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Hepatotoxic effects of mycotoxin combinations in mice

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ABSTRACT

This study was performed to assess the individual and combined toxic effects of aflatoxin B_1 (AFB_1), zearalenone (ZEA) and deoxynivalenol (DON) within the liver of mice. A total of 56 4-week-old weanling female mice were divided into seven groups ($n = 8$). For 2 weeks, each group received an oral administration of either solvent (control), AFB_1, ZEA, DON, AFB_1 + ZEA, AFB_1 + DON or ZEA + DON per day. The results showed that AFB_1, ZEA and DON induced liver injury, indicated by elevated relative liver weight, activities of alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST), as well as decreased albumin (ALB) and/or total protein (TP) concentration in the serum. These mycotoxins also decreased hepatic total antioxidant capacity (T-AOC), and/or increased the concentration of malondialdehyde (MDA). Moreover, AFB_1 + DON displayed synergistic effects, while AFB_1 + ZEA displayed antagonistic effects on those parameters previously described. Furthermore, the apoptotic potential was demonstrated associated with an upregulation of the apoptotic genes Caspase-3 and Bax, along with a downregulation of the antiapoptotic gene Bcl-2 in liver. In conclusion, this study provides a better understanding of the toxic effects of AFB_1, ZEA, DON, alone or in combinations on the liver of mice, which could contribute to the risk assessment of these mycotoxins in food and feed.

1. Introduction

Mycotoxins are secondary metabolites of fungi, which are toxic to both humans and animals and are primarily produced by five genera: *Aspergillus, Fusarium*, *Penicillium*, *Alternaria* and *Claviceps* (Steyn, 1995). Contamination with these toxins has been detected in different countries in the majority of agricultural products including maize, wheat, barley, millet, peanuts, peas, oily feedstuffs, dried fruits, and forage. These toxins have been shown to affect approximately 25% of the world's agricultural commodities each year (Casado et al., 2001; Kanora and Maes, 2009). Humans or animals ingesting the food or feed contaminated by mycotoxins can pose serious adverse effects in the health and/or productivity, and thus result in significant economic losses (Zain, 2011).

Thus far, approximately 400 mycotoxins have been identified in more than 100 fungi strains (Jard et al., 2011). Aflatoxin B_1 (AFB_1), zearalenone (ZEA) and deoxynivalenol (DON) are recognized as the major mycotoxins presented in agricultural products and therefore often coexist in food and animal feed (Monbaliu et al., 2010; Richard, 2007; Solfrizzo et al., 2014; Streit et al., 2012). Among the various toxic mycotoxins, AFB_1 is the most lethal, largely produced by the fungi *Aspergillus*, exhibiting harmful hepatotoxic, mutagenic, teratogenic and carcinogenic effects on many species of animals. It is also classified as a Group one carcinogen (International Agency for Research on Cancer (IARC), 1987). ZEA is an estrogenic mycotoxin produced by Fusarium, exhibiting a similar structure to estrogen and therefore competing with 17β-estradiol for binding to the estrogen receptor, consequently leading to problems in fertility and reproductive ability (Takemura et al., 2007). DON is a member of the trichothecenes family and mainly produced by *Fusarium*. Ingestion of DON-contaminated feed can cause anorexia, vomiting, and impair immune function in various livestock. DON also induces apoptosis in hemopoietic progenitor cells (Parent-Massin, 2004) and inhibits protein, DNA and RNA synthesis (Audenaert et al., 2013).

The symptoms of mycotoxicosis depend not only on the type of mycotoxin, but also to the relative amount and duration of exposure as well as the species, age, sex, health, environment, nutritional state and particularly for the interactions with other toxic insults (Bennett and Klich, 2003; Kanora and Maes, 2009). Since crop contamination with mycotoxins is a very complex process and often involves more than one type of mycotoxin (Murphy et al., 2006; Streit et al., 2012), many complex toxic interactions can develop as a result. A few studies have evaluated the cytotoxic effects of a

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combination of different fusarium mycotoxins such as fumonisin B_1 (FB_1), DON, and ZEA (Kouadio et al., 2007), beauvericin (BEA), T-2 toxin (T-2) and DON (Ruiz et al., 2011), enniatin B (ENB), BEA, DON, FB_1, T-2 and ZEA (Ficheux et al., 2012), as well as nivalenol (NIV), DON, ZEA and FB_1 (Wan et al., 2013a, 2013b) on various cell lines. These reports showed that the binary and ternary various combinations of the fusarium mycotoxins can result in additive, synergic, as well as antagonist toxic effects. Although AFB_1 often coexists with fusarium mycotoxins (ZEA and DON) in food and animal feed, the toxic effects of the combination of AFB_1, ZEA and DON on animals remain limited. The purpose of this research was to therefore investigate the combined toxic effects between AFB_1 + ZEA, AFB_1 + DON and ZEA + DON on mice.

2. Materials and methods

2.1. Mice, treatments, and samples collection

Our animal protocol was approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University, China. A total of 56 weanling BALB/c female mice (4-week-old; Experimental Animal Center, Wuhan University, Wuhan, China) were divided into seven groups ($n = 8$). All mice were allowed free access to a commercial rodent diet (Yamaguchi et al., 2005) and water ad libitum. After a week of acclimation, each group received an oral administration of solvent (control), AFB_1, ZEA, DON, AFB_1 + ZEA, AFB_1 + DON and ZEA + DON per day, respectively, via gavage needle. The solvent for AFB_1, ZEA and DON are 1.0% dimethyl sulfoxide (DMSO), ethanol, and water, respectively. Our preliminary experiment showed that these solvents did not have toxic effect on mice (data not shown). The administered doses of AFB_1, ZEA and DON are 2.5, 5.0 and 5.0 mg/kg BW, respectively. The subchronic toxicity test doses were chosen based on previous studies reported 1/20–1/100 LD_{50} of these toxins in mice (Pestka, 2007; Yunus et al., 2011; Zinedine et al., 2007). All the mycotoxins, DMSO and ethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). The administration continued for 2 weeks. Mice were monitored daily for mortality, along with body weights measured weekly. At the end of the study, mice from each treatment group were dissected to collect blood and liver. The serum was prepared by centrifugation of the whole blood at 1000× *g* for 15 min at 4 °C and stored at –80 °C before use. The livers were removed and weighed immediately (data expressed as relative liver weight/BW, %). Livers were then perfused with ice-cold isotonic saline immediately after being dissected and divided into aliquots, snap-frozen in liquid nitrogen, and stored at –80 °C until use (Sun et al., 2013).

2.2. Serum biochemical and hepatic antioxidant status analysis

The serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), along with concentrations of total protein (TP) and albumin (ALB) were determined in serum samples. Analyses of the serum samples were measured by an automatic biochemistry analyzer (Hitachi s7020, Tokyo, Japan) with commercial kit. Specifically, activates of ALT and AST were measured by colorimetric methods (Reitman and Frankel, 1957). TP was measured by the biuret method (Skeggs and Hochstrasser, 1964). ALB was determined by automated dye-binding method (Pinnell and Northam, 1978). Liver samples (0.5 g) were thawed in 4.5 ml isotonic saline on ice and homogenized as previously described (Wu et al., 2013b). The supernatants were then prepared by centrifugation at 12,000× *g* for 15 min at 4 °C. The activity of total antioxidant capacity (T-AOC) and the concentration of malondialdehyde (MDA) were measured by the colorimetric method through the specific assay kits (A015 and A003) purchased from the Nanjing Jiancheng Bioengineering Institute of China. Specifically, T-AOC was determined by the ferric reducing ability of plasma (FRAP) method (Benzie and Strain, 1996). The reaction measures antioxidant reduction of Fe^{3+}-TPTZ (tripyridyl triazine) to the blue colored Fe^{2+}-TPTZ. The optical density was measured at 593 nm. Thiobarbituric acid reaction (TBAR) method was used to measure the MDA which can react with thiobarbituric acid (TBA) to form a stable chromophoric production. The optical density was measured at 532 nm (Ohkawa et al., 1979).

Table 1
Primers for Q-PCR analysis of apoptosis genes in the liver.[a]

	Forward primer (5′ to 3′ direction)	Reverse primer (5′ to 3′ direction)
Bcl-2	GCTACCGTCGTGACTTCGC	CCCCACCGAACTCAAAGAAGG
Bax	CAGGATGCGTCCACCAAGAA	GCAAAGTAGAAGAGGGCAACCAC
Caspase-3	CTGACTGGAAAGCCGAAACTC	CGACCCGTCCTTTGAATTTCT
GAPDH	TGACCTCAACTACATGGTCTACA	CTTCCCATTCTCGGCCTTG

[a] *Bcl-2*, B-cell lymphoma 2; *Bax*, Bcl-2-associated X protein; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

2.3. Real-time qPCR analyses

Total RNA was prepared from liver samples by using Trizol (Invitrogen) and following the manufacturer's instructions. The quality and quantity of RNA were analyzed by Agilent Bioanalyzer 2100 using an RNA 6000 Labchip kit (Agilent Technologies, Amstelveen, Netherlands). The cDNA was synthesized from 1 μg total RNA by using Super Script III reverse transcriptase (Invitrogen) and following the manufacturer's instructions. The mRNA levels of pertaining genes (Bcl-2, Bax and Caspase-3) involved in apoptosis were determined by qPCR (7900 HT; Applied Biosystems) using the SYBR® Green PCR Master Mix (Applied Biosystems, USA) and following the manufacturer's instructions. The genes assayed and primer sequences used for each gene are presented in Table 1. The 2^{-ddCt} method was used for the quantification with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene, and the relative abundance was normalized to the control (as 1) (Sun et al., 2013).

2.4. Statistical analysis

Data were analyzed by two-way ANOVA to test the main effects of individual and interaction of AFB_1 and ZEA, AFB_1 and DON, as well as ZEA and DON, respectively. The Bonferroni *t* test was followed for multiple mean comparisons if there was a main effect. All analyses were conducted using SAS 8.2 (SAS Institute). Data are presented as means ± SD ($n = 4$–5) and significance level was set at $P < 0.05$.

3. Results

3.1. Growth rate and organ weight

Non-significant differences in initial and final body weight were observed among the seven groups (Table 2) except for relative liver weight, which was significantly affected ($P < 0.05$) by oral administration of DON alone (Table 2). Compared to the control, the relative liver weight of mice was increased ($P < 0.05$) 15.2% by administration of DON alone. No main effect of either AFB_1 alone, ZEA alone or any combinations of AFB_1, ZEA and DON was seen on the relative liver weight.

3.2. Serum biochemistry

The results showed that the serum biochemical parameters were significantly affected by administration of AFB_1, ZEA and/or DON alone, and/or their interactions (Table 3). Specifically, AFB_1, ZEA and DON individually exerted a main effect ($P < 0.05$) on ALT and AST, ALT, and ALT, AST, TP and ALB, respectively. Moreover, ALT and AST

Table 2
Effects of individual and combined AFB_1, ZEA and DON on body weight and liver weight in mice.[a]

	Control	AFB_1	ZEA	DON	AZ	AD	ZD	*P*-value					
								A	Z	D	AZ	AD	ZD
Initial weight, g	16.7 ± 1.0	16.4 ± 0.9	16.5 ± 1.0	17.0 ± 1.1	17.1 ± 0.6	16.6 ± 1.0	16.5 ± 1.1	ns	ns	ns	ns	ns	ns
Final weight, g	18.6 ± 0.7	18.1 ± 0.9	18.3 ± 1.2	18.4 ± 1.6	18.1 ± 1.3	18.2 ± 0.7	18.1 ± 0.9	ns	ns	ns	ns	ns	ns
Liver weight/BW, %	5.41 ± 0.51	5.58 ± 0.25	5.40 ± 0.33	6.23 ± 0.13	4.83 ± 0.16	6.57 ± 0.25	5.96 ± 0.40	ns	ns	*	ns	ns	ns

A, AFB_1; Z, ZEA; D, DON; AZ, AFB_1 + ZEA; AD, AFB_1 + DON; ZD, ZEA + DON.
[a] Values are means ± SD, $n = 4$.
* Means the factor or their interaction has a significant effect, $P < 0.05$.