



Insights into cellular metabolic pathways of the combined toxicity responses of Caco-2 cells exposed to deoxynivalenol, zearalenone and Aflatoxin B₁

Jian Ji^{a,1}, Qiuyun Wang^{b,1}, Hao Wu^d, Shuang Xia^a, Hongyan Guo^a, Ivana Blaženović^c, Yinzi Zhang^a, Xiulan Sun^{a,*}

^a State Key Laboratory of Food Science and Technology, National Engineering Research Center for Functional Food, Synergetic Innovation Center of Food Safety and Quality Control, Jiangnan University, Wuxi, Jiangsu, 214122, PR China

^b Wuxi Higher Health Vocational Technology School, Wuxi, Jiangsu, 214122, China

^c West Coast Metabolomics Center, UC Davis, Davis, CA, 95616, United States

^d Guangzhou GRG Metrology & Test Co., Ltd, Xiping Yun Road No. 163, Tianhe District, Whampoa Avenue, Guangzhou, 510630, PR China

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ABSTRACT

Metabolic profiling in Caco-2 cells was studied for the combined toxic effects of deoxynivalenol (DON), zearalenone (ZEN), and Aflatoxin B₁ (AFB₁) through untargeted GC-MS. The GC-MS spectra of Caco-2 cells treated with individual 6.7 μM DON, 20 μM ZEN, 20 μM AFB₁ and the combined DON + AFB₁ (6.7 + 20 μM) and DON + ZEN + AFB₁ (6.7 + 20 + 20 μM) for 24 h were deconvoluted, aligned and identified with MS DIAL. The metabolic pathway analysis was analyzed with MetaMapp and visualized with CytoScape. Results show that the combined DON + AFB₁ and DON + ZEN + AFB₁ treatment has an obvious “synergistic effect”. The apoptosis-related gene mRNA test result indicates that the combined mycotoxins downregulate Bcl-2 gene and upregulate Bax, p53, caspase-3, caspase-8 and caspase-9, more significantly than any individual mycotoxins group. The cellular metabolism illustrated that the combined mycotoxin groups, DON + ZEN + AFB₁ seriously effect glycine, serine and threonine metabolism, pyruvate metabolism, etc. while no metabolic disorders were presented in individual mycotoxin group. Our hypothesis was validated that the combined mycotoxins with low concentrations can have a synergistic effect in the metabolism, which may lead to cellular apoptosis or necrosis.

1. Introduction

Mycotoxins are ubiquitously present in agricultural commodities. Although there are almost four hundred mycotoxins known today, only a few mycotoxin groups have received widespread attention (Berthiller et al., 2013). The most important mycotoxins or mycotoxin groups are aflatoxins (AFs), zearalenone (ZEN), deoxynivalenol (DON), fumonisins (FUM) and ochratoxin A (OTA) (Streit et al., 2013). Among these, AFB₁ is the most toxic and carcinogenic, which could cause various diseases, including liver cancer, effects on the reproductive system and the immune system, encephalopathy with fatty degeneration of viscera, and pulmonary interstitial fibrosis (Marin et al., 2013; Lei et al., 2013). DON belongs to the type-B trichothecene group. Although it is not as toxic as other trichothecenes, it is one of the most common contaminants of cereals worldwide. DON directly binds to ribosome and causes translation, which activates stress kinases by ribotoxic stress response (Wen et al., 2016). Meanwhile, acute effects in humans are

abdominal pains, headache, dizziness, throat irritation, nausea and many others (Rotter, 1996). ZEN competitively associates with estrogen receptors causing reproductive disorders, genotoxicity and testicular toxicity in some animal species and possibly human as well. Among the grains for human consumption, ZEN often occur in maize (Zinedine et al., 2007).

The mycotoxin survey (2005–2012) consisting of 19,000 samples collected from all over the world have been analyzed showing that 72% of the samples contained detectable amounts of mycotoxins and 38% of all samples contained two or more mycotoxins (Schatzmayr and Streit, 2013). A full 94% of samples contained at least one mycotoxin, and 76% of samples contained two or more mycotoxins which could impact human and animal health at already low doses (Muccio, 2017). Co-occurrence of mycotoxins is likely to arise because (i) most fungi can simultaneously produce multiple mycotoxins, (ii) commodities can be contaminated by several fungi at the same time, and (iii) completed feed is made from various commodities (Jelinek et al., 1989; Streit

* Corresponding author. Jiangnan University, 1800 Lihu Avenue, Wuxi, Jiangsu 214122, China.

E-mail address: xlzxx@jiangnan.edu.cn (X. Sun).

¹ Jian Ji and Qiuyun Wang are the co-first authors.

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et al., 2012). The data on combined toxic effects of mycotoxins are limited and the health risk from multi-exposure is not well-known. Therefore, an increasing number of mycotoxin studies are devoted to their combined toxicity, especially to the exploration of the type of toxicological interaction (Lee and Ryu, 2017). DON, ZEN, and AFB₁ are so ubiquitous and co-occur in a range of commodities. However, there is few papers that study their combined cytotoxic properties. It is therefore necessary to assess the combined toxicity of these three mycotoxins. These cells are the first to be exposed to mycotoxins and at higher doses than other tissue cells. Cell submicroscopic studies have shown that Caco-2 cells are morphologically similar to human intestinal epithelial cells with identical cell polarity and tight junctions. And detection of pinocytosis function also showed that Caco-2 cells are similar to human intestinal epithelial cells. To study the effect of mycotoxin mixtures on the gastrointestinal tract, we selected the human intestinal cells Caco-2 as the *in vitro* experimental system.

Metabolomics is analytical technique that investigates small molecules present within a biological sample isolated from animal or cultured cells (Fiehn, 2002). Metabolomics is a well-established methodology which based on GC-MS and/or HPLC-MS techniques assisted with variety of statistical analysis, which can be an extremely powerful when elucidating the specific metabolic changes within a biological system in response to environmental changes such as disease, infection, or toxins (Hayton et al., 2017; Castro-Puyana and Herrero, 2013). Gas chromatography–mass spectrometry (GC-MS)–based metabolomics is ideal for identifying and quantitating small-molecule metabolites (< 650 Da), including small acids, alcohols, hydroxyl acids, amino acids, sugars, fatty acids, sterols, catecholamines, drugs, and toxins, often using chemical derivatization to make these compounds sufficiently volatile for gas chromatography (Fiehn, 2016). Metabolomics of cultured cells has the potential to uncover previously unknown information about cell biology, functions and response mechanisms (Zhang et al., 2013). However, only few studies on animal or human biological system response to mycotoxin have been reported (De Pascali et al., 2017).

Understanding the potential effects of mixtures of mycotoxins is a complex task that requires a sound theoretical framework which can be tested experimentally. The CI models were used to predict the combined effects of DON, ZEN, and AFB₁. Metabolomics is utilized to evaluate and explain the biological systems changes. To the best of our knowledge, this work is the first study which utilizes metabolomics platform to uncover cellular mechanisms and cellular toxicity in response to individual and combined mycotoxins exposure.

2. Materials and methods

2.1. Materials and apparatus

The cell culture components used, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, trypsin/EDTA solutions, phosphate buffer saline (PBS), the cell counting kit-8 (CCK8) were obtained from Gibco by Thermo Fisher Scientific and Beyotime (Jiangsu, China). 2', 7'-dichlorofluorescein diacetate (DCFH-DA), DON (purity ≥ 99%), ZEN (purity ≥ 99%) and AFB₁ (purity ≥ 99%) were obtained from Sigma-Aldrich Inc. (USA). All other chemicals used were of HPLC grade. Deionized water used for all experiments was purified with a Milli-Q system (Millipore, USA).

2.2. Cell culture and treatments

Caco-2 cells were obtained from the cell bank at Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM containing 10% FBS and antibiotics (penicillin and streptomycin) in cell culture flasks. Then cell lines were cultured at 37 °C in a humidified atmosphere containing 5% CO₂ in a CO₂ incubator (Thermo Fisher Scientific Inc., Rockford, IL, USA).

Stock solutions of DON, ZEN and AFB₁ were prepared in absolute ethanol, acetonitrile and dimethylsulfoxide (DMSO), respectively. Stock solutions were maintained at –20 °C and working dilutions were prepared in cell culture medium. The final absolute ethanol, acetonitrile and DMSO concentration in the culture medium was ≤ 0.1% (v/v) which had no adverse effect on the cellular parameters tested. The final mycotoxin concentrations tested were as follows: 0.001, 0.01, 0.1, 1, 10, 25, 50, and 100 μM. Caco-2 cells were exposed to individual mycotoxins for 24, 48, and 72 h. The cells incubated with toxins for 24 h, which reflects a realistic estimate of the human gut being exposed to mycotoxins by food consumption (Vejdovsky et al., 2016). To represent a scenario of exposure as realistic as possible, we chose 24 h as the incubation period for conducting further studies. The IC₅₀ value of each individual toxin was calculated using the software CompuSyn and Origin. These ratios were intended to yield a roughly similar toxicity for each mycotoxin combination (Gao et al., 2016).

Cell viability was determined using the CCK-8 according to the manufacturer's instruction. The optimum cell concentration, determined by the growth profile of cell, was 2.5 × 10⁴ cells/well. Cells were seeded in 96-well microplates (Corning, USA) and allowed to adhere at 37 °C for 24 h before treatment with mycotoxins. Thereafter, the culture medium was replaced by fresh medium containing working concentrations of mycotoxins and were incubated for another 24 h before adding water-soluble tetrazolium salt (WST-8) dye. Finally, the dye absorbance of the reduced WST-8 was determined at 450 nm using a multidetection microplate reader (Molecular Devices, Sunnyvale, USA). The inhibition of viability was calculated using Equation (1):

$$\text{Inhibition of viability (\%)} = \left(1 - \frac{Abs_{(treatment)} - Abs_{(blank)}}{Abs_{(control)} - Abs_{(blank)}} \right) \times 100\% \quad (1)$$

2.3. Apoptosis-related gene mRNA test

Caco-2 cells with a density of 1 × 10⁵ cells/mL were inoculated into a 12-well plate. After 24 h of culture, the culture solution was removed, the final concentrations of 6.7 μM DON, 20 μM ZEN, 20 μM AFB₁ and the combined DON + AFB₁ (6.7 + 20 μM) and DON + ZEN + AFB₁ (6.7 + 20 + 20 μM) and DMEM control group was used for cell culturing for 24 h. Use the Trizol reagent to lyse the cell reagent to prevent RNase contamination. RNA purity = A260/(A280 - background), ensuring RNA purity between 1.8 and 2.0. Primers were lysed and formulated to the appropriate concentration for reverse transcription using the PrimeScript™ RT kit. The 10 μL system was expanded according to actual needs, and the preparation process was carried out on ice, then 37 °C for 15 min, 85 °C for 5 s, and reverse transcription at 4 °C. The resulting cDNA was stored at –4 °C for subsequent experiments. Real-time fluorescent PCR was performed using SYBR Premix Ex Taq™ II kit, and 20 μL of reaction solution was set up. The program was set to: pre-denaturation: 95 °C for 30 s; PCR reaction: 95 °C for 5 s, 60 °C for 31 s, 40 cycles. The melting curve was set to 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The measured Ct value calculated the relative mRNA content of different genes according to 2^{–ΔΔCt}. The gene upstream and downstream primers and the internal reference primer sequences are shown in Table S3.

2.4. Metabolite extraction and GC-MS analysis

The cell dishes were placed on ice and washed three times with 1 mL chilled water. Except for the dosed cells and control cells, we also collected a blank sample to correct for the background signals. 1 mL of chilled 3:2 methanol/H₂O quenching solvent was added to each plate and cells scraped with a cell lifter. Extracts were transferred to 1.5 mL Eppendorf tubes and centrifuged at 4 °C for 5 min at 1000 g. Supernatant was discarded, and the cell precipitate was stored at –80 °C or analyzed immediately. Ice cold extraction solution (0.5 mL of

acetonitrile/isopropanol/H₂O (3:3:2, v/v/v) and two stainless steel beads were added into the tubes (Liu et al., 2017). Next, cells were grinded using a grinder at 1500 rpm for 30 s, for five cycles. After the homogenate was centrifuged, the supernatant was transferred into a new tube. Repeated this procedure for the precipitate. The supernatant was then divided into two 480 μ L aliquots: one for analysis and another one for preservation. At last, all samples were evaporated to dryness and frozen at -20°C until next analysis. Quality control: An equal volume (based on the number of samples) was taken from each sample into Eppendorf tubes as a mixed sample.

2.5. GCMS samples derivatization

Methoxylamine hydrochloride was added into dried samples, incubation at 30°C for 90 min. Then added N-Methyl-N-(trimethylsilyl)litrifluoroacetamide (MSTFA) with 1% trimethylchlorosilane into each sample followed by incubation at 37°C for 30 min. In the meantime, we used 1 mg/mL fatty acid/methyl ester mixture (FAMES, C8–C16: 1 mg/mL; C18–C24: 0.5 mg/mL in chloroform) as internal standard. Subsequently, the derivatized samples were analyzed by GC-MS in 24 h.

A Shimadzu QP2010 Ultra gas chromatograph coupled with a mass spectrometer was used to perform the GC-MS analysis. The system equipped with a Rxi-5 Sil MS column (30 m \times 250 μ m inner diameter, 0.25 μ m film thickness; Restek, USA). The carrier gas was helium. The column gas flow rate was 20 mL min^{-1} and the front inlet purge flow was 5 mL min^{-1} . The injection volume was 1 μ L and sample was injected on splitless mode. The initial temperature of the column was 50°C for 0.5 min, then raised to 110°C at a rate of $30^{\circ}\text{C min}^{-1}$, followed to 310°C at the rate of $10^{\circ}\text{C min}^{-1}$ and then maintained for 10 min. The temperature of injection, transfer line, and ion source were 280, 280, and 250°C , respectively. The EI voltage was -70 eV on electron impact mode. The mass spectrometry data were acquired in full-scan mode with the m/z range of 85–500 at a rate of 17 spectra per second with a solvent delay of 240 s.

2.6. Metabolite profiling analysis

The raw data was converted by GC-MS PostRun from Shimadzu and the ABF converter software (<http://www.reifycs.com/AbfConverter/index.html>), and the final format was “abf”. The MS DIAL software (Tugawa et al., 2015) with Fiehnlib (Kind et al., 2009) was used for peaks extraction, data baseline filtering, calibration of the baseline, peak alignment, deconvolution analysis, peak identification, and integration of the peak height.

2.7. Statistical analysis

Statistical analysis was performed with SPSS 17.0. The results were analyzed by *t*-test and one-way ANOVA to test the main effects. Data were expressed as the mean \pm SD. Significance was set at $p < 0.05$. In this experiment, Metaboanalyst 3.0 was implemented to perform multivariate analysis and integrating enrichment analysis (Xia et al., 2015). These included the principal component analysis (PCA), orthogonal projection to latent structures-discrimination analysis (PLS-DA) and heatmap analysis. Biomarkers analysis was performed by the plot of the PLS-DA model. The pathway mapping was analyzed with MetaMapp (Barupal et al., 2012) and visualized by CytoScape 3.4.0 (Smoot et al., 2010).

3. Results and discussion

3.1. Cytotoxicity of individual and combined mycotoxins

To identify relative toxicities of individual toxins, the cytotoxic effect of DON, ZEN, and AFB₁ in Caco-2 cells was evaluated by CCK8 assay over 24, 48, and 72 h. The results consistently demonstrated that

all mycotoxins reduce cell viability in a time- and concentration-dependent manner, especially in high concentrations (Fig. S1). However, ZEN and AFB₁ only reduced cell viability at the higher tested concentrations. According to the IC₅₀ values, the concentration of toxin that caused 50% inhibition of cell viability, DON was the most cytotoxic among the mycotoxins tested on Caco-2 cells at the three exposure times, followed by ZEN and AFB₁. IC₅₀ values were calculated by Origin (Table S1) followed by determination of the appropriate ratio of each toxin in the mixtures to obtain an equipotent toxicity.

Cytotoxic interactions were also assayed utilizing the isobologram method, which provides CI values as a quantitative measure of the three mycotoxins interaction degrees. These CI values were calculated CompuSyn software (Chou and Martin, 2005). The ratios of mycotoxins in the combinations samples were based on the ratios of the IC₅₀ values of the individual mycotoxins, 1:3:3 for DON: ZEN: AFB₁. Because ZEN and AFB₁ did not show identical monotonic sigmoid-shape response over a wide range of concentrations that did not satisfy the requirements of this method. Concentrations of the three mycotoxins were selected that showed monotonic sigmoid-shape response curves (3.3–16.7 μ M for DON, 10–50 μ M for ZEN and AFB₁), Fig. S2. The type of interaction depends on the different combinations of mycotoxins, as shown in Table 1. The binary (DON + ZEN) mixtures showed very strong antagonism effect. The binary (DON + AFB₁) showed strong synergism. Meanwhile, exposed to a combination of ZEN and AFB₁ showed nearly additive effect at low concentrations and antagonism effect at high concentrations. For the combination DON + ZEN + AFB₁, very strong antagonism effect was observed at low concentrations, whereas additive effect and synergism effect were seen at high concentrations.

3.2. Apoptosis-related gene mRNA test

The cellular toxicity test illustrated that the three kinds of

Table 1

Dose-response relationship parameters and combination index (CI) values of combined toxicity assessment based on Chou-Talaly method.

DON	ZEN	AFB ₁	Inhibition	CI	Indication
3.3	10.0		33.30%	14.224	-----
6.7	20.0		34.29%	22.806	-----
10.0	30.0		36.85%	19.676	-----
13.3	40.0		38.07%	20.318	-----
16.7	50.0		39.09%	20.588	-----
3.3		10.0	62.86%	0.064	+++++
6.7		20.0	64.25%	0.109	+++++
10.0		30.0	68.17%	0.107	+++++
13.3		40.0	68.32%	0.140	+++++
16.7		50.0	68.72%	0.169	+++++
	10.0	10.0	18.11%	1.080	\pm
	20.0	20.0	23.69%	1.556	---
	30.0	30.0	31.68%	1.600	---
	40.0	40.0	32.17%	2.090	---
	50.0	50.0	37.42%	2.113	---
3.3	10.0	10.0	28.66%	42.783	-----
6.7	20.0	20.0	58.83%	0.522	+++
10.0	30.0	30.0	55.81%	1.019	\pm
13.3	40.0	40.0	60.92%	0.896	+
16.7	50.0	50.0	65.98%	0.828	++

CI < 0.1 indicates very strong synergism (graded symbols: + + + + +); CI: 0.1–0.3 indicates strong synergism (+ + + +); CI: 0.3–0.7 indicates synergism (+ + +); CI: 0.7–0.85 indicates moderate synergism (+ +); CI: 0.85–0.90 indicates slight synergism (+); CI: 0.90–1.0 indicates nearly additive (\pm); CI: 1.10–1.20 indicates slight antagonism (–); CI: 1.20–1.45 indicates moderate antagonism (–); CI: 1.45–3.3 indicates antagonism (–); CI: 3.3–10 indicates strong antagonism (—); CI > 10 indicates very strong antagonism (——) (Chou, 2006).

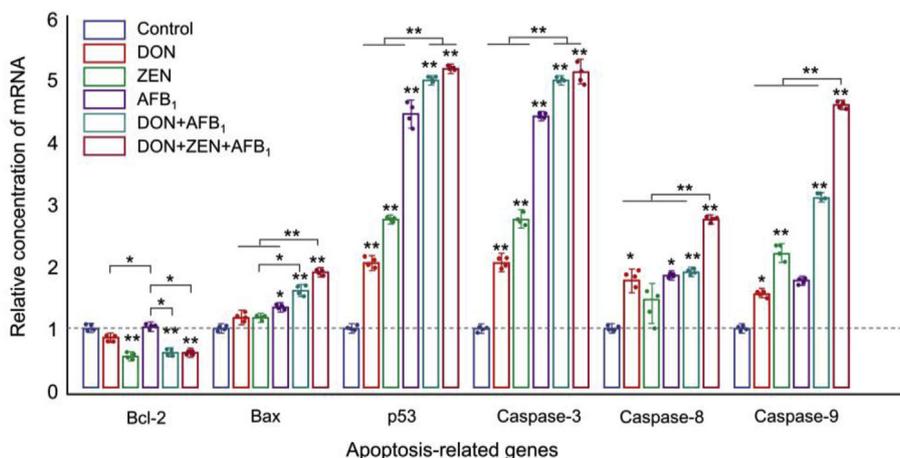


Fig. 1. Effects of mycotoxin alone and coexisting on the relative content of apoptosis-related gene mRNA in Caco-2 cells. The Dunnett's multiple comparison test was implemented for statistical analysis of the significance between these genes. Raw *p* value was labelled. “***” represents *p* value < 0.01, “*” represents *p* value < 0.05.

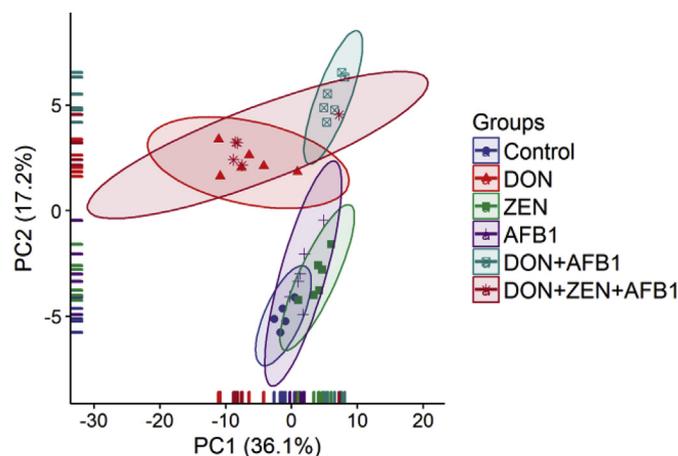


Fig. 2. Metabolomics profiles of individual and combined mycotoxins exposed groups. (A) Non-supervised principal component analysis (PCA) scores plot. Variance of PC1 is 36.1% and variance of PC2 is 17.2%.

mycotoxins co-treated Caco-2 cells would leading to combined toxicity. CI values were calculated to evaluate the combined toxicity based on Chou-Talaly method, which revealed that DON + AFB₁ and DON + ZEN + AFB₁ combined mode would result different levels of synergism. For deeper understanding of the combined effect and its mechanism of action, the effects of relative mRNA levels of six common apoptosis-related genes in mycotoxin-induced apoptosis were investigated (Kang and Reynolds, 2009). Compared with the control group, DON, ZEN and AFB₁ toxin significantly up-regulated the p53, caspase-3, caspase-8 and caspase-9 genes, but Bcl-2 and Bax fluctuated only in the combined mycotoxins groups, DON + AFB₁ and DON + ZEN + AFB₁. AFB₁ plays a major role in reducing the expression of Bcl-2 in the mixture of DON + AFB₁ and DON + ZEN + AFB₁. When DON + AFB₁ coexisted or DON and AFB₁ alone all significantly up-regulated p53, caspase-3 and caspase-9 genes, and DON + AFB₁ did not significantly revealed combined toxicity to the rest genes, as shown in Fig. 1. Furthermore, DON + ZEN + AFB₁ promoted the expression of these six genes more significantly than DON + AFB₁ and the individual toxins groups. The results showed that the coexistence of DON + AFB₁ and DON + ZEN + AFB₁ can promote apoptosis, which is closely related to down-regulation of Bcl-2 gene and up-regulation of Bax, p53, caspase-3, caspase-8 and caspase-9 genes, which was supported by the literature on the apoptosis of BRL cells. As it is published that the coexistence of DON + AFB₁ has a synergistic

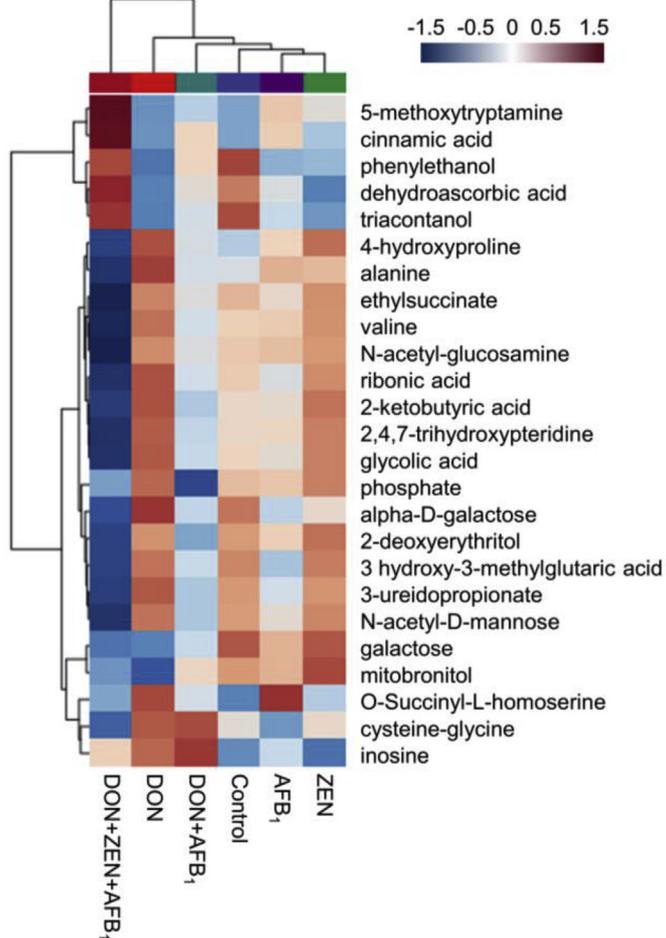


Fig. 3. Heat map of the cluster analysis of the first 20 metabolites with *p* < 0.05 calculated by one-way ANOVA. The signals corresponding to different treatments were compared after treatment of log transformation and Pareto scaling. Clustering result was calculated with distance measure using Euclidean, and clustering algorithm using ward.D.

effect on cellular cytotoxicity by up-regulating Hsp70, p53, Bax, caspase-3, caspase-8 genes, down-regulating Bcl-2 gene, inducing increasing intracellular ROS, promoting apoptosis, and decreasing cell activity (Zhou et al., 2017; Kroemer and Reed, 2000). Preliminary

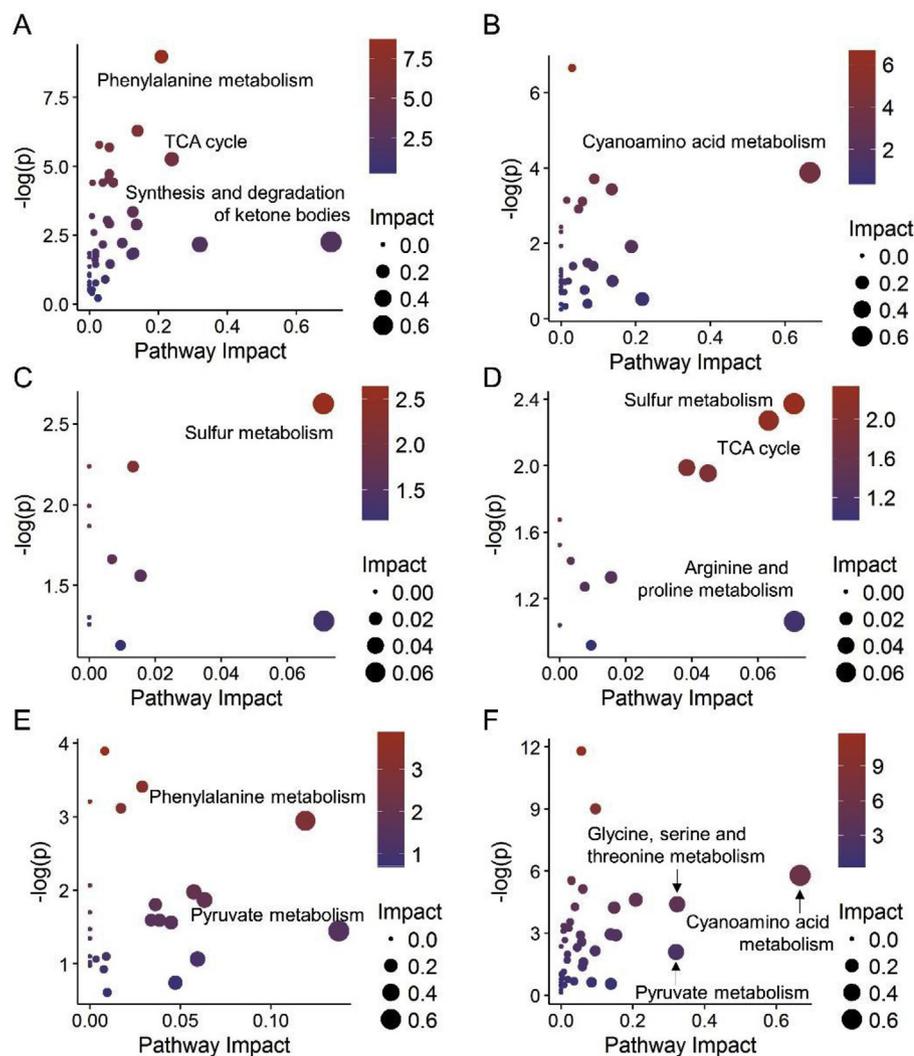


Fig. 4. The pathway analysis of the identified metabolites affected in the mycotoxins treatment groups. Significantly changed pathways based on enrichment and topology analysis are shown. The x-axis represents pathway enrichment, and the y-axis represents pathway impact. Large sizes and dark colors represent major pathway enrichment and high pathway impact values, respectively. (A) The metabolites with p value (one-way ANOVA) < 0.05, the metabolites with p value (*t*-test) < 0.05 in individual mycotoxins groups DON (B), ZEN (C), AFB₁ (D), and combined mycotoxins groups DON + AFB₁ (E) and DON + ZEN + AFB₁ (F), were selected for pathway enrichment. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

analysis of the molecular mechanism of DON, ZEN and AFB₁ on Caco-2 cytotoxicity showed that once the three mycotoxins enter Caco-2 cells, it causes oxidative stress, the mitochondrial function changes ROS, further up-regulates the pro-apoptotic gene and down-regulates genes cause cellular apoptosis and even necrosis.

3.3. Multivariate statistical analysis

Based the results of combined cytotoxicity, only the binary combination (DON + AFB₁) and tertiary combination (DON + ZEN + AFB₁) showed higher inhibition of viability than individual mycotoxins and exhibited synergistic effect. For studying the mechanism of combined cytotoxicity, we selected these two combinations in the follow-up experiments. We divided the cell samples into six groups: the control group, the DON stimulating group (6.7 μM), the ZEN stimulating group (20 μM), the AFB₁ stimulating group (20 μM), the DON + AFB₁ stimulating group (6.7 + 20 μM), and the DON + ZEN + AFB₁ stimulating group (6.7 + 20 + 20 μM). In this paper, multivariate statistical analysis included PCA, PLS-DA and hierarchical cluster analysis. The result of PCA scores plot are shown in Fig. 2. The PCA results revealed that metabolites in different groups were separated into clusters. Although the cluster of dots of the treatment groups, expect for the ZEN and AFB₁ exposed groups, did not show clear separation from controls, they were not significantly different between the four treatment groups. Obvious separation between control groups and DON, DON + AFB₁ and DON + ZEN + AFB₁ exposed groups indicates generated variation

in cellular biological systems, leading to disparity of metabolic profiles and diversity of metabolites.

3.4. Metabolites screening

Heatmap analysis was performed to construct clusters of identified metabolites based on similarity of metabolic changes and biochemical relatedities. To better display the metabolites' concentration differences and similarities in the six groups, we selected the first 20 metabolites ($p < 0.05$ by one-way ANOVA) for hierarchical Pearson clustering, as shown in Fig. 3. Each cell in the heatmap represents a metabolite and color changes of cells from blue to red indicate the downregulation and upregulation of the metabolites, respectively. The group average values were used to better visualize the variety between groups, while biochemically related metabolites were generally clustered together on the y axis. It could be clearly noticed that the relative concentrations of most metabolites of the control group were elevated. The individual groups of ZEN, AFB₁ and DON, show a similar pattern compared to a control group. However, in the two combined groups, DON + AFB₁ and DON + ZEN + AFB₁, majority of metabolites shows down-regulation which indicated that the individual groups might have a weaker toxicity. On the other hand, the result of combined groups is contrary to the control, which suggests that combined mycotoxins have a greater effect on metabolic responses. These data support, once more, the hypothesis that the combined mycotoxins can lead to a synergistic or additive effect in the metabolism of Caco-2 cells. To determine the type of

4. Conclusions

It was found that DON + AFB₁ and DON + ZEN + AFB₁ were more toxic than the single toxicity at most dose points; CI method showed that DON + AFB₁ and DON + ZEN + AFB₁ have strong synergistic effect. The apoptosis-related gene mRNA in Caco-2 cells revealed that the coexistence of DON + AFB₁ and DON + ZEN + AFB₁ can promote apoptosis, which is closely related to down-regulation of Bcl-2 gene and up-regulation of Bax, p53, caspase-3, caspase-8 and caspase-9 genes. The cellular metabolic pathway analysis was implemented for discovering the synergistic effect on metabolism. Results revealed that The DON + AFB₁ group and the DON + ZEN + AFB₁ group, which display synergism with CI mode analysis, effected the amino acid, organic acid, and fuel and energy metabolism, which may be on the key factor of leading to the cellular apoptosis and even necrosis.

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Appendix A. Supplementary data

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Transparency document

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