Modulation Of Fumonisin B1 Toxic Action-Induced By Zearalenone In Human Intestinal Cells Caco-2

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Abstract: The natural co-occurrence of Fusarium toxins fumonisin B1 (FB1) and Zearalenone (ZEA) in cereal grains is well known. However, a few studies have been reported that address to the toxicity of Fusarium toxins mixtures. Thus, the aim of the present study was to investigate in the interactive effect of binary Fusarium toxins FB1 and ZEA on intestinal cells line Caco-2 using several cellular endpoints such as caspase-3 activity modulation, malondialdehyde (MDA) production, lactate dehydrogenase (LDH) leakage as necrosis measure and cells viability as evaluated by lysosome and mitochondria integrities. As results, ZEA + FB1 led to an antagonistic effect on lysosome and mitochondria damage but, the necrosis-induced was more potent when compared to ZEA or FB1 alone showing an additive effect. Moreover, MDA production induced by ZEA + FB1 was higher than additive effect but not synergistic effect. At last, the combined effect of toxins on key apoptosis enzyme caspase-3 kinetic activity was an additive effect after 6 h and 24 h but, after 3 h, ZEA tended to exert its anti-apoptotic action by reducing the enzyme activation by FB1. Taken together, the results were contrasted and results suggested that combined effects of binary Fusarium toxins ZEA and FB1, 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 toxins.

Index Terms: Fusarium-toxins, interactive-effect, caspace-3-kinetic-activity, cytotoxicity, oxidative-stress

1 INTRODUCTION
Zearalenone (ZEA) and Fumonisins B1 (FB1) 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. Fumonisins B1 causes liver and kidney cancer, and neural tube defects in rodents, leukoencelephalomalacia in horses and pulmonary oedema in pigs [1]. Of major concern is the association of FB1 with elevated incidence of human oesophageal cancer in parts of South Africa, North Eastern Iran and China, upper gastrointestinal tract cancer in Northern Italy [2, 3] and neural tube defects in human babies [4]. The structures of FB1 and sphingolipids show marked similarities [5], which may be the reason why FB1 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 caspase-3 dependant [5-9]. In addition, FB1 also induces lipid peroxidation in Vero cells [6], in primary rat hepatocytes [8] in C6 glioma cells [9] and human intestinal Caco-2 cells [10]. On the other hand, ZEA have estrogenic and anabolic activities in several species (rodents, pigs and monkeys), being able to cause alterations in the reproductive tract of laboratory animals [11]. 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 [11]. Cellular mechanism of ZEA has been described by its high binding affinity to oestrogen receptors [12] and DNA adducts formation in female mousse tissues and carcinogenic disorders [11]. It has also been reported that ZEA inhibits DNA and protein synthesis and induces oxidative stress mediated cell death [11-14]. Consequently, the intracellular generation of reactive oxygen species (ROS) by ZEA is likely responsible for its cytotoxic and genotoxic effects [11, 13]. ZEA causes cells death by apoptosis via caspase-independent and mitochondria/AIF-mediated pathways [15] with a key role of activations of p53 and JNK/p38 [16]. ZEA and FB1 could be found in the same commodities as secondary metabolites of different Fusarium species [17]. For example, co-occurrence of Fusarium mycotoxins FB1 and Zearalenone ZEA had been found in rice, maize and peanut from West Africa countries such as Côte d’Ivoire [18]. Thus, in preliminary study on possible interactive effect of combination of FB1 and ZEA, we had reported an antagonistic cytotoxic effect as measured only by cell lysosomse integrity but an additive effect on lipid peroxidation [10]. In order to understand the best possible interactive effect of these Fusarium toxins, we investigated in their combined effect regarding several cellular endpoints including the key apoptosis enzyme caspase-3 kinetic activity, lactate dehydrogenase (LDH) release as necrosis measure, lipid peroxidation, lysosome and mitochondria damage.

2 MATERIAL AND METHODS
2.1 Material
Chemicals: ZEA and FB1 were obtained from Sigma Chemical Company (St Louis, MO, USA) and were dissolved in ethanol/water (90:10). Dulbeccco’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.

Cells 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) [19]. 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), 8mM L-glutamine, 1% of mixture penicillin (100 IU/ml) and streptomycin (100μg/ml) incubated at 37 °C in an atmosphere of 5% CO2—95% air mixture. For cell counting and subculture, the cells were dispersed with a solution of 0.05% trypsin and 0.02% EDTA.

2.2 Methods
Cytotoxicity assay by NR test: The NR test was performed to assess cell viability, as previously described [10, 13]. Viable cells actively transport this dye across their cell membrane by the system Endosome-lysosome; 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%
Different lourless salt to a blue –, as previously described [21–23]. Freshly prepared dialysed in the presence of each mycotoxin in content of cellular homogenates, determined using the colorimetric method of Bradford [23]. Leakage was expressed as percentage difference from controls.

**Cytotoxicity assay by Lactate dehydrogenase measure:** The LDH assay was performed to assess cytotoxicity or cell death by necrosis, as previously described [20]. Caco-2 cells (1 x 105 cells/ml/well) were preincubated in 24-well multidishes (Polylabo, France) for 24 h at 37°C. Cell viability was assessed by LDH leakage through the membrane into the medium. After 48 h of incubation in presence of toxins (ZEA and FB1) 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 FB1) and their mixture. The amount of LDH measured is related to the protein content of cellular homogenates, determined using the colorimetric method of Bradford [21]. Leakage was expressed as percentage difference from controls.

**Extraction and determination of malondialdehyde (MDA)–thiobarbituric acid (TBA) adduct:** Cells (105 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 (0.1M NaNH, 20mM EDTA, 50mM Tris–HCl, pH 8.0). As previously described [6, 13], extraction and determination of the malondialdehyde (MDA)–thiobarbituric acid (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 [21].

**Caspase-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+FB1, cells (106 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-nitrianiiline (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 analyze was carried out using a non-parametric statistical test, Mann–Whitney test for significance of differences. Acceptable limit is set from p < 0.05* to p < 0.01** [22].

### 3 Results

#### 3.1 Cells viability and necrosis:

**Cells viability:** Fig.1 showed the viability of Caco-2 cells measured by MTT test after incubation of either individual mycotoxin FB1, or ZEA or their mixture and the results were diminishing of cell viability. ZEA (10µM) reduced slightly cell viability by about 20–25%, which is lower than 10µM FB1 does (35–38%). Thus, ZEA appears weakly cytotoxic. Strikingly, FB1 and ZEA mixed did not affect cell viability higher than FB1 alone. Indeed, combined effect observed of these toxins is lower than expected additive effect of their mixture. Fig.2 showed Caco-2 cells viability by Neutral Red test. Cells incubation with mycotoxin FB1 or ZEA alone or their mixture revealed reduction of cell viability. But, the induction of cell viability induced by FB1 (10µM) is low i.e. only by about 10–15%. ZEA (10µM) reduced Caco-2 cells viability by about 23–25%. Surprisingly, addition of FB1 to ZEA leads to reduction of cell viability similarly to those induced by FB1 alone.

**Fig. 1. Cytotoxicity effect of ZEA (10µM) or FB1 (10µM) 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.**
**Cell necrosis:** Fig. 3 showed necrosis resulted from incubation of cells Caco-2 with mycotoxins FB1 or ZEA alone or their mixture. All toxins and their mixture increased leakage of LDH into the culture medium compared with controls. Effects produced by both *Fusarium* toxins were similar and the mixture induced higher increasing of leakage of LDH probably as additive effect.

**3.2 Oxydative stress:**
Fig. 4 showed the oxidative damage induced by ZEA, FB1, or their mixture as measured by MDA production resulting from lipid peroxidation. ZEA and FB1, 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%. A rapid comparison between experimental values of MDA increases (80%) and theoretical values calculated from observed individual ones (33% + 36%) showed that the addition of ZEA and FB1 increased MDA production in a more additive way.

**3.3 Caspase-3 kinetic activity:**
Fig. 5 revealed the modulation of caspase-3 activation after ZEA (10μM) or FB1 (10μM) or their mixture exposure on Caco-2 cells according to variable duration namely 3 h, 6 h and 24 h. ZEA (10μM) or FB1 (10μM) or their mixture increased activity of caspase-3. But, the modulation of caspase-3 activity varied for the same toxin according to the duration of its incubation with Caco-2 cells. Thus, the activation of caspase-3 by ZEA increased proportionally to the duration of Caco-2 cells exposure. Indeed, ZEA was more potent at 24h > 6h > 3h. FB1 also increased caspase-3 activity proportionally to the duration of cells exposure until 6h but decreased caspase-3 activation at 24h. The mixture of ZEA and FB1 was more potent at 6 h and 24 h where compared to ZEA or FB1 alone but at 3h of cells incubation, FB1 alone appeared stronger than the mixture. Thus, the mixture of toxins led to additive effect at 6h and 24h of cells exposure but not at 3h where ZEA tended to reduce FB1 effect-induced.
4 Discussion
Concentrations of toxins ZEA and FB$_1$ used in the present study were 10μM for each mycotoxin and are those that allow distinguishing synergistic or antagonistic effects of toxins when used in mixture following previous data [10, 13]. Additionally, these concentrations are the combinations that could be reached in animals or possibly in human tissues following ingestion of 2–4 mg/kg BW of FB$_1$ or ZEA in foods or feed. In previous studies focused on interactive effect of Fusarium toxins ZEA, FB$_1$ and Deoxynivalenol (DON), it had 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 antagonistic effect in contrast to additive effect observed on lipid peroxidation and protein synthesis [10]. Such interactive effect must be clarified by using several cellular specific endpoints. Thus, the binary toxins had been tested on mitochondrial succinate dehydrogenase activity and the results revealed ZEA undoubtly reduced FB$_1$ inhibition mitochondria enzyme-induced. Similarly, results in the present study have confirmed the antagonistic effect of ZEA and FB$_1$ mixture on cell lysosome damage [10]. In contrast to both anterior specific cell endpoints, the combination of ZEA and FB$_1$ produced an additive effect on leakage of lactate dehydrogenase (LDH), an enzyme marker of cell membrane damage or cells necrosis [23, 24]. 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 cytotoxicity [25-27]. But, the differences between cell damage pathways evaluated or measured in 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 regulatory mechanism or activation of executive caspases [28]. Thus, the amount of LDH release resulted from cell disorders induced by individual effect of each mycotoxin leading necrotic 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 [28, 27]. Previously, it had 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 [13]. Surprisingly, in the present study, it has been observed an antagonistic effect. In fact, the concentrations of mycotoxins could modulate their interactive effect leading to unexpected findings as previously reported on growth of yeast [29]. These authors [29] reported antagonism for low concentrations but synergy for high concentrations for mixture of ZEA and DON. Concerning lipid peroxidation, association of ZEA and FB$_1$ led to more additive effect as reported previously [10]. These findings traduce the capability of ZEA or FB$_1$ to produce reactive oxygen species (ROS) but the nature of ROS and the mechanism involved in their 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 [7, 8] and the intracellular generation of ROS is likely or partly responsible for their cytotoxic and genotoxic effects [10, 14]. Although, both ZEA and FB$_1$ target the same cellular organelle i.e. mitochondria in the production of ROS [13], but any chemical interaction is not at stake. On the other hand, since ZEA or FB$_1$ had been shown to induce apoptosis casapse-3 dependent [7], it appeared interesting to test their possible interactive effect on caspase-3 activity modulation. The present study confirmed clearly modulation of caspase-3 activity induced by ZEA or FB$_1$ [9]. It had been reported that FB$_1$ modules caspase-3 activity by several pathways involving mitochondria disturbs following by cytochrome-c release and cell membrane TNF-R1 receptor activation by TNF-α [30]. Concerning ZEA, studies reported that ZEA induced apoptosis caspase-3 dependant or not [14, 16] and apoptosis caspase independent induced by ZEA could be related to AIF-mediated and ROS-dependent pathways, in which p53 and JNK/p38 MAPK play crucial roles as upstream effectors [31]. In the present study, FB$_1$ has been showed to induce early apoptosis caspase-3 dependent in contrast to ZEA which was very potant 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 leaded to additive effect on caspase-3 activation at 6h and 24h of cells exposure in contrast to 3h 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 probable 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.

5 Conclusion
Fusarium toxin ZEA or FB$_1$ alone had been found to cause cytotoxicity, oxidative damage as measured by MDA production and key apoptosis enzyme caspase-3 kinetic activity modulation. That indicated their capability to target the same cell endpoints but caspase-3 activity modulation was precocious with FB$_1$, when compared to ZEA. Despite the same targets, the combined effect of ZEA and FB$_1$ on cells Caco-2 seemed to be unpredictable. ZEA antagonized FB$_1$ toxic action on mitochondria and lysosome integrities but enhanced its effect in necrosis, apoptosis and oxidative damage. Since ZEA reduced or enhanced FB$_1$, caspase-3 activation-induced according to the duration of cells incubation with toxins, it was plausible that the interactive of binary ZEA and FB$_1$ varied according to several parameters such as the cellular endpoints and the duration of cells incubation with toxins.

REFERENCES


