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Acute oral toxicity test and assessment of combined toxicity of cadmium and aflatoxin B_1 in kunming mice

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ABSTRACT

Cadmium and aflatoxin B_1 (AFB_1) are both common and widespread pollutants in food and feed. There are several reports on toxicity induced by Cadmium or AFB_1 alone, but few address the toxicity caused by co-exposure to the two substances. In this study, 42 female and 42 male Kunming (KM) mice were divided into seven groups to test the acute oral toxicity of $CdCl_2$ and AFB_1, using Karber's method. The combined toxicity was assessed using the Keplinger evaluation system. Acute toxicity symptoms, deaths, and body and organ weights were evaluated, and hematological, blood biochemical, and histopathological analyses were conducted. The results revealed the following median lethal doses (LD_{50}): $LD_{50(\text{Female KM mice})}$ = 62.56 mg/kg; $LD_{50(\text{Male KM mice})}$ = 48.79 mg/kg; $LD_{50(\text{KM mice})}$ = 55.27 mg/kg. The combined toxicity of AFB_1 and $CdCl_2$ showed an additive effect in mice, and an increase in the mixed dose of AFB_1 and $CdCl_2$ resulted in greater toxicity. These results demonstrated that the combined toxicity of AFB_1 and $CdCl_2$ was greater than the toxicities of the individual components in mice; thus, this may cause particular challenges when addressing these hazards in food and feed and the associated risk to human and animal health.

1. Introduction

Cadmium and AFB_1 are both widespread toxicants found in a variety of animal feed and human foods (Zhang et al., 2015), and are recognized as severely hazardous pollutants to animal and human health (IRAC, 2012). Cd is a toxic heavy metal that has a variety of adverse effects on the health of humans and animals (Qixiao Zhai et al., 2014), it generally exists as a divalent cation, complexed with other elements (e.g., $CdCl_2$) (Bernhoft, 2013). Cd has a strong toxic effect, is an ubiquitous environmental pollutant with an elimination half-life of 10–30 years, and accumulates in humans and animals (Nawrot et al., 2006). Previous studies have found that Cd accumulates predominantly in the kidney and liver, which are the critical targets for acute Cd toxicity (Gunnar F. Gunnar et al., 2007), and has resulted in public health concerns (Nordberg, 2009). After absorption, Cd is transported throughout the body, usually bound to a sulfhydryl group-containing protein, such as metallothionein (Bernhoft, 2013). Approximately 30% is deposited in the liver and 30% in the kidney, with the remainder distributed throughout the body (T.M. Davis et al., 2014). Cd is known to increase oxidative stress by acting as a catalyst for the formation of reactive oxygen species, increasing lipid peroxidation, and depleting glutathione and protein-bound sulfhydryl groups (Bagchi, 1995; CSEM, 2008); in addition, it can also stimulate the production of inflammatory cytokines and downregulate the protective action of nitric oxide formation (Navas-Acien et al., 2004). Although there are very strict limits to control the residual levels of Cd in the food or feed, it is still a common metal contaminant (Satarug et al., 2017).

Aflatoxins (AFs) are naturally-occurring mycotoxins that are produced by various Aspergillus species including *A. flavus*, *A. parasiticus*, and *A. nominus*. As secondary metabolites of these fungi, AF may contaminate a variety of foods and feedstuffs (Rawal et al., 2010). Among the known AFs, AFB_1 is the most commonly encountered, is considered

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the most toxic, and is classified as a human carcinogen (Wogan, 1992; Yunus et al., 2011). AFB_1 is hepatoxic and hepatocarcinogenic in several animal species and epidemiological studies suggest that it may also be an important factor in the etiology of human liver cancer (Steven A. Leadon et al., 1983; Wogan, 1973). AFB_1 is metabolically biotransformed in the liver by CYP1A2 and 3A (3A4, 3A5, and 3A7) enzymes into several highly reactive electrophilic metabolites that interact with cellular macromolecules, which are either secreted out of the body or react with cellular macromolecules such as DNA and proteins (Eaton and Gallagher, 1994; Guengerich et al., 1998; Nayak and Sashidhar, 2010; Partanen et al., 2010).

The potential for the combined toxic action of chemicals has been recognized by diverse groups: scientists, toxicologists, health risk assessors, and environmentalists (Parvez. et al., 2009). As common food and feed contaminants, AFB_1 and Cd may exist in the same food or feed (Eskandari and Pakfetrat, 2014; Gloag, 1981; Silano and Silano, 2017). However, to the best of our knowledge, there are no toxicity data for the combined toxicity of a mixture of AFB_1 and Cd.

The KM mouse is an outbred stock derived from Swiss albino mice with a high level of genetic heterogeneity (Sun, 2001); the original colony of mice in China was started from Swiss mice brought to Kunming city, China, from the Indian Haffkine Institute in 1944. It was subsequently dispersed to researchers and commercial dealers nationwide. Thereafter, these mice were called Kunming mice (Shang et al., 2009). After being bred for several decades, the KM mouse is now considered a native breed (Cui et al., 2013). These mice are internationally recognized and were included in International Index of Laboratory Animals and named KM mice in 1993. Because of the high resistance of these animals to disease, their good adaptive capacity, high breeding coefficient, and good survival rates, this line has been widely utilized in pharmacological (Yang. et al., 2001), toxicological (Jiang et al., 2017, 2018; Liu et al., 2013), pathogenicity (Huang et al., 2013; Li et al., 2012; Ti et al., 2016), neuroscience (Tang et al., 2015), immunology (Guo. et al., 2018; Xu et al., 2014), and genetic (Shang et al., 2009) research and testing in Chinese laboratories.

However, to our knowledge, few data are available about the combined acute toxicity of AFB_1 and $CdCl_2$ in KM mice. Thus, in this study, we used KM mice as our experimental animals to explore the acute oral toxicity and the combined toxicity effect of $CdCl_2$ and AFB_1. In addition, acute toxicity symptoms, mortality rates, and body and organ weights as well as hematological, blood biochemical, and histopathological changes were examined. It is expected that the results should provide valuable information on the $CdCl_2$ and AFB_1 co-exposure for the assessment of acute toxicity and the subsequent effects on human and animal health.

2. Materials and methods

2.1. Animals and treatment

Healthy male and female KM mice, 20 ± 2 g, were supplied from Chengdu Dashuo Experimental Animal Company (Chengdu, Sichuan Province, China). KM mice were housed in clean polypropylene cages and maintained in an air-conditioned animal house at 20 ± 2 °C, 50–70% relative humidity, under a 12 h light/dark cycle; six male or female mice were housed per cage. The cleaning processes of the cages, including checking of the water supply, were performed daily. The animals were provided with a standard commercial mouse pellet diet (Chengdu Dashuo Experimental Animal Company, Chengdu, Sichuan Province, China) and ultrapure water ad libitum. The Cd content of the commercial mouse pellet diet was 0.08 ppm and the content of AFB_1 was ≤0.02 ppm, which met the quality requirements for laboratory animals. The general quality standard for formula feeds (National Standardization of China, GB 14924.1-2002) by graphite furnace atomic absorption spectrometry was used for the determination of Cd (National Standardization of China, GB/T 5009.15-2014) and enzyme-linked immunosorbent assay (National Standardization of China, GB/T 17480-2008) was used for the determination of AFB_1.

2.2. Reagents

A standard sample of AFB_1 was purchased from Sigma-Aldrich (Shanghai, China). Cadmium chloride ($CdCl_2$.2.5 H_2O) was purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Corn oil was purchased from Shanghai Jingchun Reagent Co., Ltd. (Shanghai, China). Sterile stock solutions of AFB_1 (5 mM) were prepared in corn oil and $CdCl_2$ (10 mM) was prepared in ultrapure water.

2.3. Acute oral toxicity

Several methods are described to compute LD_{50} values, including the Limit Tests, Dose-Probing Tests, Up-and-Down Tests, and Pyramiding Tests (FDA, 2000a,b; OECD, 2001). Among the several fixed-sample methods, the most widely used Karber estimator is not only quite simple to use but is also considered efficient (Jung. and Choi., 1994; Klassen., 1991; Rath. et al., 2011; Zhang. et al., 2012). The acute oral toxicity for calculating LD_{50} was performed in accordance with the National Health and Family Planning Commission of the People's Republic of China (NHFPC) standard GB15193.3–2014 "Karber's method" (NHFPC, 2014), which are detailed therein.

2.3.1. Dosage selection

In this study, $CdCl_2$ and AFB_1 were used in an equitoxic mixture ratio. The fixed mixture ratio was based on single toxicant LD_{50} value for an oral acute test. Based on previous research, the LD_{50} of $CdCl_2$ for mouse is 150 mg/kg (Zhang, 2007), and the LD_{50} of AFB_1 for mouse is 9 mg/kg (Chen. et al., 2001).

Following Karber's method, before the formal acute oral toxicity test, a preliminary acute oral toxicity experiment was conducted and the highest dose at which all animals died was $2\ LD_{(50,\ AFB1)} + 2\ LD_{(50,\ CdCl2)} = 18 + 300$ (mg/kg), and the maximum nonfatal dose was $0.1\ LD_{(50,\ AFB1)} + 0.1\ LD_{(50,\ CdCl2)} = 0.9 + 15$ (mg/kg). The endpoint doses were converted into a common logarithm, and then the logarithmic difference between the highest and maximum nonfatal dose was divided into several logarithmic isometric dose groups based on the required number of groups.

From the results of this preliminary experiment of mixed AFB_1 and $CdCl_2$ for equal-toxicity ratio and five dose levels, the mice grouping and dose levels were set as shown in Table 1.

2.3.2. Experimental groups

After a 5-day acclimation period, 84 mice were randomly divided into seven groups, with six female mice and six male mice in each group, showed in Table 1. The mice were fasted for 12 h before treatment.

Mice in Group 1 to Group 5 were administered 0.9 + 15, 1.9 + 31.7, 4.0 + 66.9, 8.4 + 141.2, and 17.7 + 297.9 mg/kg·bw AFB_1 + $CdCl_2$, respectively, Group 6 was administered corn oil as the

Table 1
Mice grouping and dose level.

Group	Dose levle (20 mL/kg.bw)	Male + Female (number)
1	$AFB_1 + CdCl_2$ = 0.9 + 15 (mg/kg)	6 + 6
2	$AFB_1 + CdCl_2$ = 1.9 + 31.7 (mg/kg)	6 + 6
3	$AFB_1 + CdCl_2$ = 4.0 + 66.9 (mg/kg)	6 + 6
4	$AFB_1 + CdCl_2$ = 8.4 + 141.2 (mg/kg)	6 + 6
5	$AFB_1 + CdCl_2$ = 17.7 + 297.9 (mg/kg)	6 + 6
6	Corn oil	6 + 6
7	Ultrapure water (Control)	6 + 6

Note: Combined dosages were equitoxic ratio mixing of AFB_1 and $CdCl_2$.

Table 2
Effect of AFB_1 and $CdCl_2$ on deaths and mortality rates in KM mice [n(d)].

Group	Female		Male		Female + Male	
	Death counts (day)	Mortality rate (n = 6)(%)	Death counts (day)	Mortality rate (n = 6)(%)	Death counts (day)	Mortality rate (n = 12)(%)
1	0(14)	0	0(14)	0	0(14)	0
2	1(0)	16.7	2(0)	33.3	3(0)	25.0
3	1(1),2(2)	50.0	1(0),3(1)	66.7	1(0),4(1),2(2)	58.3
4	2(0),4(1)	100	1(0),5(1)	100	3(0),9(1)	100
5	6(0)	100	6(0)	100	12(0)	100
6	0(14)	0	0(14)	0	0(14)	0
7	0(14)	0	0(14)	0	0(14)	0

solvent group, and Group 7 was administered ultrapure water as the control group. The intragastric intubation dose level was based on 0.2 mL suspension liquid per 10 g body weight. After administration, the mice were fasted for 2 h, and then allowed to consume water or feed ad libitum. They were then observed continuously for signs of morbidity and mortality during the 14 day treatment period.

2.3.3. Body weight, organ weights, hematological analysis, blood biological analysis, histopathological analysis, and LD_{50} measurement

Body weights were measured before administration and at daily intervals after administration.

Clinical signs and mortality rates of the mice were observed daily for 14 days with each group.

The mice were drugged, and after 4 h, blood samples were collected via the ocular vein (approximately 0.8–1.0 mL from each mouse) for serum chemistry tests and determination of hematological parameters. The hematological parameters were measured by using V-52D reagent kit (Mindray, P.R. China) and a BC-5000Vet Auto Hematology Analyzer (Shenzhen Mindray Bio-Medical Electronics Co., Ltd. Nanshan, Shenzhen, China). The blood biochemical analysis was conducted by using IDEXX Catalyst™ Chem CLIP test packages and an automated IDEXX Catalyst™ Chemistry Analyzer (IDEXX Laboratories, Inc., Westbrook, Maine, USA).

After 14 days, all mice were sacrificed by cardiac exsanguination under anesthesia and gross pathological examination was performed. Body and relative organ weights for the heart, liver, spleen, lung, and kidney were measured and calculated. Two small blocks of the organ tissues from each group were fixed in 10% paraformaldehyde solution for further histopathological examinations. The tissue samples were embedded in paraffin blocks, sliced, and placed on glass slides. After haematoxylin and eosin (HE) staining, the pathological changes were observed by using an optical microscope.

The LD_{50} at the 95% confidence limit was calculated by using Karber's method, and the combined toxicity effect was determined using the Keplinger evaluation system (NHFPC, 2014).

2.4. Statistical analysis

Data from the control group and treated groups were statistically evaluated by using SPSS 22.0 software package program for Windows. The means and standard deviations were calculated for the measurement of data in each group, which included body weight, organ weights, and clinical pathological data. All results are expressed as the mean ± standard deviation (± SE). Levene's test of the homogeneity of variance was performed: if the variances were homogeneous, the single factor analysis of variance was performed for inter-group comparison; if Analysis of Variance (ANOVA) showed significant differences, Dunnett's test was performed, and if the variances were not homogeneous, a non-parametric test was performed for inter-group comparisons.

2.5. Ethic statement

The animal experiment was conducted in strict accordance with the guiding principles of the Regulations for the Administration of Affairs Concerning Experimental Animals in China (Revised in 2017), and the experimental protocol was approved by Animal Health and Care Committee of Sichuan Agricultural University (License No.: SYXK, 2014-187), Sichuan, China.

3. Results

3.1. Acute toxicity symptoms

After treatment for 1 h, the mice in Groups 1 to 5 showed varying degrees of symptoms of listlessness and sluggishness, weak heartbeat, experienced convulsions, drooping upper eyelids, shortness of breath, and lack of perception of stimulus. This was especially noticeable in the highest-dose Group 5 in which the symptoms were more severe for female mice than for male mice.

In Group 1, mice recovered after 16 h, and all female and male mice survived. In Groups 6 and 7, throughout the whole experiment, all female and male mice were observed to be normal.

3.2. Mortality

Mortality rate is a measure of the number of deaths of the total number of mice in each group. The results of deaths and mortality rates are shown in Table 2. The first sign of death was observed after 4 h in male mice and after 4.5 h in female mice in the highest dose Group 5. All mice in Group 5 died within 4–24 h of treatment. For Groups 6 and 7, no deaths were observed.

3.3. LD_{50} and combined toxicity effect

The LD_{50} values for the equal toxicity mixture of AFB_1 and $CdCl_2$ by the oral route for KM mice are shown in Table 3.

The combined toxicity effect was determined by using the Keplinger evaluation system. The expected LD_{50} was 79.81 mg/kg, calculated from the equation of Finney (FDA, 2000a,b) (Equation (1)).

Table 3
LD_{50} values for combined doses of Cd and AFB1 in male and female Kunming Mice.

Gender	LD_{50}	95% confidence interval	
		Lower	Upper
Male	48.79	31.54	75.48
Female	62.56	40.44	96.77
Female + male	55.27	41.38	73.83

$$\frac{1}{\text{The expected LD50 of mixture}} = \frac{a}{\text{The LD50 of test substance A}} + \frac{b}{\text{The LD50 of test substance B}} + \cdots + \frac{n}{\text{The LD50 of test substance N}} \quad (1)$$

where,

a, b, …n = The mass ratio of each A or B or … … or N test substance to the mixture, and a + b + … + n = 1

The LD $_{50}$ for AFB_1 was 9 mg/kg and the LD_{50} for $CdCl_2$ was 150 mg/kg, therefore, the expected LD_{50} of the mixture for AFB_1 and $CdCl_2$ was 79.81 mg/kg.

Keplinger and Deichmann (M.L.Keplinger. and Deichmann., 1967) reported that when the combined effect coefficient K was between 0.57 and 1.75, this was indicative of a definite additive effect. In this study, the combined effect coefficient K was 1.27 for female, 1.63 for male, and 1.44 for both female and male KM mice, these results indicated that the AFB_1 with $CdCl_2$ had an additive acute oral toxicity effect in both female and male KM mice.

3.4. Body weight and organ/body weight ratio (g/100 g)

The body weight of each mouse was measured before administration and on Days 7 and 14 after administration (Fig. 1). The body weight of female and male mice from Groups 6 and 7 gradually increased. In Groups 1 to 3, the body weight of the surviving female and male mice increased more slowly than in Group 7. The increase in body weight in Groups 1 to 3 was significantly slower at the higher doses of AFB_1 and $CdCl_2$, particularly in male mice.

The organ/body weight ratio (g/100 g) of mice in each group is shown in Table 4. The relative weights of heart, liver, spleen, lung, and kidney of Group 6 (administered corn oil) were not significantly different from the Group 7 in female or male mice; this result confirmed that using corn oil as the solvent did not affect the relative organ weights. The heart/body weight ratios of female mice in all treatment groups (Group 1 to 5) were significantly higher than that of female mice in Group 7 ($p < 0.05$). For male mice, Groups 1, 4, and 5 showed a significantly higher heart/body weight ratios than in Group 7 ($p < 0.05$).

The liver/body weight ratios of female mice in Groups 2 and 3 and male mice in Group 1 were significantly higher than Group 7 ($p < 0.05$).

The spleen/body weight ratios of male mice in Groups 1 to 5 and female mice in Group 2 were significantly higher than that in Group 7 ($p < 0.05$).

The lung/body weight ratios of female and male mice in Groups 4 and 5 were both significantly higher than those mice in Group 7 ($p < 0.05$).

The kidney/body weight ratios showed that in Group 2 only, female and male mice were significantly different from the ratio in Group 7 ($p < 0.05$).

3.5. Hematological and blood biochemical analysis

The results of the hematological and blood biochemical analysis are shown in Table 5. The source of reference ranges in each hematological examination indicator were the service manuals of BC-5000Vet Auto Hematology Analyzer and automated IDEXX Catalyst™ Chemistry Analyzer for mice (see Table 5).

Eosinophils (EOS) of female and male mice in Groups 3 to 5 all were above the reference range and increased in a dose-related manner. Female mice in Groups 3 to 5 and male mice in Groups 4 to 5 were significantly different to Group 7 ($p < 0.05$).

Neutrophil percentages (NEUT %) of male mice in Groups 4 and 5, and female mice in Group 5 was above the reference range. Compared with Group 7, the female and male mice in Groups 4 and 5 were significantly different ($p < 0.05$). As the dose increased, NEUT % followed an increasing trend, male mice were higher than female mice in all groups.

Hematocrit (HCT) and Platelet count (PLT) were below the reference range in female and male mice in Groups 3 to 5, HCT showed a decreasing trend, and PLT showed a increasing trend as the dose increased. Mice in Groups 3 to 5 were significantly different than Group 7 ($p < 0.05$).

Compared with Group 7, the values for UREA, PHOS, ALT, ALKP and TBIL were above the reference range in Groups 3 to 5, and increased with an increase in dose, in both female and male mice. In contrast, the values for GLU (Groups 3 to 5) and CA (Groups 4 to 5) were below the reference range, and increased with a decrease in dose, in both female and male mice.

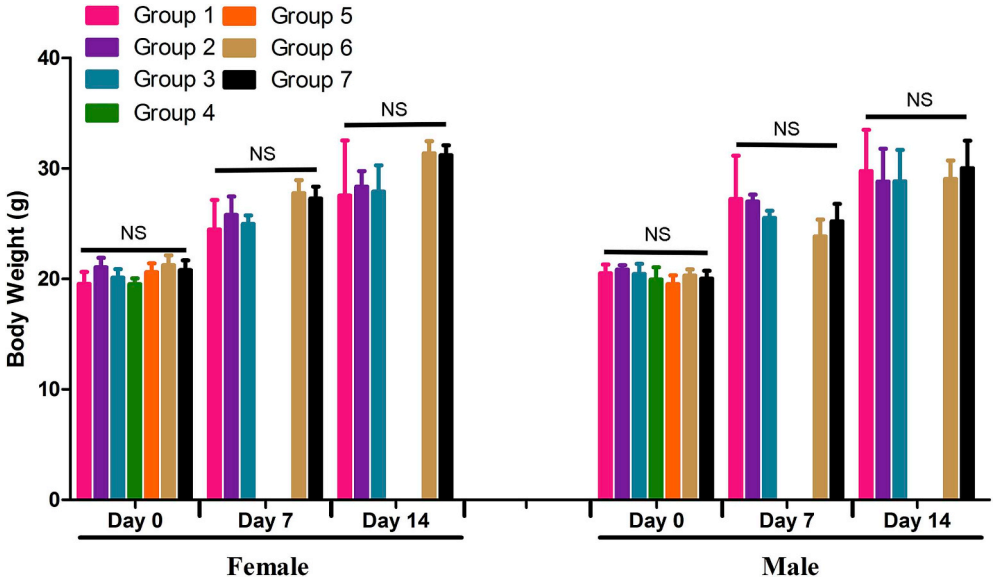

Fig. 1. Effect of treatment on body weight in mice (g). NS, not significant.

Table 4
Effects of treatment on organ/body weight ratios (g/100 g) in KM mice.

Group	Gender	Heart/body weight ratio	Liver/body weight ratio	Spleen/body weight ratio	Lung/body weight ratio	Kidney/body weight ratio
Group 1	Female	0.631 ± 0.065[a]	5.389 ± 0.292	0.463 ± 0.061	0.764 ± 0.146	1.262 ± 0.157
	Male	0.675 ± 0.025[b]	6.363 ± 0.534[b]	0.437 ± 0.018[b]	0.708 ± 0.067	1.735 ± 0.126
Group 2	Female	0.606 ± 0.036[a]	5.985 ± 0.073[a]	0.623 ± 0.021[a]	0.778 ± 0.017	2.136 ± 0.469[a]
	Male	0.613 ± 0.028	6.007 ± 0.081	0.641 ± 0.029[b]	0.743 ± 0.051	2.149 ± 0.140[b]
Group 3	Female	0.614 ± 0.023[a]	6.056 ± 0.387[a]	0.503 ± 0.031	0.639 ± 0.031	1.506 ± 0.023
	Male	0.528 ± 0.047	6.020 ± 0.122	0.482 ± 0.082[b]	0.642 ± 0.014	1.413 ± 0.199
Group 4	Female	0.628 ± 0.036[a]	5.839 ± 0.307	0.498 ± 0.018	1.724 ± 0.037[a]	1.204 ± 0.096
	Male	0.727 ± 0.077[b]	4.426 ± 0.165	0.577 ± 0.022[b]	1.153 ± 0.110[b]	1.903 ± 0.192
Group 5	Female	0.666 ± 0.082[a]	4.473 ± 0.306	0.428 ± 0.020	1.263 ± 0.404[a]	1.352 ± 0.202
	Male	0.656 ± 0.066[b]	4.474 ± 0.501	0.422 ± 0.021[b]	1.166 ± 0.110[b]	1.790 ± 0.119
Group 6	Female	0.452 ± 0.015	5.280 ± 0.424	0.460 ± 0.031	0.654 ± 0.030	1.242 ± 0.091
	Male	0.494 ± 0.041	5.350 ± 0.725	0.310 ± 0.022	0.614 ± 0.034	1.656 ± 0.151
Group 7	Female	0.436 ± 0.091	5.024 ± 0.412	0.431 ± 0.122	0.626 ± 0.028	1.276 ± 0.054
	Male	0.526 ± 0.139	5.223 ± 1.373	0.295 ± 0.123	0.610 ± 0.159	1.625 ± 0.417

Note.
[a] Compared with the female normal control group (Group 7), the mean difference is significant at the 0.05 level.
[b] Compared with the male normal control group (Group 7), the mean difference is significant at the 0.05 level.

Table 5
Effect of AFB_1 and $CdCl_2$ on hematological and blood biochemistry parameters in KM mice.

Parameters (Units)	Reference ranges	Group 3		Group 4		Group 5		Group 7	
		Female	Male	Female	Male	Female	Male	Female	Male
EOS(10^9/L)	0.00 ~ 0.51	1.15 ± 0.21[a]	0.59 ± 0.04	1.28 ± 0.31[a]	1.17 ± 0.25[b]	1.81 ± 0.41[a]	1.67 ± 0.25[b]	0.04 ± 0.01	0.24 ± 0.05
NEUT% (%)	6.5 ~ 50.0	30.9 ± 5.6	30.3 ± 6.5	40.5 ± 5.8[a]	51.4 ± 6.1[b]	53.4 ± 6.2[a]	76 ± 5.3[b]	18.8 ± 2.7	18.9 ± 3.2
HCT (%)	35.0 ~ 55.0	24.0 ± 4.0[a]	13.9 ± 3.2[b]	22.2 ± 3.8[a]	11 ± 4.5[b]	17 ± 4.2[a]	2.3 ± 3.2[b]	44.5 ± 4.1	42.1 ± 3.4
PLT(10^9/L)	400 ~ 1600	186 ± 73[a]	203 ± 99[b]	95 ± 67[a]	125 ± 58[b]	87 ± 68[a]	57 ± 44[b]	570 ± 63	565 ± 78
UREA (mmol/L) (mmol/L)	6.4 ~ 10.4	21.4 ± 2.3[a]	19.7 ± 1.9[b]	32.9 ± 1.7[a]	26.4 ± 1.5[b]	39.7 ± 1.1[a]	34.1 ± 1.2[b]	7.3 ± 2.1	7.7 ± 1.6
PHOS(mmol/L) (mmol/L)	1.97 ~ 3.26	4.30 ± 0.26[a]	3.67 ± 0.22	5.16 ± 0.44[a]	4.13 ± 0.38[b]	> 5.20 ± 0[a]	> 5.20 ± 0[b]	3.14 ± 0.53	2.85 ± 0.41
ALT (U/L)	28 ~ 132	218 ± 16[a]	197 ± 18[b]	449 ± 13[a]	342 ± 11[b]	476 ± 17[a]	426 ± 15[b]	80 ± 17	89 ± 12
ALKP (U/L)	62 ~ 209	270 ± 27[a]	238 ± 24[b]	262 ± 28[a]	251 ± 30[b]	284 ± 34[a]	268 ± 30[b]	168 ± 32	144 ± 29
TBIL (μmol/L) (μmol/L)	2 ~ 15	22 ± 4[a]	28 ± 7[b]	55 ± 2[a]	32 ± 2[b]	67 ± 2[a]	40 ± 5[b]	2 ± 2	6 ± 1
GLU(mmol/L) (mmol/L)	5.00 ~ 10.68	3.45 ± 0.87[a]	4.85 ± 1.03[b]	2.25 ± 1.14[a]	4.53 ± 0.95[b]	1.93 ± 1.01[a]	3.11 ± 1.31[b]	5.31 ± 1.41	7.91 ± 1.63
CA(mmol/L) (mmol/L)	1.48 ~ 2.35	1.96 ± 0.06	1.85 ± 0.07	0.69 ± 0.06[a]	1.58 ± 0.05[b]	0.54 ± 0.04[a]	1.13 ± 0.08[b]	2.21 ± 0.28	2.31 ± 0.21

Note.
[a] Means compared with control female mice, the results are significantly different ($p < 0.05$).
[b] Means compared with control male mice, the results are significantly different ($p < 0.05$).

3.6. Histopathological analysis

All of the mice treated with AFB_1 and $CdCl_2$ from Groups 1 to 5 showed various degrees of liver and kidney damage in both sexes. Representative microphotographs of the liver and kidney tissue of male mice are presented in Fig. 2.

There were hepatic lobule central vein congestion, hepatocyte nuclear condensation and hepatocyte nuclear lysis (black, white and green arrows in Fig. 2-A, respectively) in Group 5. In Group 7 (control group), the cross-sections of livers showed a normal appearance (Fig. 2-B).

There were glomerular congestion and granular degeneration of renal tubular epithelial cells (yellow and blue arrows in Fig. 2-C, respectively) in Group 5. In Group 7, the appearances of kidney, glomerulus, and renal tubule structures were normal (Fig. 2-D).

These histologic changes indicate that $CdCl_2$ and AFB_1 may affect permeability of the cell membrane in hepatocytes and renal tubular epithelial cells.

4. Discussion

The acute toxicity of combined metals and mycotoxins is poorly understood and may be different from the summed concentration responses of the individual chemical food safety hazards. Acute toxicity tests provide preliminary information on the toxic nature of a material for which no other toxicology information is available. Such information can be used to deal with cases of accidental ingestion of a large amount of the material (e.g., for poison control information), to determine possible target organs that should be scrutinized and/or the special tests that should be conducted in repeated-dose toxicity evaluation and to select doses for short-term and subchronic toxicity tests when no other toxicology information is available (FDA, 2000a,b). The acute combined toxicity (LD_{50}) test provided information, in addition to suggestions about the dose range that could be used in subsequent toxicity testing, this could equally reveal the possible clinical signs induced by the substance under investigation. It is also a useful parameter for the estimation of the therapeutic index (i.e., LD_{50} or ED_{50}) of drugs and xenobiotics (Aniagu. et al., 2005; Rang. et al., 2015; Yuan et al., 2014).

In an acute toxicology study, interference may affect the LD_{50} value by factors such as the route of exposure, animal species, age, sex, experimental techniques, and environmental conditions (Liju. et al., 2013). To ensure the credibility and accuracy of the test results, we tested seven experimental groups of SPF-grade female and male mice; Group 6 was the solvent-treated group and Group 7 was the control-treated group; these groups were administered reagent grade corn oil and ultrapure water, separately.

This study was the first to investigate the combined acute toxicity of AFB_1 and $CdCl_2$ in KM mice. In this study, the acute toxicity and mortality rates showed significant dose-related effects as the dose increased; here were no obvious effects on body weight. The LD_{50} values showed that male mice were more sensitive than female mice. The sex specificity of Cd toxicity (Wolkowski-Tyl. and Preston, 1979) and AFB_1

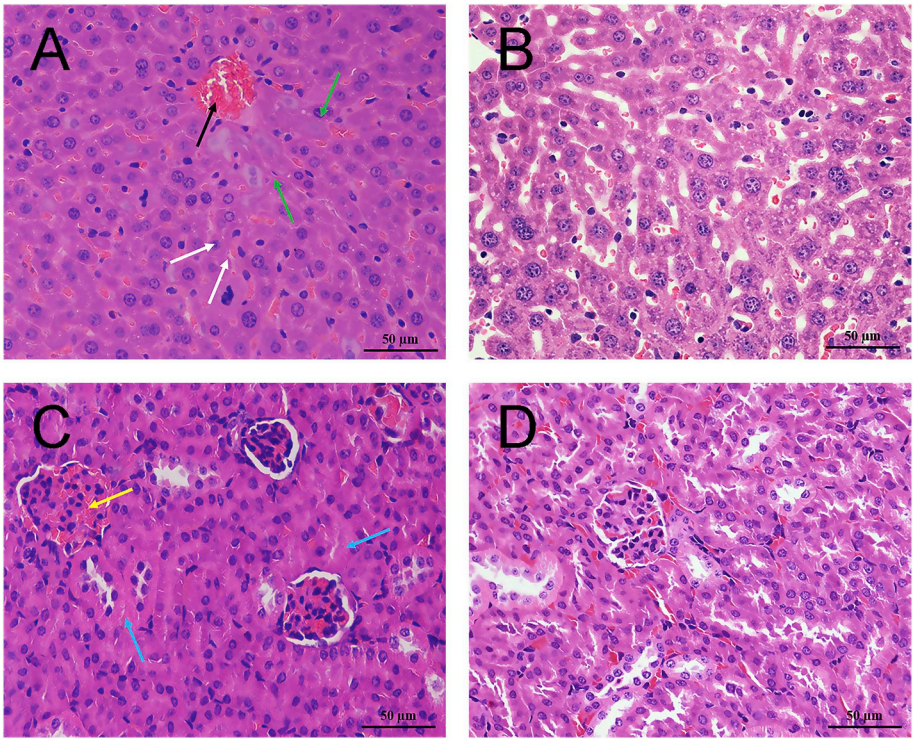

Fig. 2. Representative histological images of liver and kidney slices of male mice after administration for acute toxicity test (strained with haematoxylin and eosin). Panel A and B show images from liver slice of Group 5 ($AFB_1 + CdCl_2$ = 17.7 + 297.9 mg/kg, HE 400X) and Group 7 (control group, 0 mg/kg, HE 400X) respectively. Hepatocyte nuclear condensation (white arrows), hepatocyte nuclear lysis (green arrows), and the hepatic lobule central vein congestion (black arrow). Panel C and D show images from Kidney slice of Group 5 ($AFB_1 + CdCl_2$ = 17.7 + 297.9 mg/kg, HE 400X) and Group 7 (control group, 0 mg/kg, HE 400X) respectively. Glomerular congestion(yellow arrow), and granular degeneration of renal tubular epithelial cells (blue arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

toxicity (Gurtoo. and Motycka., 1976) may explain the result. The acute combined toxicity test (LD_{50}) provided information on the range of doses that could be used in subsequent toxicity testing and for the estimation of the therapeutic index of xenobiotics (Aniagu. et al., 2005; Yuan et al., 2014).

Our findings indicated that the co-exposure of female and male KM mice to AFB_1 and $CdCl_2$ resulted in an additive effect on toxicity. This result not only complements new scientific data on acute toxicity, but also provides a reference for the development of standard limit standards for mixture residues and for risk assessment. The greatest concern is that the mixture of AFB_1 and $CdCl_2$ may elicit additive toxicity that is undetected in the evaluation of individual chemical toxicity, but the specific mechanism is still not clear and should be investigated further.

The liver/body weight ratio and the kidney/body weight ratio were significantly different in the lower dose groups. In the high-dose groups (Groups 4 and 5), there were no significantly difference in the liver and kidney/body weight ratios ($p < 0.05$). The liver and kidney are the main target organs for the poison of AFB_1 and $CdCl_2$. The reason may be attributed to the limited experimental time (2 weeks), and as Groups 4 and 5 were administered a high dose of AFB_1 and $CdCl_2$ the mice died rapidly, so the organ/body weight ratios should not be used to evaluate the high-dose and acute damage caused to the animals.

The hematopoietic system is one of the most sensitive parameters to assess the toxicity of drugs in humans and animals (Liju. et al., 2013; Yuan et al., 2014). This study indicated that co-exposure to AFB_1 and $CdCl_2$ can cause decreases in the level of HCT, and PLT, whereas EOS and NEUT % tended to increase as the AFB_1 and $CdCl_2$ concentrations were increased as previously described (Demir. et al., 2006; Ibrahim. et al., 2018; Ohsawa. and Kawai., 1981; Sharma. et al., 2011).

Results from significant differences in GLU, CREA, UREA, PHOS, CA, ALT, ALKP, TBIL and LIPA levels were found in the treated animals compared with the control group. The liver enzymes ALT and ALKP are cellular enzymes that are present at low concentrations in serum under normal conditions (Yuan et al., 2014). The presence of ALT in serum is considered as the first sign of damage to cells and the liver (Liju. et al., 2013; Mukinda and Eagles, 2010). CREA and UREA levels are indicators of renal function, and an increase in CREA indicates obvious damage to functional nephrons (Liju. et al., 2013), which suggests that the co-exposure to AFB_1 and Cd may induce liver and kidney damage, and the synthesis functions of the liver and kidney were more affected when mice were exposed to a high combination dose.

The characteristic histopathological findings included mainly granular degeneration, hepatic lobule central vein congestion, and hepatocyte nuclear lysis in liver, renal interstitial congestion, renal tubular epithelial cell granular degeneration and glomerular congestion in kidney, all of which indicated that AFB_1 and $CdCl_2$ co-exposure could lead to body damage. These findings are consistent with the hematology and biochemical findings.

5. Conclusion

The LD_{50} value of the equal toxicity mixture for AFB_1 and $CdCl_2$ administered by the oral route to male and female KM mice was 48.695 mg/kg and 62.675 mg/kg, respectively, and the combined effect coefficient K was 1.27 and 1.64 for female and male mice, respectively. AFB_1 and $CdCl_2$ co-exposure had an additive effect on acute oral toxicity both female and male mice.

The main target organs of the toxic effects are the blood, liver, and kidney. Meanwhile, the changes of hematological, and blood biochemical indicators were more sensitive in kidney.

This investigation has provided information that will assist future research into the molecular mechanism of the combined toxicity, which should provide a scientific basis for the risk assessment of mixed AFB_1 and Cd pollutants. Further studies on animals and observational studies on humans are necessary to fully characterize the nature and extent of the organ system toxicity caused by concomitant exposure to mycotoxins and heavy metals.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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References

FDA, 2000a. Guidelines for Toxicity Tests-Acute Oral Toxicity Tests. FDA, USA.
Aniagu, S.O., Nwinyi, F.C., Akumka1, D.D., Ajoku, G.A., Dzarma, S., Izebe, K.S., Ditse, M., Nwaneri, P.E.C., Wambebe, C., Gamaniel, K., 2005. Toxicity studies in rats fed nature cure bitters. Afr. J. Biotechnol. 1, 72–78.
Bagchi, S.J.S.D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radical Biol. Med. 18, 321–336.
Bernhoft, R.A., 2013. Cadmium toxicity and treatment. Sci. World J. 2013, 394652.
Chen, B., Liu, Z., Wang, M., 2001. Modern Food Hygiene. People's Medical Publishing House, Beijing.
CSEM, 2008. Agency for Toxic Substances and Disease Registry Case Studies in Environmental Medicine (CSEM) Cadmium Toxicity. pp. 1.
Cui, L.B., Zhou, X.Y., Zhao, Z.J., Li, Q., Huang, X.Y., Sun, F.Z., 2013. The Kunming mouse: as a model for age-related decline in female fertility in human. Zygote 21, 367–376.
Davis, T.M., G, J.L., Moos, L.P., 2014. Argonne National Laboratory Site Environmental Report for Calendar Year 2013. Argonne National Laboratory, USA, pp. 202.
Demir, H., Kanter, M., Uz, Y.H., Coskun, O., Koc, A., Yildiz, A., 2006. Effect of black cumin (Nigella sativa) on heart rate, some hematological values, and pancreatic β-cell damage in cadmium-treated rats. Biol. Trace Elem. Res. 110, 151–162.
Eaton, D.L., Gallagher, E.P., 1994. Mechanisms of aflatoxin carcinogenesis. Annu. Rev. Pharmacol. Toxicol. 34, 135–172.
Eskandari, M.H., Pakfetrat, S., 2014. Aflatoxins and heavy metals in animal feed in Iran. Food Addit. Contam. Part B Surveill 7, 202–207.
FDA, 2000b. Revised July 2007. Guidance for Industry and Other Stakeholders Toxicological Principles for the Safety Assessment of Food Ingredients Redbook 2000. FDA, College Park, MD,USA.
Gloag, D., 1981. Contamination of food: mycotoxins and metal. Br. Med. J. 282, 879–882.
Guengerich, F.P., Johnson, W.W., Shimada, T., Ueng, Y.-F., Yamazaki, H., Langouët, S., 1998. Activation and detoxication of aflatoxin B1. Mutat. Res. 402, 121–128.
Gunnar, F., Nordberg, K.N., Nordberg, Monica, Friberg, Lars T., 2007. Chapter 23 cadmium. In: Gunnar, B.A.F., Nordberg, F., Nordberg, Monica, Friberg, Lars T. (Eds.), Handbook on the Toxicology of Metals, 3th Edition ed. Academic Press, USA, pp. 992 Third Edition.
Guo, L., He, Y., Li, H., Chen, Y., Zhu, F., Yang, M., Yang, C., Dai, Q., Shi, H., Liu, L., 2018. Monitoring and evaluation of immune status of female Kunming mice maintained in different biosafety level laboratories. Biol. Open 7, o35006–o35010.
Gurtoo, H.L., Motycka, L., 1976. Effect of sex difference on the in vitro and in vivo metabolism of aflatoxin B_1 by rat. Cancer Res. 4663–4671.
Huang, C., Lei, H., Zhao, X., Tang, H., Wang, Y., 2013. Metabolic influence of acute cyadox exposure on Kunming mice. J. Proteome Res. 12, 537–545.
Ibrahim, M.A., Almaeen, A.H., Moneim, M.A.E., Tammam, H.G., Khalifa, A.M., Nasibe, M.N., 2018. Cadmium-induced hematological, renal, and hepatic toxicity: the amelioration by spirulina platensis. Saudi J. Forensic Med. Sci. 1, 1–13.
IRAC, 2012. Cadmium and Cadmium Compounds IARC Monographs on the Evaluation of Carcinogenic Risks to Humans 100C. pp. 121–145.
Jiang, P., Ni, Z., Wang, B., Ma, B., Duan, H., Li, X., Ma, X., Wei, Q., Ji, X., Liu, Q., Xing, S., Li, M., 2017. Acute toxicity, twenty-eight days repeated dose toxicity and genotoxicity of vanadyl trehalose in kunming mice. Regul. Toxicol. Pharmacol. 85, 86–97.
Jiang, P., Liu, Q., Ni, Z., Wei, Q., Li, X., Xing, S., Kong, D., Li, M., 2018. Primary study on the toxic mechanism of vanadyl trehalose in Kunming mice. Regul. Toxicol. Pharmacol. 94, 1–7.
Jung, H., Choi, S.C., 1994. Sequential method of estimating the LD_{50} using a modified up-and-down rule. J. Biopharm. Stat. 1, 19–30.
Keplinger, M.L., Deichmann, W.B., 1967. Acute toxicity of combinations of pesticides. Toxicol. Appl. Pharmacol. 10, 586–895.
Klassen, C., 1991. Principles of toxicology. In: G. AG, T. TW., N. AS., T. P. (Eds.), Pharmacological Basis of Therapeutics. McGraw-Hill, New York, NY, USA, pp. 49–61.
Leadon, Steven A., Zolan, Miriam E., C.Hanawalt, P., 1983. Restricted repair of aflatoxin B_1 induced damage in alpha DNA of monkey cells. Nucleic Acids Res. 11, 5675–5689.
Li, S.A., Lee, W.H., Zhang, Y., 2012. Efficacy of OH-CATH30 and its analogs against drug-resistant bacteria in vitro and in mouse models. Antimicrob. Agents Chemother. 56, 3309–3317.
Liju, V.B., Jeena, K., Kuttan, R., 2013. Acute and subchronic toxicity as well as mutagenic evaluation of essential oil from turmeric (Curcuma longa L). Food Chem. Toxicol. 53, 52–61.
Liu, J., Law, W.-C., Liu, J., Hu, R., Liu, L., Zhu, J., Chen, H., Wang, J., Hu, Y., Ye, L., Yong, K.-T., 2013. Toxicity assessment of phospholipid micelle-encapsulated cadmium-based quantum dots using Kunming mice. RSC Adv. 3, 1768–1773.
Mukinda, J.T., Eagles, P.F., 2010. Acute and sub-chronic oral toxicity profiles of the aqueous extract of Polygala fruticosa in female mice and rats. J. Ethnopharmacol. 128, 236–240.
Navas-Acien, A., Selvin, E., Sharrett, A.R., Calderon-Aranda, E., Silbergeld, E., Guallar, E., 2004. Lead, cadmium, smoking, and increased risk of peripheral arterial disease. Circulation 109, 3196–3201.
Nawrot, T., Plusquin, M., Hogervorst, J., Roels, H.A., Celis, H., Thijs, L., Vangronsveld, J., Van Hecke, E., Staessen, J.A., 2006. Environmental exposure to cadmium and risk of cancer: a prospective population-based study. Lancet Oncol. 7, 119–126.
Nayak, S., Sashidhar, R.B., 2010. Metabolic intervention of aflatoxin B_1 toxicity by curcumin. J. Ethnopharmacol. 127, 641–644.
NHFPC, 2014. Acute Oral Toxicity Test. China Standard Press, Beijing, pp. 1–24.
Nordberg, G.F., 2009. Historical perspectives on cadmium toxicology. Toxicol. Appl. Pharmacol. 238, 192–200.
OECD, 2001. Test No. 423 OECD Guideline for Testing of Chemicals-Acute Oral Toxicity – Acute Toxic Class Method. pp. 1–14.
Ohsawa, M., Kawai, K., 1981. Cytological shift in lymphocytes induced by cadmium in mice and rats. Environ. Res. 192–200.
Partanen, H.A., El-Nezami, H.S., Leppanen, J.M., Myllynen, P.K., Woodhouse, H.J., Vahakangas, K.H., 2010. Aflatoxin B_1 transfer and metabolism in human placenta. Toxicol. Sci. 113, 216–225.
Parvez, S., Venkataraman, C., Mukherji, S., 2009. Nature and prevalence of non-additive toxic effects in industrially relevant mixtures of organic chemicals. Chemosphere 75, 1429–1439.
Rang, H.P., Ritter, James M., R.J.F, Henderson, G., 2015. Rang & Dale's Pharmacology, 8 ed. IE. Churchill Livingstone.
Rath, S., Sahu, M.C., Dubey, D., Debata, N.K., Padhy, R.N., 2011. Which value should be used as the lethal concentration 50 (LC_{50}) with bacteria? Interdiscip. Sci. Comput. Life Sci. 2, 138–143.
Rawal, S., Kim, J.E., Coulombe Jr., R., 2010. Aflatoxin B_1 in poultry: toxicology, metabolism and prevention. Res. Vet. Sci. 89, 325–331.
Satarug, S., Vesey, D.A., Gobe, G.C., 2017. Health risk assessment of dietary cadmium intake: do current guidelines indicate how much is safe? Environ. Health Perspect. 125, 284–288.
Shang, H., Wei, H., Yue, B., Xu, P., Huang, H., 2009. Microsatellite analysis in two populations of Kunming mice. Lab. Anim. 43, 34–40.
Sharma, V., Gupta, R., Mishra, N., Sharma, S., 2011. Influence of tinospora cordifolia root extract supplementation on hematological and serological parameters of male mice exposed to aflatoxin B_1. Int. J. Pharmacol. 5, 659–663.
Silano, M., Silano, V., 2017. Food and feed chemical contaminants in the European Union: regulatory, scientific, and technical issues concerning chemical contaminants occurrence, risk assessment, and risk management in the European Union. Crit. Rev. Food Sci. Nutr. 57, 2162–2217.
Sun, J.-F., 2001. Experimental Methodology in Animals (Chinese). People Healthy House, Beijing.
Tang, J., Yuan, Y., Wei, C., Liao, X., Yuan, J., Nanberg, E., Zhang, Y., Bornehag, C.-G., Yang, X., 2015. Neurobehavioral changes induced by di(2-ethylhexyl) phthalate and the protective effects of vitamin E in Kunming mice. Toxicol. Res. 4, 1006–1015.
Ti, J., Zhang, M., Li, Z., Li, X., Diao, Y., 2016. Duck tembusu virus exhibits pathogenicity to kunming mice by intracerebral inoculation. Front. Microbiol. 7, 190.
Wogan, G.N., 1973. Aflatoxin carcinogenesis. In: Busch, J. (Ed.), Methods in Cancer Research. Academic Press, New York.
Wogan, G.N., 1992. Aflatoxins as risk factors for hepatocellular carcinoma in humans. Cancer Res. 52, 2114s–2118s.
Wolkowski-Tyl, R., Preston, S., 1979. The interaction of cadmium-binding proteins (Cd-bp) and progesterone in cadmium-induced tissue and embryo toxicity. Teratology 3, 341–352.
Xu, D., Tian, Y., Li, R., Li, H., Sun, X., Wang, Q., Shi, J., Zhou, P., Sun, X., 2014. Stochastic food deprivation increases cellular immunity in kunming mice. Health 06, 1099–1107.
Yang, Q., Lu, J.-T., Zhou, A.-W., Wang, B., He, G.-W., Chen, M.-Z., 2001. Antinociceptive effect of astragaloside and its mechanism of action. Acta Pharmacol. Sin. 9, 809–812.
Yuan, G., Dai, S., Yin, Z., Lu, H., Jia, R., Xu, J., Song, X., Li, L., Shu, Y., Zhao, X., 2014. Toxicological assessment of combined lead and cadmium: acute and sub-chronic toxicity study in rats. Food Chem. Toxicol. 65, 260–268.
Yunus, A.W., Razzazi-Fazeli, E., Bohm, J., 2011. Aflatoxin $B(_1)$ in affecting broiler's performance, immunity, and gastrointestinal tract: a review of history and contemporary issues. Toxins 3, 566–590.
Zhai, Qixiao, G.W, Zhao, Jianxin, Liu, Xiaoming, Narbad, Arjan, Chen, Yong Q., Zhang, Hao, Tian, Fengwei, Chena, Wei, 2014. Protective effects of lactobacillus plantarum CCFM8610 against chronic cadmium toxicity in mice indicate routes of protection besides intestinal sequestration. Appl. Environ. Microbiol. 80, 4063–4071.
Zhang, H., 2007. Safety Techniques for Hazardous Chemicals Volumn, second ed. vol 1 Chemical Industry Press, Beijing.
Zhang, X., Liu, F., Chen, B., Li, Y., Wang, Z., 2012. Acute and subacute oral toxicity of polychlorinated diphenyl sulfides in mice : determining LD_{50} and assessing the status of hepatic oxidative stress. Environ. Toxicol. Chem. 31, 1485–1493.
Zhang, J., Zheng, N., Liu, J., Li, F.D., Li, S.L., Wang, J.Q., 2015. Aflatoxin B_1 and aflatoxin M_1 induced cytotoxicity and DNA damage in differentiated and undifferentiated Caco-2 cells. Food Chem. Toxicol. 83, 54–60.