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Full Length Research Paper

Interactive effect of Zeralenone and Fumonisin B_1 on caspace-3 kinetic activity, lactate dehydrogenase leakage, malondialdehyde levels and viability in Caco-2 cells

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Several *Fusarium* toxins are often found in combination in infested cereal grains such as peanuts, rice, wheat, maize and sorghum. Our previous study reported co-occurrence of *Fusarium* toxins fumonisin B_1 (FB_1) and Zearalenone (ZEA) in all food analyzed from Côte d'Ivoire namely rice, maize, peanut and millet. However, a few studies have been reported that address the toxicity of *Fusarium* toxins mixtures. In our preliminary previous study on combination of FB_1 and ZEA on human intestinal cell line Caco-2 results indicated that their interactive effects seemed to be antagonist effect. The aim of the present study was to investigate in the FB1+ZEA-induced antagonist effect on intestinal cells line Caco-2 using several cellular endpoints including Caspace-3 activity modulation, malonedialdehyde (MDA), cells viability as evaluated by lysosome and mitochondria integrities and cell lactate dehydrogenase (LDH) leakage. Taken together, our results were contrasted. Concerning lysosome and mitochondria integrities, combined effect of binary mycotoxins is an antagonist effect but by LDH release as a measure of cytoplasmic leakage, the interactive effect seems to be an additive effect. MDA production induced by ZEA+FB_1 was more additive effect but not synergistic effect. Caspace-3 activity modulation by ZEA+FB_1 after 6 and 24 h of incubation toxins with cells was additive effect, but after 3 h of incubation ZEA have a tendency to reduce the effect of FB_1. Our results suggest that combined effects of binary *Fusarium* toxins ZEA and FB_1 in cell line Caco-2 were unpredictable and varied according to several parameters such as the cellular endpoints and the duration of cells incubation with toxicants.

Key words: *Fusarium* toxins, interactive effect, caspace-3 kinetic activity, cells viability, malonedialdehyde (MDA), production.

INTRODUCTION

Zearalenone (ZEA) and Fumonisin B_1 (FB_1) are secondary metabolites of some toxigenic species of *Fusarium* genera. They pose a health risk not only to humans but also to livestock and, as a consequence, may cause economical losses either by unfavorable effects on domestic animals themselves or by an increased potential for health effects in human beings from consuming mycotoxin-contaminated edible animal

products. FB_1 causes liver and kidney cancer, and neural tube defects in rodents, leukoencephalomalacia in horses and pulmonary oedema in pigs (Dutton, 1996). Of major concern is the association of FB_1 with elevated incidence of human oesphageal cancer in parts of South Africa, North Eastern Iran and China, upper gastrointestinal tract cancer in Northern Italy (Chu and Li, 1994; Rheeder et al., 1992; Sydenham et al.1990) and neural tube defects in human babies (Hendricks, 1999; Marasas et al., 2004). The structures of FB1 and sphingolipids show marked similarities (Bezuidenhout et al., 1988), which may be the reason why FB_1 drastically disrupts the normal sphingolipid metabolism leading to an intracellular accumulation of sphingoid bases (mainly sphinganine relative to sphingosine), which mediate several key biological processes including inhibition of protein, DNA synthesis and apoptosis caspace-3 dependant (Abado-Bécognée et al., 1998; Soriano et al., 2005; Seefelder et al., 2003; Gopee and Sharma, 2004). In addition, FB_1 also induces lipid peroxidation in Vero cells, in primary rat hepatocytes (Abado-Bécognée et al., 1998) in C6 glioma cells (Mobio et al., 2003) and human intestinal Caco-2 cells (Kouadio et al., 2007).

On the other hand, ZEA have estrogenic and anabolic activities in several species (rodents, pigs and monkeys) (Kuiper-Goodman et al., 1987; Etienne and Dourmad, 1994), being able to cause alterations in the reproductive tract of laboratory animals (Kuiper-Goodman et al., 1987; Abid-Essefi et al., 2004). ZEA is associated with outbreaks of precocious pubertal changes in children in Puerto Rico, and has been suggested to have a possible involvement in human cervical cancer (Zinedine et al., 2007). Cellular mechanism of ZEA has been described by its high binding affinity to oestrogen receptors (Shier et al., 2001) and DNA adduct formation in female mousse tissues (Pfohl-Leszkowicz et al., 1995) and carcinogenic disorders (NTP, 1982). It has also been reported that ZEA inhibits DNA and protein synthesis and induces oxidative stress mediated cell death (Abid-Essefi et al., 2003; Ouanes et al., 2005; Kouadio et al., 2005, 2007). Consequently, the intracellular generation of reactive oxygen species (ROS) by ZEA is likely responsible for its cytotoxic and genotoxic effects (Hassen et al., 2007). ZEA causes cells death by apoptosis via caspase-independent and mitochondria/AIF-mediated pathways with a key role of activations of p53 and JNK/p38 (Yu et al., 2011).

ZEA and FB_1 could be found in the same commodities as secondary metabolites of different *Fusarium* species (Scudamore et al., 1998; Desjardins et al., 2000; Dawlatana et al., 2002). Our previous study reported co-occurrence of *Fusarium* mycotoxins FB_1 and ZEA in all food analyzed from Côte d'Ivoire such as rice, maize peanut (Sangare et al., 2006). Thus, in preliminary study on possible interactive effect of combination of FB_1 and ZEA, we reported cytotoxic effect as evaluated by cell membrane transport integrity of co-occurrence of binary FB_1 + ZEA seemed to be antagonist effect but we observed additive effect on lipid peroxydation (Kouadio at al., 2007). In order to understand the best possible interactive effect of these *Fusarium* toxins, we investigated in their combined effect regarding several cellular endpoints.

MATERIALS AND METHODS

Chemicals

ZEA and FB_1 were obtained from Sigma Chemical Company (St Louis, MO, USA) and were dissolved in ethanol/water (90:10). Dulbecco's Modified Eagle Medium (DMEM), foetal calf serum (FCS) and neutral red (NR) solution were provided from Sigma-Aldrich (Saint Quentin Falavier, France). All other chemicals used were of analytical grade and provided by Sigma-Aldrich (Saint Quentin Falavier, France).

Cell culture and treatment

Caco-2 cells, a human colon cancer cell line, were obtained from Dr. Jing Yu, Tufts School of Medicine (Medford, MA, USA) (Rousset et al., 1985). The cells were grown as monolayer culture in a high glucose concentration (4.5 g/l) DMEM medium supplemented with 10% foetal calf serum (FCS), 8 mM L-glutamine, 1% of mixture penicillin (100 IU/ml) and streptomycin (100 µg/ml) incubated at 37°C in an atmosphere of 5% CO_2—95% air mixture. For cell counting and subculture, the cells were dispersed with a solution of 0.05% trypsin and 0.02% EDTA.

Cytotoxicity assay by NR test

The NR test was performed to assess cytotoxicity, as described by Kouadio et al. (2005). Viable cells actively transport this dye across their cell membrane; therefore, after subsequent lyses absorbance can be used as a measure of cell viability. The solution stock of NR (3.3 g/l) was diluted to 1/100 in the cell culture medium and the extract solution consisted of 50% (v/v) ethanol in Milli-Q water with 1% (v/v) acetic acid. After 72 h of incubation in presence of each mycotoxin alone and their mixture (ZEA+FB1) or the vehicle, 150 ml of freshly prepared NR solution pre-warmed to 37°C was added to each well and all plates returned to the incubator at 37°C for 4 h. The cells were washed two times and 150 µl of the extract solution were added in each well and plates were shaken for 15 min. The absorbance at 540 nm was determined using a Microplate Reader DYNATECH MR 4000 manufactured by DYNATECH and provided by MICROPLATE in Business & Industrial, Healthcare, Lab & Life Science.

Cytotoxicity assay by tetrazolium-based colorimetric assay (MTT test)

MTT test was used to assess cell viability based on the capacity for

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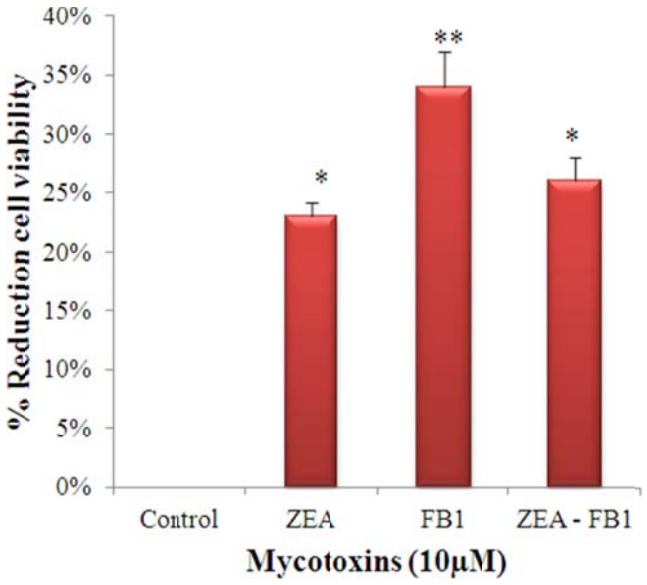

Figure 1. Cytotoxicity effect of ZEA (10 µM), FB_1 (10 µM) or their mixture on Caco-2 cells after 72 h incubation evaluated by MTT test. Results are given as mean ± SD from three independent experiments. *Different from control at $p < 0.05$ and **$p < 0.001$.

viable cells to metabolise a tetrazolium colourless salt to a blue formazan in mitochondria (Kouadio et al., 2005). After 72 h of incubation in presence of toxins ZEA, FB_1 and their mixture or the vehicle, 100 µl of 0.5% solution of thiazolyl blue tetrazolium bromide (MTT) were added to each well and 2 h later the medium was eliminated. Subsequently, 100 µl of dimethyl sulfoxide (DMSO) were added to the wells to extract the formazan formed in the viable cells. After 5 min of continuous stirring, the absorbance was determined at 540 nm using a Microplate Reader DYNATECH MR 4000. The absorbance is proportional to the number of viable cells.

Cytotoxicity assay by lactate dehydrogenase measure (LDH assay)

The LDH assay was performed to assess cytotoxicity, as described by Yusup et al. (2005). Caco-2 cells (1 x 10^5 cells/ml/well) were preincubated in 24-well multidishes for 24 h at 5% CO_2 - 95% air at 37°C. Cell viability was assessed by LDH leakage through the membrane into the medium. After 48 h of incubation in presence of mixture of toxins (ZEA and FB_1) or the vehicle, cells supernatant were used for the presence of LDH by LDH assay kit (Biomerieux, Lyon, France). In this test, three wells were used for each mycotoxin (ZEA and FB_1) and their mixture. The amount of LDH measured is related to the protein content of cellular homogenates, determined using the colorimetric method of Bradford (1976). Leakage was expressed as percentage difference from controls.

Extraction and determination of malonedialdehyde (MDA)-thiobarbituric acid (TBA) adduct

Cells (10^5 cells/ml) were cultured in 24-well multidishes (Polylabo, France) for 24 h at 37°C as described above, and then cultures were incubated in the presence of each mycotoxin alone and their mixture ZEA+FB1 for 24 h at 37°C. After this incubation, cells were trypsinised, centrifuged and resuspended in SET buffer (0.1 M NaCl, 20 mM EDTA, 50 mM Tris-HCl, pH 8.0). As described by Ennamany et al. (1995) and Abado-Bécognée et al. (1998), extraction and determination of the MDA- TBA adduct by HPLC and fluorimetric detection after extraction in n-butanol (50 µl injected for analysis) was performed. The amount of MDA measured is related to the protein content of cellular homogenates, determined using the colorimetric method of Bradford (1976).

Caspace-3 activity assay

The assay was performed according to the manufacturer's instructions, Promega, USA. After 3, 6 or 24 h of incubation at 37°C in the presence of each mycotoxin alone or their mixture ZEA+FB_1, cells (10^6 cells/ml) were disrupted by incubation ice-cold lysing buffer for 10 min and then centrifuged at 15,000 x *g* for 20 min. Supernatants (cell extracts containing caspase-3) were retrieved and 50 µl aliquots (100-200 µg total protein) along with Ac-DEVD substrate labeled with the chromophore p-nitrianiline (pNA) were added in a 96-well flat bottomed microplate. In presence of active caspase-3, cleavage and release of pNA from substrate occurs. Free pNA produced a yellow color that can be detected by spectrophotometer Microplate Reader DYNATECH MR 4000 at 405 nm. Additional controls, some free from cell lysates and others lacking substrate, were included. The results were expressed as caspase-3 specific activity (IU/mg protein).

Statistical analysis of data

The data are expressed as mean standard deviation (SD) for at least three independent determinations in triplicate or quadruplicated for each experimental point. The statistical differences between treated groups and control groups were determined by Student's t-test using SPSS 11.19 statistical software, and $p = 0.05$ was considered the limit for significance.

RESULTS AND DISCUSSION

Cytotoxicity assay by MTT test

The viability of Caco-2 cells measured with MTT tests after incubation of either individual mycotoxin FB_1 or ZEA or their mixture revealed diminishing of cell viability (Figure 1). ZEA (10 µM) reduced slightly cell viability by about 20-25%, which is lower than 10 µM FB_1 does (35-38%). Thus, ZEA appears weakly cytotoxic. Strikingly, FB_1 and ZEA mixed did not affect cell viability higher than FB_1 alone (Figure 1). Indeed, combined effect observed of these toxins is lower than expected additive effect of their mixture.

Cytotoxicity assay by NR test

The measure of Caco-2 cells viability with Neutral Red tests after incubation of mycotoxin FB_1 or ZEA alone or their mixture revealed reduction of cell viability (Figure 2). But, the induction of cell viability induced by FB_1 (10 µM) is low that is only by about 10-15%. ZEA (10 µM) reduced Caco-2 cells viability by about 23-25%.

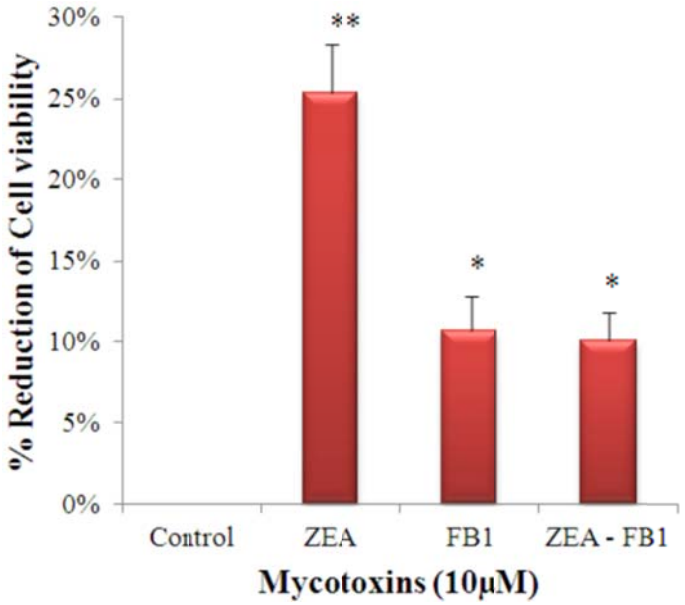

Figure 2. Cytotoxicity effect of ZEA (10 µM), FB_1 (10 µM) or their mixture on Caco-2 cells after 72 h incubation evaluated by NR test. Results are given as mean ± SD from three independent experiments.*Different from control at $p < 0.05$ and **$p < 0.001$.

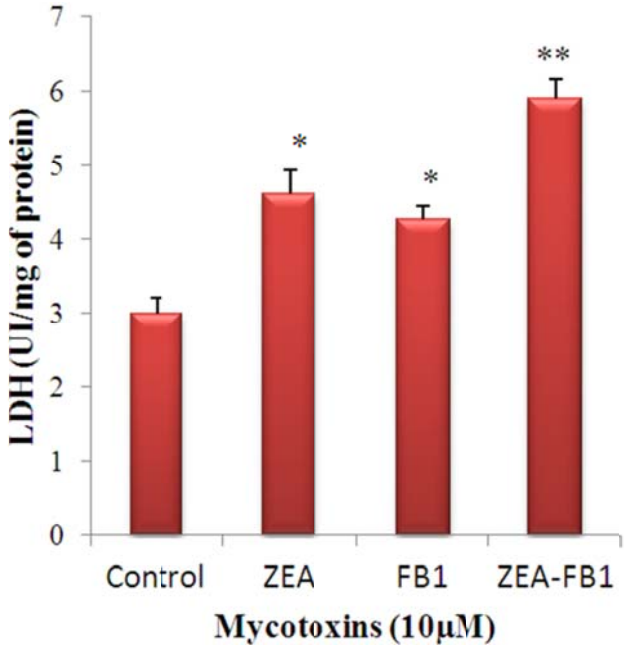

Figure 3. Increasing of LDH leakage into the cell culture medium after incubation of Caco-2 cells with ZEA (10 µM) or FB_1 (10 µM) or their mixture by 24 h. Results are given as mean ± SD from three independent experiments. *Different from control at $p<0.05$ and **$p<0.001$.

Surprisingly, addition of FB_1 to ZEA leads to reduction of cell viability similarly to those induced by FB_1 alone.

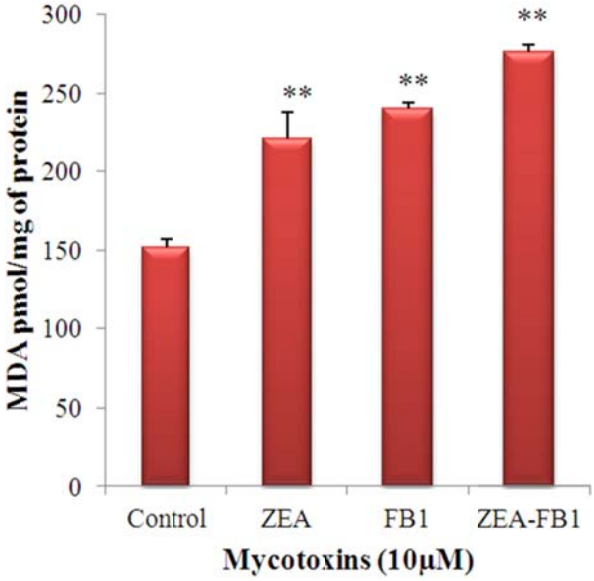

Figure 4. Lipid peroxidation as measured by MDA-TBA adduct after incubation of Caco-2 cells with ZEA (10 µM) or FB_1 (10 µM) or their mixture by 24 h. Results are given as mean ± SD from three independent experiments.*Different from control at $p<0.05$ and **$p<0.001$.

Cytotoxicity assay by LDH assay

Incubation with mycotoxin FB_1 or ZEA alone or their mixture resulted in increasing of leakage of LDH into the culture medium compared with controls (Figure 3). Effects produced by both *Fusarium* toxins were similar and the mixture induced higher increasing of leakage of LDH.

Extraction and determination of MDA- TBA adduct

ZEA and FB_1, all at a concentration of 10 µM, increased MDA production in Caco-2 cells by 33%, 36%, respectively. Their mixture also increased MDA production by about 80% (Figure 4). A rapid comparison between experimental values of MDA increases and theoretical values calculated from observed individual ones showed the addition of ZEA and FB_1 increased MDA production in a more additive way.

Caspace-3 activity assay

The modulation of activation of caspase-3 after ZEA (10 µM) or FB1 (10 µM) or their mixture exposure on Caco-2 cells was evaluated according to variable duration namely 3 h, 6 h and 24 h. As shown in Figure 5, ZEA (10 µM) or FB_1 (10 µM) or their mixture increased activity of caspase-3. But, the modulation of caspase-3 activity was

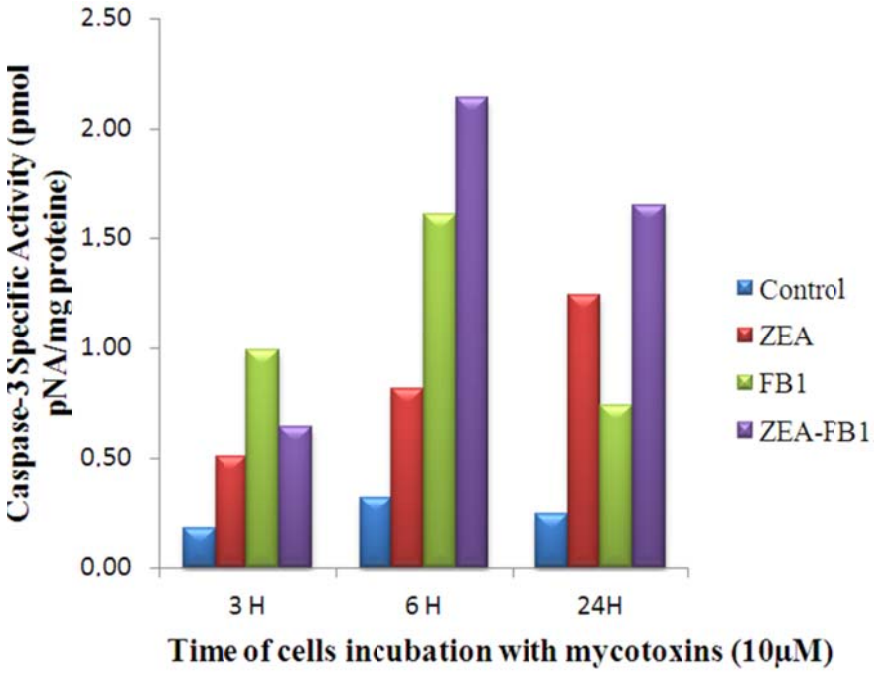

Figure 5. Activation of caspase-3 after ZEA (10 µM) or FB1 (10 µM) or their mixture exposure on Caco-2 cells for variable duration namely 3 , 6 and 24 h.

variable for the same toxin according to the duration of incubation of Caco-2 cells with toxins. The activation of caspase-3 by ZEA increased proportionally to the duration of Caco-2 cells exposure. Thus, ZEA was more potent at 24 h > 6 h > 3 h. FB_1 increased caspase-3 activity proportionally to the duration of cells exposure until 6h but the FB_1-effect induced decreased at 24 h. The mixture of ZEA and FB_1 was more potent in activation of caspase-3 at 6 h of cell exposure. In addition, the mixture of toxins leaded to additive effect on caspase-3 activation at 6 and 24 h of cells exposure in contrast to 3 h of incubation where FB_1 alone is stronger than the mixture.

DISCUSSION

Concentrations of toxins ZEA and FB_1 used in the present study were 10 µM for each mycotoxin and are those that allow distinguishing synergistic or antagonistic effects of toxins when used in mixture following our previous data (Kouadio et al., 2005; 2007). ZEA and FB_1 were tested alone at concentrations from 1 to 150 µM (Kouadio et al., 2005) and their mixture at concentrations between 4 and 40 µM (Kouadio et al., 2007). Additionally, these concentrations are the concentrations that could be reached in animals or possibly in human tissues following ingestion of 2-4 mg/kg BW of FB1 or ZEA in foods or feed (Creppy, 2002).

In our previous studies focused on interactive effect of *Fusarium* toxins ZEA, FB_1 and Deoxynivalenol (DON). It has been reported that the cytotoxic effect as evaluated by DNA synthesis and cell membrane transport integrity of combination of ZEA and FB_1 seemed to be antagonist effect in contrast to additive effect observed on lipid peroxydation and protein synthesis (Kouadio et al., 2007). In order to understand the best possible interactive effect of these *Fusarium* toxins, we investigated in their combined effect regarding several cellular specific endpoints. Thus, the binary toxins have been tested on mitochondrial succinate dehydrogenase activity and the results reveal that ZEA undoubtedly reduced FB_1 inhibition mitochondria enzyme-induced. Similarly, we have confirmed the antagonist effect of ZEA and FB_1 mixture on cell lysosome integrity (Kouadio et al., 2007). In contrast to both previous cases, the combination of ZEA and FB_1 produced an additive effect on leakage of LDH, an enzyme marker of cell membrane damage or cells necrosis (Galluzzi et al., 2009; Fotakis and Timbrell, 2006). These findings were surprising because the three cells endpoints namely lysosome integrity, mitochondrial succinate dehydrogenase activity and leakage of LDH have been always considered as makers of cells viability or cytoxicity (Babich and Borenfreund, 1987; Kouadio et al., 2005; Smith et al., 2011). Although, the differences between cell damage pathways evaluated or measured by the three assays could explain such findings. Indeed, cell membrane leakage of LDH is known as a marker of necrosis which represents passive cell death without an underlying regu-

latory mechanism or activation of executive caspases (Wyllie et al., 1980). Thus, the amount of LDH release resulted from cell disorders induced by individual effect of each mycotoxin leading necrosis cell death. In contrast, membrane transport integrity and mitochondrial succinate dehydrogenase activity represent cell specific vital functions implying regulatory or organized mechanisms which also could be targets of toxicants (Babich and Borenfreund, 1987; Kouadio et al., 2005; Smith et al., 2011). Previously, it has been reported that ZEA and FB_1 targeted the same cellular organelles namely mitochondria and/or lysosomes consequently authors have hypothesized that combinations of these toxins would lead to additive or synergistic effects (Kouadio et al., 2005). Surprisingly, in the present study, antagonist effect was observed. In fact, the concentrations of mycotoxins could modulate their interactive effect leading to unexpected findings as reported by Boeira et al. (2000) on growth of yeast. These authors reported antagonism for low concentrations and synergism for high concentrations for mixture of ZEA and DON. Concerning lipid peroxydation, association of ZEA and FB_1 leaded to additive effect as reported previously (Kouadio et al., 2007). These findings traduce the capably of ZEA and FB_1 to produce reactive oxygen species (ROS) but the nature of ROS and their mechanism of production remains unclear. However, it appears probable that FB_1 or ZEA induces ROS production in mitochondria and/or by inflammatory disorders involving TNF-α (Soriano et al., 2005; Seefelder et al., 2003; Ayed-Boussema et al., 2008; Bouaziz et al., 2008) and the intracellular generation of ROS is likely or partly responsible for their cytotoxic and genotoxic effects (Hassen et al., 2007; Mobio et al., 2003; Kouadio et al., 2007). Although, both ZEA and FB_1 target the same cellular organelle that is mitochondria, in the production of ROS (Kouadio et al., 2005), but any chemical interaction is not at stake. On the other hand, since ZEA and FB_1 have been shown to induce apoptosis caspace-3 dependent (Ayed-Boussema et al., 2008; Bouaziz et al., 2008; Soriano et al., 2005 ; Seefelder et al., 2003 ; Gopee and Sharma, 2004), we have tested their possible interactive effect on caspase-3 activity modulation. Our results have confirmed clearly modulation of caspase-3 activity induced by ZEA or FB_1 (Ayed-Boussema et al., 2008; Soriano et al., 2005). It has been reported that FB_1 modules caspase-3 activity by several pathways involving mitochondria and disturbs cytochrome-c release and cell membrane TNF-R1 receptor activation by TNF-α (Di Pietro et al., 2005). Concerning ZEA, studies reported ZEA induced apoptosis caspase-3 dependence or not (Bouaziz et al., 2008; Yu et al., 2011) and apoptosis caspase independence induced by ZEA could be related to apoptosis-inducing factor-mediated and ROS-dependent pathways, in which p53 and JNK/p38 MAPK play crucial roles as upstream effectors (Yu et al., 2011). In the present study, FB_1 has been showed to induce early apoptosis caspase-3 dependent in contrast to ZEA which was found very potent later until after 24 h of cells incubation. In contrast to FB_1, ZEA could module caspase-3 activity by long processes. The mixture of toxins led to additive effect on caspase-3 activation at 6 and 24 h of cells exposure in contrast to 3 of incubation where FB_1 alone is stronger than the mixture. In fact, ZEA tended to counterbalance FB_1-caspase-3 activity modulation by a mechanism of functional antagonism which contrasted with additive effect observed subsequently. The effect of mixture of ZEA and FB_1 on caspase-3 activity was unpredicted or in the present study, this effect is linked to the duration of cells incubations with mycotoxins.

In conclusion, our findings reveal that the combined effect of ZEA and FB_1 on cells Caco-2 seems to be unpredictable. Although, these mycotoxins could provoke the same disorders in cells, it is very difficult to predict whether their mixture can lead to antagonism, additive or synergistic effect.

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Conflict of Interest

The author(s) have not declared any conflict of interests.

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