Abid-Essefi et al. (2004) the cytotoxic potential of ZEN was determined by the MTS assay (a modified version of MTT) using Vero kidney and Caco-2 cells and the determined IC_{50} values were 7 μM and 15 μM (2.2 μg/ml and 4.8 μg/ml), respectively. Surprisingly, Chinese hamster ovarian cells (CHO-K1) were less sensitive to ZEN with IC_{50} values of > 100 μM (31.8 μg/ml), 60.3 μM (19.2 μg/ml), 68 μM (21.6 μg/ml) for 24-h, 48-h and 72-h exposure, respectively (Tatay et al., 2014).

In the present study, combinations of FB_1, DON and ZEN resulted in antagonistic effects regarding cytotoxicity. The ternary mixture (DFZ) exerted significant antagonism after all incubation periods, which was most pronounced after 72 h. A similar trend was observed for the rest of the mixtures which exerted interactive effects (FZ) or increased the degree of interaction (DF and DZ) in a time-dependent manner. Ficheux et al. (2012) used myelotoxicity as an endpoint and observed an antagonistic effect of the mixture of DON and FB_1 on haematopoietic progenitor cells. Another study investigating the effects of binary and ternary mixtures of DON, FB_1 and ZEN on porcine intestinal (jejunal) epithelial cells demonstrated antagonistic effects of the mixture when the lowest dose was used (Wan et al., 2013*a*). DON and ZEN were tested in combination on human colon carcinoma cells and their effects were sub-additive for all the endpoints used (cell viability and cycle, mitochondrial inner membrane potential and permeability transition pore opening) (Bensassi et al., 2014). On the contrary, Dąbrowski et al. (2016) reported a synergistic effect of DON and ZEN in porcine subpopulations of lymphocytes. Lymphocytes were isolated from pigs after dietary exposure to no observed effect level (NOAEL) doses of DON and ZEN. The immunomodulatory effect that the single mycotoxins exerted was stronger when they were combined. Kouadio et al. (2007) observed additive effects for the mixtures of DF and DFZ but an antagonistic effect for FZ. Kouadio et al. (2013) reported an antagonistic effect for FB_1 and ZEN for lysosomal and mitochondrial damage but an additive effect for necrosis.

FZ was the most cytotoxic among the binary mixtures, and DFZ was significantly antagonistic at all incubation time points. The order of cell viability for mixtures (in an increasing order) was consistent after 24 and 48 h of exposure (DF < DZ < DFZ < FZ) but it was reversed after 72 h (DFZ < FZ < DF < DZ). This phenomenon could be due to the delayed cytotoxic effect of FB_1 in a similar manner as with the single exposure (IC_{50} calculated for 72 h only). Differences found in the interactions between different time points indicate the difficulty to foresee the effects of chronic multimycotoxin exposure. Kouadio et al. (2007) reported that the reduction of cell viability as assessed by the neutral red (NR) test was FZ < DF < DZ < DFZ in increasing order. These results are partly consistent with the findings of the present study regarding DFZ and DF combinations, whereas DZ and FZ showed opposite results. In the study of Wan et al. (2013*a*) the binary and ternary mixtures of FB_1, DON and ZEN were cytotoxic although non-cytotoxic concentrations of the individual mycotoxins were used. The least toxic mixture was FZ after 48 h of incubation, like in our study. From the data of the present study it can be concluded that in the case of porcine lymphocytes ZEN does not determine the interactions of the binary mixtures as strongly as does FB_1 and/or DON.

To the best of our knowledge there are no studies addressing the combined genotoxic effects of FB_1, DON and ZEN. Two different series of experiments were performed using a set of lower and higher concentrations, respectively. When the lower set of concentrations (i.e. 5, 0.07 and 5 μg/ml for FB_1, DON and ZEN, respectively) were used, antagonism was the main interaction observed. The ternary mixture showed a significant antagonism after 24 h and 48 h but the antagonism became moderate after 72 h. Occasionally the interactions vary among different exposure times, like in the case of the DF mixture which exerted antagonistic effects after 24 h and 72 h and synergistic effects after 48 h of exposure. DZ showed a non-significant antagonism for all exposure times.

Since the DNA damage observed was only slightly higher than the normal range for control cells, following the proposition of Gopalan et al. (2011) higher concentrations were used (25, 0.21 and 10 μg/ml for FB_1, DON and ZEN, respectively) in order to confirm/reject the interactions observed. The antagonistic effect of DFZ was confirmed. It has been observed that the antagonism exerted by the combinations of mycotoxins in lower concentrations can convert to synergism in higher concentrations (Alassane-Kpembi et al., 2015). Likewise, the effects of DZ and FZ were synergistic in a time-dependent manner. Like in the experiment performed with lower concentrations, the DF mixture showed inconsistency among different exposure periods. It can be concluded that ZEN acts synergistically with DON and FB_1, especially after a prolonged exposure (72 h). However, this synergism was not exerted when all three mycotoxins were combined. This hypothesis should be further investigated since there are no con-

firmed data on the mechanism of genotoxicity for any of the three toxins on porcine lymphocytes.

Yang et al. (2014) investigated the expression of a heme oxygenase gene (HO-1) in human lymphocytes after exposure to DON. A reduction in the expression of HO-1 was observed and it was suggested that this could probably be related to the mechanism of action. Human lymphocytes which were exposed to FB_1 showed DNA strand breaks only at the highest concentration (i.e. 20 μg/ml) (Domijan et al., 2015). These results suggest that FB_1 exerts weak genotoxicity, which is in accordance with our results since FB_1 reached a maximum tail intensity of 12.8%. Although it was stated that the exact mechanisms responsible for the genotoxicity of FB_1 are not clear, the authors suggested that oxidative stress could account for the genotoxic effects, at least partially (Domijan et al., 2015). Oxidative stress mediated genotoxicity is has been suggested by Theumer et al. (2010), but the authors could not explain the exact mechanisms for the observed antagonistic effect of FB_1 against aflatoxin B1. According to several authors, ZEN induces DNA damage through oxidative stress (Abid-Essefi et al., 2003; Bouaziz et al., 2013). However, Gao et al. (2013) suggested that oxidative stress does not play a key role in ZEN-induced DNA strand breaks on human embryonic kidney cell line, and they showed that the lysosome is the main determining factor for the ZEN-induced DNA strand breaks.

In conclusion, the mixtures of DON, FB_1 and ZEN exert mostly antagonistic effects on porcine lymphocytes with the interactions being more evident after 72 h of exposure. Further studies with additional endpoints (e.g. DNA fragmentation, protein synthesis) should be performed in order to elucidate the mechanisms underlying the interactions observed. Furthermore, an antioxidant agent such as vitamin E could be used in combination with these mycotoxins in order to assess whether oxidative damage plays a significant role in genotoxicity.

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