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Fumonisin B_1 Induces Immunotoxicity and Apoptosis of Chicken Splenic Lymphocytes

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Fumonisin B_1 (FB_1), produced by *Fusarium*, is among the most abundant and toxic mycotoxin contaminations in feed, causing damages to the health of livestock. However, the mechanisms of FB_1 toxicity in chickens are less understood. As splenic lymphocytes play important roles in the immune system, the aim of this study was to investigate the immunotoxic effects and mechanisms of FB_1 on chicken splenic lymphocytes. In the present study, the chicken primary splenic lymphocytes were harvested and treated with 0, 2.5, 5, 10, 20 and 40 μg/mL FB_1. Then, the cell proliferation, damage, ultrastructure, inflammation and apoptosis were evaluated. Results showed that the proliferation rate of splenic lymphocytes was decreased by FB_1 treatments. The activity of lactate dehydrogenase (LDH) was increased by FB_1 treatments in a dose-dependent manner, implying the induction of cell damage. Consistently, the ultrastructure of splenic lymphocytes showed that FB_1 at all the tested concentrations caused cell structure alterations, including nuclear vacuolation, mitochondrial swelling and mitochondrial crest fracture. Besides, immunosuppressive effects of FB_1 were observed by the decreased concentrations of interleukin-2 (IL-2), IL-4, IL-12 and interferon-γ (IFN-γ) in the cell culture supernatant. Furthermore, apoptosis was observed in FB_1-treated cells by flow cytometry. The mRNA expressions of apoptosis-related genes showed that the expression of *Bcl-2* was decreased, while the expressions of the *P53*, *Bax*, *Bak-1*, and *Caspase-3* were increased with FB_1 treatment. Similar results were found in the concentrations of apoptosis-related proteins in the cell supernatant by ELISA assay. Moreover, regression analysis indicated that increasing FB_1 concentration increased LDH activity, concentrations of Bax, Bak-1 and mRNA expression of *Bak-1* linearly, increased M1 area percentage quadratically, decreased concentration of IFN-γ, mRNA expression of *Bcl-2* linearly, and decreased concentrations of IL-2 and IL-4 quadratically. Besides, regression analysis also showed reciprocal relationships between IL-12 concentration, *Caspase-3* mRNA expression and increasing FB_1 concentration. The increasing FB_1 concentration could decrease IL-12 concentration and increase *Caspase-3* mRNA expression. Altogether, this study reported that FB_1 induced the immunotoxicity of chicken splenic lymphocytes and caused splenic lymphocytes apoptosis by the Bcl-2 family-mediated mitochondrial pathway of caspase activation.

Keywords: fumonisin B_1, chicken, splenic lymphocytes, immunotoxicity, apoptosis

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INTRODUCTION

Mycotoxin fumonisins (FBs) are the secondary metabolites produced by *Fusarium verticillioides* and *Fusarium proliferatum* (1). Among the fumonisin homologs, fumonisin B_1 (FB_1) is the most prevalent and abundant mycotoxin contamination in stale corn. It is reported that FB_1 has great potential health hazards to humans and animals (2, 3). FB_1 can lead to intestinal damage (4, 5), neurotoxity (6, 7) and various cancers (8–10). Moreover, in chickens, FB_1 exposure lead to reduced performance, nutrient digestibility, immune function, and increased diarrhea as well as mortality (11–14).

The underlying cellular mechanisms of FB_1-induced toxicity include the induction of oxidative stress, apoptosis and immunotoxicity (15, 16). The immunotoxicity of FB_1 in chickens may partially be due to the impairment of lymphatic organs and lymphocyte (17, 18). Spleen, a secondary lymphoid organ, is the main filter for blood-borne pathogens and antigens, playing an important role in maintaining immune homeostasis. Circulating T and B cells often gain access to secondary lymphoid organs to search for their cognate antigens (19). Harmful substances, such as cadmium and atrazine can impair chicken spleens (20, 21). It is also reported that exposure to FB_1 reduced basal rate of splenic lymphocyte proliferation in female mice (22). In chickens, although Todorova et al. showed that FB_1 affected the immune function by damaging the ultrastructure of splenic lymphocyte (18), the detailed immunotoxicity and molecular mechanisms is still unclear. Thus, in this study, we investigated the immunotoxicity of FB_1 on splenic lymphocytes by evaluating the proliferation rate, cell damage, the expressions of cytokines, and further tried to examine the potential molecular mechanisms related to apoptosis to provide reference for further research on the toxicity of FB_1.

MATERIALS AND METHODS

Materials

FB_1 and RPMI 1640 medium were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Fetal bovine serum (FBS) was purchased from Sijiqing Biological Engineering Materials Co. Ltd. (Hangzhou, China). The methyl thiazolyl tetrazolium (MTT) and lactate dehydrogenase (LDH) assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The chicken interleukin-2 (IL-2), IL-4, IL-6, IL-12, and interferon-γ (IFN-γ) ELISA kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The propidium iodide (PI) apoptosis detection kit was purchased from BD Pharmingen (Lexington, KY, USA). The chicken B cell CLL/lymphoma-2 (Bc1-2), P53, Bcl-2 associated X (Bax), Bcl-2 antagonist/killer 1 (Bak-1), and cysteinyl aspartate specific proteinase-3 (Caspase-3) ELISA kits were obtained from Qiyi Biological Technology Co. Ltd. (Shanghai, China). RNA extraction kit and the SYBR PremixScript RT-PCR Kit II were purchased from Takara (Shiga, Japan). All other reagents used were of analytical grade.

Cell Culture and Treatment

The 40-day-old healthy male specific pathogen free (SPF) White Leghorn chickens were obtained from Shandong Academy of Agricultural Sciences for splenic lymphocytes isolation and culture. The use of animals was approved and performed in accordance with the guidelines of Ethics and Animal Welfare Committee of Qingdao Agricultural University. Chickens were given intramuscular injections of ketamine-846 anesthesia mixture (Shengda Animal Medicine Co., Ltd., Dunhua, China) prior to splenic lymphocytes harvesting. Chicken splenic lymphocytes were prepared and cultured according to previous method (23). Briefly, spleen samples were removed from the chickens, washed with sterile cooled phosphate buffered saline (PBS) and ground on ice. The mixture was filtered through a 200-mesh sieve into a Petri dish to collect spleen cell suspension. The lymphocytes were collected by centrifuging at 2,000 *g* for 15 min at room temperature in Histopaque 1077 (Sigma-Aldrich, USA). Then, lymphocytes were washed twice with cooled PBS and re-suspended in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin, and counted using a hemocytometer. Based on trypan blue dye exclusion, when the lymphocytes viability was more than 95%, the cells could be used for the experiments.

In this study, the splenic lymphocytes were treated with 0, 2.5, 5, 10, 20, and 40 μg/mL FB_1 according to previous studies (24–26), in which 2.5–50 μg/mL FB_1 suppressed the proliferation of chicken primary cells, such as splenocytes and peripheral lymphocytes. The FB_1 was dissolved in deionized water to obtain a 40 mg/mL concentration solution. Then, various dilutions of the 40 mg/mL FB_1 solution were added to cell cultures with final concentrations of 0, 2.5, 5, 10, 20 and 40 μg/mL FB_1. For MTT assay, splenic lymphocytes were cultured in 96-well microplates (1×10^4 cells/mL) under 5% CO_2 at 42°C, and stimulated with 10 μg/mL concanavalin A (ConA) to induce cell proliferation and treated with 0, 2.5, 5, 10, 20 and 40 μg/mL FB_1 for 72 h, with 6 parallel holes in each treatment group. For other assays, splenic lymphocytes were cultured in 24-well microplates (1×10^5 cells/mL) under 5% CO_2 at 42°C, and treated with 0, 2.5, 5, 10, 20, and 40 μg/mL FB_1 for 48 h. Four parallel holes in each treatment group were set for quantitative real-time PCR (RT-PCR) and flow cytometry, and 6 parallel holes were set in each treatment group for the measurement of proliferation rate, inflammatory cytokine levels, LDH activity and apoptosis protein concentrations.

MTT Assay

After 72 h treatment with ConA and FB_1, 10 μL MTT solution (5 mg/mL) was added in each well and then incubated for 4 h. After incubated with 100 μL of DMSO, the optical density (OD) was measured at 490 nm using a microplate reader (ThermoFisher MK_3, USA). Proliferation rate (%) = (OD of cells treated with FB_1-OD of cells without FB_1 treatment)/OD of cells without FB_1 treatment × 100%.

Transmission Electron Microscopy (TEM)

Cells were fixed in a fresh solution of 0.1 M sodium cacodylate buffer containing 2.5% glutaraldehyde and 2% formaldehyde

followed by a 2 h fixation at 4°C with 2% osmium tetroxide in 50 mM sodium cacodylate (pH 7.2). Staining was performed overnight with 0.5% aqueous uranyl acetate. Specimens were dehydrated, embedded in Epon 812 and sectioned into ultrathin slices. The sections were examined on CCD camera system (AMT Corp., USA) (27).

Analysis of Inflammatory Cytokines and LDH

The concentrations of inflammatory cytokines, including IL-2, IL-4, IL-6, IL-12, IFN-γ and the activity of LDH were determined spectrophotometrically using commercial kits (Nanjing Jiancheng Bioengineering Institute, China) according to manufacturer's protocol.

Apoptosis Assay by PI Staining

After adding pancreatin (HyClone, USA), the cells were harvested by centrifuging at 1,000 *g*. The cell pellet was fixed in 1.5 mL cold 75% ethanol at 4°C for 8 h. Then, cells were centrifuged, washed in 1 mL PBS and re-suspended in 0.5 mL PBS. To a 0.5 mL cell sample, 0.5 mL RNase A (Sigma-Aldrich, USA) was added, followed by mixing by 1 mL PI (Sigma-Aldrich, USA) solution. The mixed cells were incubated in the dark at room temperature for 30 min and kept at 4°C in the dark until measured. The PI fluorescence was measured using a FC500 flow cytometer (Beckman Coulter, Fullerton, USA).

RNA Extraction and RT-PCR

Total RNA extraction and reverse transcription were performed according to Mao et al. (28). Primer and Oligo softwares were used for PCR primer sequences (**Table 1**) design. RT-PCR was performed using Premix Ex TaqTM with SYBR Green (TaKaRa, Dalian, China) and ABI Stepone Real-Time PCR System 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The thermocycle protocol was 30-s at 95°C followed by 40 cycles of 5-s denaturation at 95°C, 34-s annealing/extension at 60°C, and then a final melting curve analysis to monitor purity of the PCR product. The mRNA abundances of *Bcl-2*, *Bax*, *Bak-1*, *P53* and *Caspase-3* were determined by $2^{-\Delta\Delta Cq}$ method. Relative gene expression concentrations were normalized by eukaryotic reference gene *GAPDH*.

Analysis of Apoptosis-Related Proteins by ELISA

The concentrations of Bcl-2, Bax, Bak-1, P53 and Caspase-3 in the cell culture supernatant were measured by ELISA kits according to the manufacture instructions (Qiyi Biological Technology Co. Ltd., Shanghai, China). Briefly, standard solutions were prepared. Then, the specimens were thawed at room temperature and 50 μL aliquots were added to the wells covered with a layer of monoclonal antibody, then incubated at 37°C for 30 min together with the standard solutions. Subsequently, the wells were washed and 50 μL of conjugate reagent was added. The wells were further incubated at 37°C for 30 min at the end of which the wells were washed and 100 μL of streptavidin–horseradish peroxidase (HRP)-tagged antibodies were added. The wells were incubated at 37°C for 30 min. Following another round of washing, 100 μL of tetramethylbenzidine was added to the wells. The wells were incubated at room temperature for 30 min in the dark. The reaction was stopped by adding 50 μL stop solution to the wells. The absorbances of the solutions in the wells were measured by a spectrophotometer at 450 nm. A standard curve was plotted from the absorbance values of the standard solutions and the protein concentrations of the samples were calculated from the standard curve.

TABLE 1 | RT-PCR primer sequences and products.

Gene	Primer sequences (5′ → 3′)	Product size (bp)	GeneBank accession No.
GAPDH	TCCTGGTATGACAATGAGTTTGGA	199	NM_204305
	GGGGAGACAGAAGGGAACAGA		
Bcl-2	ATCGTCGCCTTCTTCGAGTT	150	Z11961.1
	ATCCCATCCTCCGTTGTCCT		
Bax	GTGATGGCATGGGACATAGCTC	90	XM_422067.2
	TGGCGTAGACCTTGCGGATAA		
Bak-1	ATGGATGCCTGTCTGTCCTGTTC	106	NM_001030920.1
	GCAGAGCAGTCCAAAGACACTGA		
P53	GAGATGCTGAAGGAGATCAATGAG	145	X13057.1
	GTGGTCAGTCCGAGCCTTTT		
Caspase-3	ACTCTGGAATTCTGCCTGATGACA	129	NM_204725.1
	CATCTGCATCCGTGCCTGA		

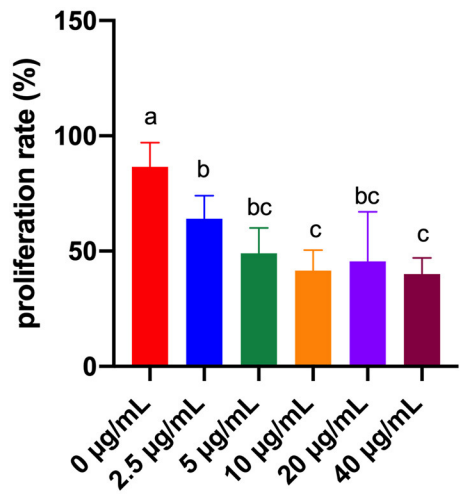

FIGURE 1 | Effects of different concentrations of FB_1 on the proliferation rate of splenic lymphocytes. Data are presented as the means ± SD for $n = 6$. End-point means without a common letter are significantly different (a, b, or c, $P < 0.05$).

Statistical Analysis

One-way ANOVA was performed with the GLM procedure by SAS (SAS Institute Inc, USA, version 9.3), and multiple comparisons were performed with Duncan's test. Results were presented as the means ± standard deviation (SD). The Reg procedure of SAS (SAS Institute Inc, USA, version 9.3) was used

for performing the regression of increasing FB_1 concentration on the measurements. One-dimensional linear equation, one-dimensional quadratic equation, and reciprocal equation were fit. The significant one with the largest R^2 was selected as the most appropriate equation. The differences were considered significant at $P < 0.05$.

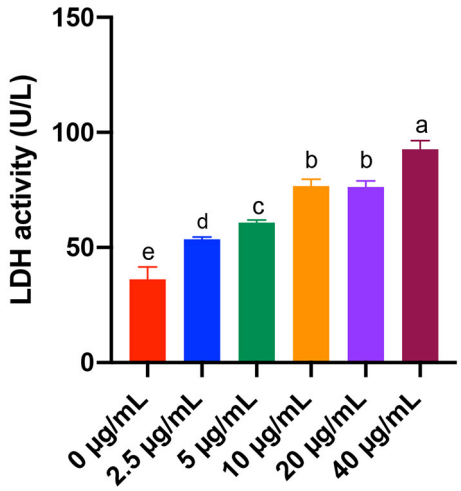

FIGURE 2 | Effects of different concentrations of FB_1 on the LDH activity in the culture supernatant of splenic lymphocytes. Data are presented as the means ± SD for $n = 6$. End-point means without a common letter are significantly different (a, b, c, d, or e, $P < 0.05$).

RESULTS

Effects of Different Concentrations of FB_1 on the Splenic Lymphocytes Proliferation Rate

According to **Figure 1**, compared to the FB_1-non-treated cells, the lymphocytes proliferation rate was significantly decreased by FB_1 at 2.5, 5, 10, 20 and 40 μg/mL ($P < 0.05$). Moreover, compared to cells treated with 2.5 μg/mL FB_1, cells treated with 10 and 40 μg/mL FB_1 had much lower lymphocytes proliferation rate ($P < 0.05$).

Effects of Different Concentrations of FB_1 on the Activity of LDH

As for the LDH, we found that 2.5, 5, 10, 20, and 40 μg/mL FB_1 significantly increased the LDH activity ($P < 0.05$) in a dose-dependent manner, although there was no significant difference between the 10 and 20 μg/mL FB_1 treatment groups ($P > 0.05$) (**Figure 2**).

Effects of Different Concentrations of FB_1 on the Ultrastructure of Splenic Lymphocytes

TEM results indicated that the non-treated cells had intact cell membrane and nucleus envelope. Moreover, mitochondria also

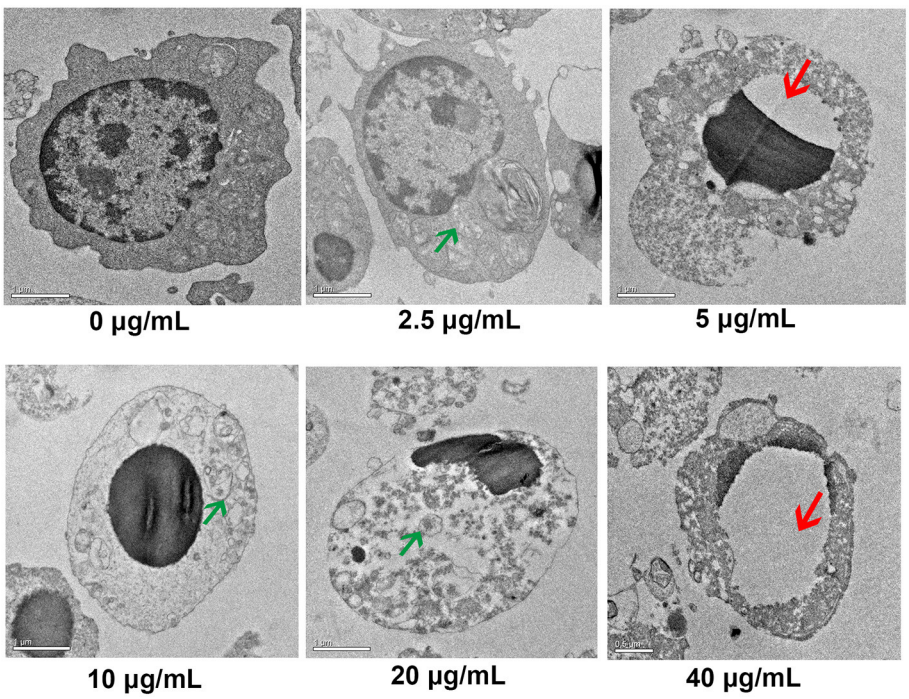

FIGURE 3 | Ultrastructure of splenic lymphocytes by TEM. Green arrows indicate the mitochondrial swelling and mitochondrial crest fracture. Red arrows indicate nuclear vacuolation.

TABLE 2 | Effects of different concentrations of FB_1 on the inflammatory cytokines.

FB_1 concentrations (μg/mL)	IL-2 (ng/L)	IL-4 (ng/L)	IL-6 (ng/L)	IL-12 (ng/L)	IFN-γ (ng/L)
0	107.12 ± 2.13[a]	123.37 ± 14.24[a]	38.35 ± 1.63	140.24 ± 37.59[a]	84.73 ± 2.84[a]
2.5	95.25 ± 0.64[b]	116.77 ± 2.20[a]	36.02 ± 4.39	123.52 ± 20.52[a]	73.48 ± 7.15[b]
5	87.04 ± 4.62[c]	105.31 ± 3.59[b]	37.30 ± 5.58	92.17 ± 8.43[b]	77.35 ± 3.13[b]
10	51.96 ± 10.16[d]	93.91 ± 4.21[c]	37.58 ± 2.87	91.10 ± 6.61[b]	73.67 ± 2.05[b]
20	37.76 ± 5.12[e]	79.60 ± 4.12[d]	35.23 ± 5.73	76.39 ± 3.01[bc]	72.66 ± 5.30[b]
40	34.54 ± 0.82[e]	62.47 ± 2.83[e]	37.02 ± 1.03	62.01 ± 2.43[c]	65.05 ± 2.44[c]

Data are presented as the means ± SD for n = 6.
[a,b,c,d,e]Mean value within a row with no common superscript differ significantly (P < 0.05).

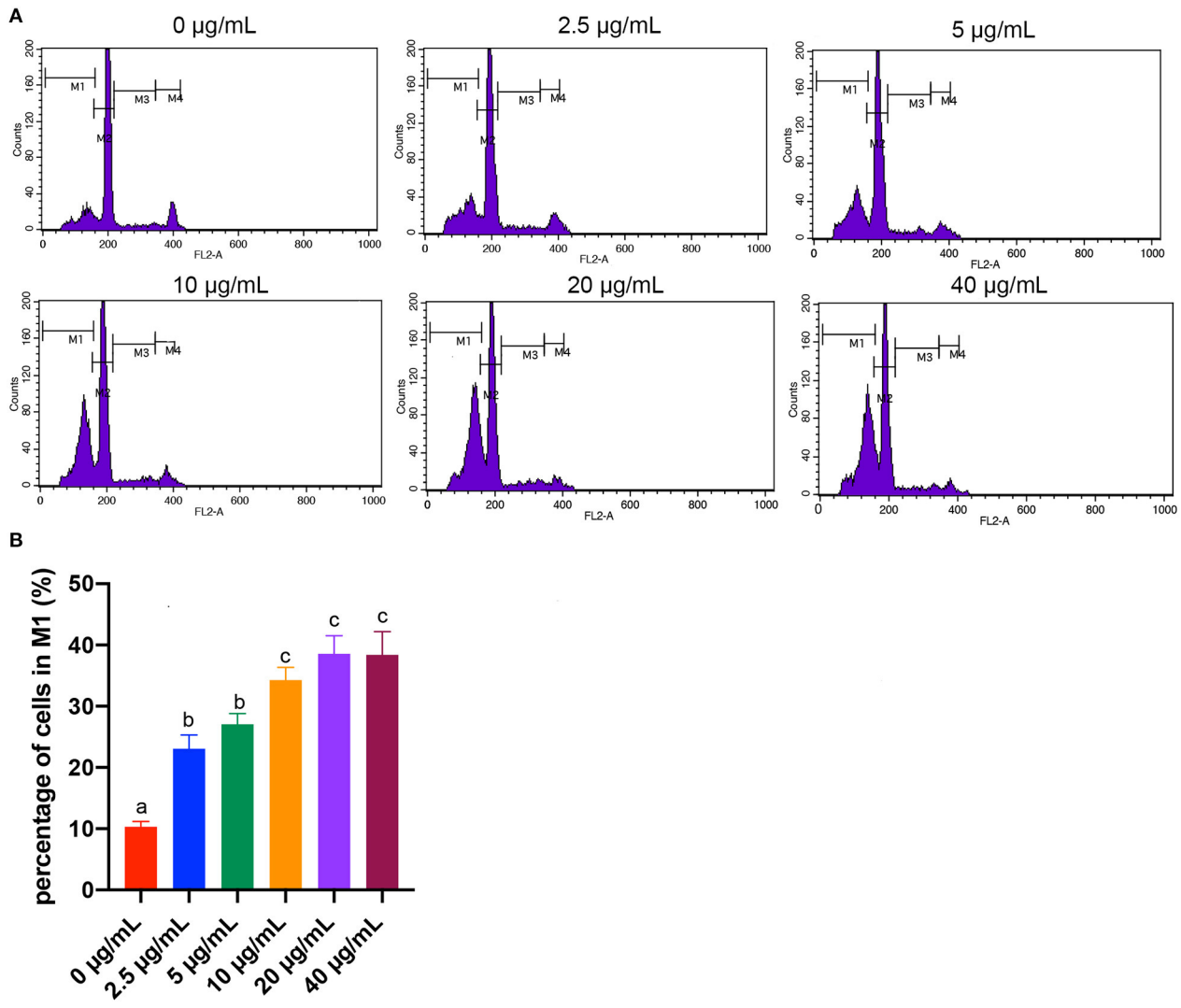

FIGURE 4 | Effects of different concentrations of FB_1 on the apoptosis of splenic lymphocytes. **(A)** Cell apoptosis was analyzed by flow cytometry. M1 gate indicates the pre-G0/G1 (apoptotic) population, M2 gate indicates the G0/G1 phase, M3 gate indicates the S phase and M4 gate indicates the G2/M phase. **(B)** Percentage of cells in M1 gate. Data are presented as the means ± SD for $n = 4$. End-point means without a common letter are significantly different (a, b, or c, $P < 0.05$).

TABLE 3 | Effects of different concentrations of FB_1 on the relative expression of apoptosis-related genes.

FB_1 concentrations (μg/mL)	*Bcl-2*	*P53*	*Bax*	*Bak-1*	*Caspase-3*
0	1.01 ± 0.06^{a}	1.01 ± 0.12^{b}	1.03 ± 0.03^{bc}	1.01 ± 0.08^{c}	1.03 ± 0.18^{b}
2.5	0.96 ± 0.04^{ab}	1.09 ± 0.11^{ab}	0.98 ± 0.04^{c}	1.09 ± 0.12^{bc}	1.18 ± 0.31^{ab}
5	0.86 ± 0.07^{b}	1.05 ± 0.10^{b}	1.09 ± 0.04^{b}	1.27 ± 0.18^{b}	1.36 ± 0.29^{ab}
10	0.91 ± 0.09^{ab}	1.14 ± 0.08^{ab}	1.17 ± 0.07^{a}	1.19 ± 0.22^{bc}	1.65 ± 0.21^{a}
20	0.66 ± 0.10^{c}	1.01 ± 0.02^{b}	1.07 ± 0.02^{b}	1.31 ± 0.03^{b}	1.52 ± 0.45^{ab}
40	0.54 ± 0.02^{d}	1.24 ± 0.13^{a}	1.22 ± 0.04^{a}	1.54 ± 0.10^{a}	1.70 ± 0.57^{a}

Data are presented as the means ± SD for n = 4. mRNA expression was standardized to GAPDH expression.
[a,b,c]Mean value within a row with no common superscript differ significantly (P < 0.05).

TABLE 4 | Effects of different concentrations of FB_1 on the apoptosis-related proteins.

FB_1 concentrations (μg/mL)	Bcl-2 (μg/mL)	P53 (μg/mL)	Bax (μg/mL)	Bak-1 (μg/mL)	Caspase-3 (μg/mL)
0	2.50 ± 0.13^{a}	281.75 ± 10.68^{c}	3.45 ± 0.17^{e}	15.07 ± 1.19^{b}	1.58 ± 0.15^{bc}
2.5	2.39 ± 0.04^{ab}	336.34 ± 29.56^{b}	3.74 ± 0.19^{d}	13.98 ± 0.98^{b}	1.70 ± 0.14^{b}
5	2.21 ± 0.23^{b}	379.58 ± 28.42^{b}	3.91 ± 0.14^{cd}	14.29 ± 1.09^{b}	1.51 ± 0.12^{c}
10	2.35 ± 0.09^{ab}	340.76 ± 30.42^{b}	4.23 ± 0.20^{b}	14.55 ± 0.96^{b}	1.72 ± 0.14^{b}
20	2.35 ± 0.22^{ab}	469.84 ± 17.63^{a}	4.11 ± 0.19^{bc}	17.48 ± 0.40^{a}	1.48 ± 0.05^{c}
40	2.16 ± 0.23^{b}	445.86 ± 55.15^{a}	5.12 ± 0.29^{a}	17.52 ± 1.57^{a}	1.94 ± 0.13^{a}

Data are presented as the means ± SD for n = 6.
[a,b,c,d]Mean value within a row with no common superscript differ significantly (P < 0.05).

had intact structure and were not swollen. However, 2.5, 5, 10, 20, and 40 μg/mL FB_1 caused cell structure damage, including nuclear vacuolation, mitochondrial swelling and mitochondrial crest fracture (**Figure 3**).

Effects of Different Concentrations of FB_1 on the Concentrations of Inflammatory Cytokines

With the treatments of different concentrations of FB_1, the concentration of IL-2 was significantly decreased compared to the non-treated group ($P < 0.05$). Moreover, a dose-dependent effects of FB_1 were observed for the reduction of IL-2 concentration. In addition, 5, 10, 20, and 40 μg/mL FB_1 also significantly down-regulated the concentration of IL-4 in a dose-dependent manner compared to the non-treated and 2.5 μg/mL FB_1-treated cells ($P < 0.05$). Furthermore, the concentration of IL-12 was also significantly reduced by 5, 10, 20, and 40 μg/mL FB_1 ($P < 0.05$). Compared to 5 and 10 μg/mL FB_1, 40 μg/mL FB_1 treatment led to a much lower IL-12 concentration ($P < 0.05$). Besides, the concentration of IFN-γ was significantly reduced by the FB_1 at all the tested concentrations ($P < 0.05$), and 40 μg/mL FB_1 induced the lowest IFN-γ concentration compared to that of other FB_1 treatments ($P < 0.05$). However, the concentration of IL-6 was not significantly affected by the treatments of FB_1 ($P > 0.05$) (**Table 2**).

Effects of Different Concentrations of FB_1 on the Apoptosis of Splenic Lymphocytes

Apoptotic cells were quantified by flow cytometry analysis. Early apoptotic cells appear in the cell cycle distribution as cells with a hypodiploid DNA. This alteration in DNA content results from degradation of cellular DNA by activation of endogenous endonucleases during apoptosis (29). Thus, cells in the pre-G0/G1 phase (M1) were therefore defined as apoptotic cells (**Figure 4**). Results showed that the percentages of cells in M1 area of all the FB_1 treatment groups were significantly increased compared to the non-treated ones ($P < 0.05$). Moreover, compared to the 2.5 and 5 μg/mL FB_1 treatments, 10, 20, and 40 μg/mL FB_1 treatments significantly increased the percentages of cells in M1 ($P < 0.05$).

Effects of Different Concentrations of FB_1 on the mRNA Expressions of Apoptosis-Related Genes

The expression of *Bcl-2* was significantly down-regulated by 5, 20, and 40 μg/mL FB_1 compared to the cells treated with 0, 2.5 and 10 μg/mL FB_1 ($P < 0.05$). And 40 μg/mL FB_1 led to the lowest *Bcl-2* mRNA expression ($P < 0.05$). The expression of *P53* was only up-regulated in the 40 μg/mL FB_1 treatment group ($P < 0.05$). Besides, the expression of *Bax* was significantly up-regulated by 10 and 40 μg/mL FB_1 compared to other groups ($P < 0.05$). In addition, 10, 20 and 40 μg/mL FB_1 significantly elevated the expression of *Bak-1* ($P < 0.05$). And 40 μg/mL FB_1 induced the highest expression of *Bak-1* among groups ($P < 0.05$). Furthermore, 10 and 40 μg/mL FB_1 significantly up-regulated the *Caspase-3* expression compared to the non-treated cells ($P < 0.05$) (**Table 3**).

TABLE 5 | Regression equations for FB_1 concentrations as a function for biochemical indices of splenic lymphocytes.

Biochemical indices	Equation	R^2	*P*-value
Proliferation Rate (%)	$y = 50.47/x + 42.28$	0.74	0.06
IL-2 (ng/L)	$y = 0.10x^2\text{-}5.71x + 108.49$	0.96	0.04
IL-4 (ng/L)	$y = 0.04x^2\text{-}2.87x + 121.02$	0.99	0.01
IL-12 (ng/L)	$y = 143.20/x + 66.84$	0.91	0.01
INF-γ (ng/L)	$y = -0.27x + 76.64$	0.84	0.03
LDH (U/L)	$y = 0.94x + 57.32$	0.85	0.03
M1 (%)	$y = -0.03x^2 + 1.50x + 20.24$	0.98	0.02
Bax (μg/mL)	$y = 0.03x + 3.71$	0.90	0.01
Bak-1 (μg/mL)	$y = 0.10x + 13.96$	0.78	0.05
Bcl-2	$y = -0.01x + 0.96$	0.90	0.01
Bak-1	$y = 0.01x + 1.12$	0.87	0.02
Caspase-3	$y = -1.37/x + 1.70$	0.84	0.03

Effects of Different Concentrations of FB_1 on the Concentrations of Apoptosis-Related Proteins

ELISA results showed that the concentration of Bcl-2 in the cell supernatant was significantly decreased in 5 and 40 μg/mL FB_1 treatment groups compared to the non-treated group ($P < 0.05$). But there was no significant difference for the Bcl-2 concentration among cells treated with FB_1 ($P > 0.05$). Moreover, the concentration of P53 was significantly elevated by FB_1 at all the tested concentrations compared to the non-treated group ($P < 0.05$), in addition, P53 concentration was much higher in 20 and 40 μg/mL FB_1-treated groups than that of the 2.5 and 5 μg/mL FB_1-treated groups ($P < 0.05$). FB_1 at all the tested concentrations also significantly increased the Bax concentration ($P < 0.05$), and 40 μg/mL FB_1 induced the highest Bax concentration among the groups. Besides, the Bak-1 concentration in the 20 and 40 μg/mL FB_1-treated groups was much higher than that of the other groups ($P < 0.05$). Furthermore, 40 μg/mL FB_1 significantly increased Caspase-3 concentration compared to other groups ($P < 0.05$) (**Table 4**).

Regression Analysis

Regression equations are presented in **Table 5**. Results indicated that increasing FB_1 concentration increased the activity of LDH, the concentrations of Bax, Bak-1 and mRNA expression of *Bak-1* linearly, increased percentage of M1 area quadratically, decreased concentration of IFN-γ, mRNA expression of *Bcl-2* linearly, and decreased concentrations of IL-2 and IL-4 quadratically. Rregression analysis also showed reciprocal relationship between IL-12 concentration, *Caspase-3* mRNA expression and increasing FB_1 concentration. The increasing FB_1 concentration could decrease IL-12 concentration and increase *Caspase-3* expression. Proliferation rate tended to decrease with increasing FB_1 concentration ($P = 0.06$).

DISCUSSION

Fusarium mycotoxins such as FB_1 are the major contaminants in animal feed and induce subclinical symptoms. The spleen of birds is a central immune organ for the proliferation of T and B cells.

In the present study, we aimed to determine the toxic effects and mechanisms of FB_1 on splenic lymphocytes. We found the proliferation rate of splenic lymphocytes was significantly decreased by treatments of FB_1 at 2.5, 5, 10, 20, and 40 μg/mL. Similarly, Johnson and Sharma also reported that FB_1 exposure was able to reduce the lymphocyte proliferation (22). LDH, a key feature of cells undergoing cellular damage, is a stable cytoplasmic enzyme that is found in all cells (30). In the present study, FB_1 significantly increased the LDH activity in a dose-dependent manner. Regression

analysis also showed that the LDH activity was increased by increasing FB_1 concentrations. Consistent with this result, the ultrastructure of FB_1-treated lymphocytes indicated cell impairments, including the mitochondrial swelling, mitochondrial crest fracture and nuclear vacuolation. Cell ultrastructure changes were also observed in a study by Todorova et al. (31). According to this study, the splenic lymphatic nodules were with pale centers, reduced cell number and contained large undifferentiated lymphoblasts or cells with pycnotic nuclei in chickens consuming FB_1 and deoxynivalenol.

The immunotoxicity of FB_1 has been associated with decreased immune responses (32). Here, the concentrations of IL-2, IL-4, IL-12, and IFN-γ in the cell culture supernatant were decreased by FB_1 treatments, and the reductions of IL-2 and IL-4 were in a dose-dependent manner. However, the concentration of IL-6 was not significantly changed by FB_1. Regression analysis further demonstrated that the concentrations of IL-2, IL-4, IL-12, and IFN-γ were reduced by increasing FB_1 concentrations. Studies demonstrated that splenocytes from female mice exposed to FB_1 had a reduced expression of *IL-2* mRNA (22). *In vitro* treatment of swine lymphocytes with FB_1 significantly decreased IL-4 production (33). Moreover, the secretions of IL-12 induced by LPS exposure of murine bone marrow-derived dendritic cells were suppressed by FB_1 in a dose dependent manner (34). The mRNA concentration of *IFN-γ* in macrophages was also reduced by supplementation of FB_1 in chickens (35). Similar with our results, Bhandari et al. reported that the IL-6 concentration in the spleen of FB_1-treated mice was not significantly altered (36). Unfortunately, no other studies have shown related data on splenic lymphocytes to serve for comparison with our results.

The decrease of cell proliferation might not only be associated with the FB_1 direct cytotoxicity effects but also be due to apoptosis of lymphocytes (37). Therefore, the pro-apoptotic role of FB_1 in splenic lymphocytes was further investigated. Previously, studies indicated that FB_1 induced apoptosis of porcine kidney cells (38), human gastric epithelial cells (39) and turkey peripheral blood lymphocytes (40). Currently, we also observed splenic lymphocytes apoptosis with FB_1 treatment. Two major apoptotic pathways have been identified: (i) the extrinsic pathway, in which activation of a death receptor by a ligand leads to the activation of initiator Caspase-8; and (ii) the intrinsic

pathway, which is caused by cellular stress and cytochrome c release from mitochondria, leading to Caspase-9 activation (41). Once active, Caspase-9 directly cleave and activate Caspase-3 (42). The apoptotic processes that occur before cytochrome c release require a variety of effector molecules, including Bcl-2 family proteins (43, 44). The Bcl-2 family is composed of two subfamilies: one consisting of anti-apoptotic proteins (e.g., Bcl-2, Bcl-XL, Bcl-w, etc.) and the other of pro-apoptotic proteins (e.g., Bax, Bak, Bcl-XS, etc.) (45). In the current study, results of RT-PCR and ELISA demonstrated that the expression of Bcl-2 was decreased, while the expressions of the P53, Bax, Bak-1 and Caspase-3, which can promote apoptosis, were increased with FB_1 treatment. Regression analysis suggested that the M1 area percentage, concentrations of Bax, Bak-1 and mRNA expressions of *Bak-1* and *Caspase-3* were increased, while the *Bcl-2* mRNA expression was reduced by increasing FB_1 concentrations. These results indicated that FB_1 can induce splenic lymphocytes apoptosis by the Bcl-2 family-mediated mitochondrial pathway.

In conclusion, data in this study imply that FB_1 strongly suppressed the chicken splenic lymphocytes proliferation and caused cell damage, especially the impairment of structure of mitochondria. The immunosuppressive effect of FB_1 was also found by the decreased concentrations of inflammatory cytokines. Moreover, FB_1 induced splenic lymphocytes apoptosis through the Bcl-2 family-mediated mitochondrial pathway of caspase activation. Overall, our data provide new evidence for the toxic effects and mechanisms of FB_1 on chicken and provide new targets for regulating the FB_1-related subclinical symptoms with possible unfavorable economic outcome.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics and Animal Welfare Committee of Qingdao Agricultural University.

AUTHOR CONTRIBUTIONS

FZ designed the study. YW analyzed data and wrote the manuscript. FZ and YW performed the research and contributed to revision of the manuscript. Both authors read and approved the final manuscript, contributed to the article and approved the submitted version.

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REFERENCES

1. Lumsangkul C, Chiang HI, Lo NW, Fan YK, Ju JC. Developmental toxicity of mycotoxin fumonisin B1 in animal embryogenesis: an overview. *Toxins.* (2019) 11:114. doi: 10.3390/toxins11020114
2. Schelstraete W, Devreese M, Croubels S. Comparative toxicokinetics of Fusarium mycotoxins in pigs and humans. *Food Chem Toxicol.* (2020) 137:111140. doi: 10.1016/j.fct.2020.111140
3. Ochieng PE, Scippo ML, Kemboi DC, Croubels S, Okoth S, Kang'ethe EK, et al. Mycotoxins in poultry feed and feed ingredients from sub-Saharan Africa and their impact on the production of broiler and layer chickens: a review. *Toxins*. (2021) 13:633. doi: 10.3390/toxins13090633
4. Oswald IP, Desautels C, Laffitte J, Fournout S, Peters SY, Odin M, et al. Mycotoxin fumonisin B1 increases intestinal colonization by pathogenic *Escherichia coli* in pigs. *Appl Environ Microbiol.* (2003) 69:5870. doi: 10.1128/AEM.69.10.5870-5874.2003
5. Bouhet S, Hourcade E, Loiseau N, Fikry A, Martinez S, Roselli M, et al. The mycotoxin fumonisin B1 alters the proliferation and the barrier function of porcine intestinal epithelial cells. *Toxicol Sci.* (2004) 77:165–71. doi: 10.1093/toxsci/kfh006
6. Doi K, Uetsuka K. Mechanisms of mycotoxin-induced neurotoxicity through oxidative stress-associated pathways. *Int J Mol Sci.* (2011) 12:5213–37. doi: 10.3390/ijms12085213
7. Domijan AM. Fumonisin B(1): a neurotoxic mycotoxin. *Arh Hig Rada Toksikol.* (2012) 63:531–44. doi: 10.2478/10004-1254-63-2012-2239
8. Wang H, Wei H, Ma J, Luo X. The fumonisin B1 content in corn from North China, a high-risk area of esophageal cancer. *J Environ Pathol Toxicol Oncol.* (2000) 19:139–41. Available online at: https://europepmc.org/article/med/10905519
9. Alizadeh AM, Rohandel G, Roudbarmohammadi S, Roudbary M, Sohanaki H, Ghiasian SA, et al. Fumonisin B1 contamination of cereals and risk of esophageal cancer in a high risk area in northeastern Iran. *Asian Pac J Cancer Prev.* (2012) 13:2625–8. doi: 10.7314/APJCP.2012.13.6.2625
10. Müller S, Dekant W, Mally A. Fumonisin B1 and the kidney: modes of action for renal tumor formation by fumonisin B1 in rodents. *Food Chem Toxicol.* (2012) 50:3833–46. doi: 10.1016/j.fct.2012.06.053
11. Sobrane Filho ST, Junqueira OM, de Laurentiz AC, da Silva Filardi, da Silva Rubio M, Duarte KF, et al. Effects of mycotoxin adsorbents in aflatoxin B 1-and fumonisin B 1-contaminated broiler diet on performance and blood metabolite. *R Bras Zootec.* (2016) 45:250–256. doi: 10.1590/S1806-92902016000500007
12. Henry MH, Wyatt RD, Fletchert OJ. The toxicity of purified fumonisin B1 in broiler chicks. *Poult Sci.* (2000) 79:1378–84. doi: 10.1093/ps/79.10.1378
13. Grenier B, Dohnal I, Shanmugasundaram R, Eicher SD, Selvaraj RK, Schatzmayr G, et al. Susceptibility of broiler chickens to coccidiosis when fed subclinical doses of deoxynivalenol and fumonisins—special emphasis on the immunological response and the mycotoxin interaction. *Toxins*. (2016) 8:231. doi: 10.3390/toxins8080231
14. Liu JD, Doupovec B, Schatzmayr D, Murugesan GR, Bortoluzzi C, Villegas AM, et al. The impact of deoxynivalenol, fumonisins, and their combination on performance, nutrient, and energy digestibility in broiler chickens. *Poult Sci.* (2020) 99:272–9. doi: 10.3382/ps/pez484
15. Stockmann-Juvala H, Savolainen K. A review of the toxic effects and mechanisms of action of fumonisin B1. *Hum. Exp. Toxicol.* (2008) 27:799–809. doi: 10.1177/0960327108099525

16. Bhatti SA, Khan MZ, Saleemi MK, Saqib M, Khan A, ul-Hassan Z. Protective role of bentonite against aflatoxin B1- and ochratoxin A-induced immunotoxicity in broilers. *J Immunotoxicol.* (2017) 14:66–76. doi: 10.1080/1547691X.2016.1264503
17. Mwanza M, Kametler L, Bonai A, Rajli V, Kovacs M, Dutton KMF. The cytotoxic effect of fumonisin B1 and ochratoxin A on human and pig lymphocytes using the Methyl Thiazol Tetrazolium (MTT) assay. *Mycotoxin Res.* (2009) 25:233–8. doi: 10.1007/s12550-009-0033-z
18. Todorova KS, Kril AI, Dimitrov PS, Gardeva EG, Toshkova RA, Tasheva YR, et al. Effect of fumonisin B1 on lymphatic organs in broiler chickens-pathomorphology. *Bull Vet Inst Pulawy.* (2011) 55:801–5. doi: 10.2376/0005-9366-124-83
19. Bronte V, Pittet MJ. The spleen in local and systemic regulation of immunity. *Immunity.* (2013) 39:806–918. doi: 10.1016/j.immuni.2013.10.010
20. Chen D, Zhang Z, Yao H, Liang Y, Xing H, Xu S. Effects of atrazine and chlorpyrifos on oxidative stress-induced autophagy in the immune organs of common carp (Cyprinus carpio L.). *Fish Shellfish Immun.* (2015). 44:12–20. doi: 10.1016/j.fsi.2015.01.014
21. Chen M, Li X, Fan R, Yang J, Jin X, Hamid S, et al. Cadmium induces BNIP3-dependent autophagy in chicken spleen by modulating miR-33-AMPK axis. *Chemosphere.* (2017) 194:396–402. doi: 10.1016/j.chemosphere.2017.12.026
22. Johnson VJ, Sharma RP. Gender-dependent immunosuppression following subacute exposure to fumonisin B1. *Int Immunopharmacol.* (2001) 1:2023–34. doi: 10.1016/S1567-5769(01)00131-X
23. Xiao Y, Xu S, Zhao S, Liu K, Lu Z, Hou Z. Protective effects of selenium against zearalenone-induced apoptosis in chicken spleen lymphocyte via an endoplasmic reticulum stress signaling pathway. *Cell Stress Chaperon.* (2019) 24:77–89. doi: 10.1007/s12192-018-0943-9
24. Wu W, Liu T, Vesonder RF. Comparative cytotoxicity of fumonisin B1 and moniliformin in chicken primary cell cultures. *Mycopathologia.* (1995) 132:111–6. doi: 10.1007/BF01103783
25. Li YC, Ledoux DR, Bermudez AJ, Fritsche KL, Rottinghaus GE. Effects of fumonisin B1 on selected immune responses in broiler chicks. *Poult Sci.* (1999) 78:1275–82. doi: 10.1093/ps/78.9.1275
26. Keck BB, Bodine AB. The effects of fumonisin B1 on viability and mitogenic response of avian immune cells. *Poult Sci.* (2006) 85:1020–4. doi: 10.1093/ps/85.6.1020
27. Hackenberg S, Friehs G, Kessler M, Froelich K, Ginzkey C, Koehler C, et al. Nanosized titanium dioxide particles do not induce DNA damage in human peripheral blood lymphocytes. *Environ Mol Mutagen.* (2011) 52:264–8. doi: 10.1002/em.20615
28. Mao Y, Wang B, Xu X, Du W, Li W, Wang Y. Glycyrrhizic acid promotes M1 macrophage polarization in murine bone marrow-derived macrophages associated with the activation of JNK and NF-kappaB. *Mediators Inflamm.* (2015) 2015:372931. doi: 10.1155/2015/372931
29. Liu KZ, Li J, Kelsey SM, Newland AC, Mantsch HH. Quantitative determination of apoptosis on leukemia cells by infrared spectroscopy. *Apoptosis.* (2001) 6:269–78. doi: 10.1023/A:1011383408381
30. Kumar P, Nagarajan A, Uchil D. Analysis of cell viability by the lactate dehydrogenase assay. *Cold Spring Harb Protoc.* (2018) 2018:6. doi: 10.1101/pdb.prot095497
31. Todorova K, Dimitrov P, Toshkova R, Lazaroza S, Gardeva E, Yossifova L, et al. Influence of fumonisin B1 and deoxynivalenol on the immune system of chickens after application in quantities, naturally presented in fodders. *Medecine.* (2014) 67:139–44. Available online at: https://www.researchgate.net/publication/281775009
32. Li YC, Ledoux DR, Bermudez AJ, Fritsche KL, Rottinghaus GE. The individual and combined effects of fumonisin B1 and moniliformin on performance and selected immune parameters in turkey poults. *Poult Sci.* (2000) 79:871–8. doi: 10.1093/ps/79.6.871
33. Taranu I, Marin DE, Bouhet S, Pascale F, Bailly JD, Miller JD, et al. Mycotoxin fumonisin B1 alters the cytokine profile and decreases the vaccinal antibody titer in pigs. *Toxicol Sci.* (2005) 84:301–7. doi: 10.1093/toxsci/kfi086
34. Li Y, Fan Y, Xia B, Xiao Q, Wang Q, Sun W, et al. The immunosuppressive characteristics of FB1 by inhibition of maturation and function of BMDCs. *Int Immunopharmacol.* (2017) 47:206–11. doi: 10.1016/j.intimp.2017.03.031
35. Cheng YH, Ding ST, Chang MH. Effect of fumonisins on macrophage immune functions and gene expression of cytokines in broilers. *Arch Anim Nutr.* (2006) 60:267–76. di: 10.1080/17450390600785079 doi: 10.1080/17450390600785079
36. Bhandari N, Brown CC, Sharma RP. Fumonisin B1-induced localized activation of cytokine network in mouse liver. *Food Chem Toxicol.* (2002) 40:1483–91. doi: 10.1016/S0278-6915(02)00075-3
37. Jones C, Ciacci-Zanella JR, Zhang Y, Henderson G, Dickman M. Analysis of fumonisin B1-induced apoptosis. *Environ Health Perspect.* (2001) 109(Suppl. 2):315–20. doi: 10.1289/ehp.01109s2315
38. Chen J, Yang S, Huang S, Yan R, Wang M, Chen S, et al. Transcriptome study reveals apoptosis of porcine kidney cells induced by fumonisin B1 via TNF signalling pathway. *Food Chem Toxicol.* (2020) 139:111274. doi: 10.1016/j.fct.2020.111274
39. Yu S, Jia B, Yang Y, Liu N, Wu A. Involvement of PERK-CHOP pathway in fumonisin B1-induced cytotoxicity in human gastric epithelial cells. *Food Chem Toxicol.* (2020) 136:111080. doi: 10.1016/j.fct.2019.111080
40. Dombrink-Kurtzman MA. Fumonisin and beauvericin induce apoptosis in turkey peripheral blood lymphocytes. *Mycopathologia.* (2003) 156:357–64. doi: 10.1023/B:MYCO.0000003607.69016.d2
41. Fuchsluger TA, Jurkunas U, Kazlauskas A, Dana R. Corneal endothelial cells are protected from apoptosis by gene therapy. *Hum Gene Ther.* (2011) 22:549–58. doi: 10.1089/hum.2010.079
42. Brentnall M, Rodriguez-Menocal L, De Guevara RL, Cepero E, Boise LH. Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. *BMC Cell Biol.* (2013) 14:32. doi: 10.1186/1471-2121-14-32
43. Scorrano L, Korsmeyer SJ. Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. *Biochem Biophys Res Commun.* (2003) 304:437–44. doi: 10.1016/S0006-291X(03)00615-6
44. Burlacu A. Regulation of apoptosis by Bcl-2 family proteins. *J Cell MolMed.* (2003) 3:249–57. doi: 10.1111/j.1582-4934.2003.tb00225.x
45. Wu X. Study of anti-tumor and immunomodulatory activities of two annonaceous acetogenins: microcarpacin A and microcarpacin B. [dissertation/doctoral thesis]. Hong Kong: Chinese University of Hong Kong.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.