Fumonisin B₁ Induces Immunotoxicity and Apoptosis of Chicken Splenic Lymphocytes

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Fumonisin B₁ (FB₁), 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 FB₁ 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 FB₁ 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 FB₁. Then, the cell proliferation, damage, ultrastructure, inflammation and apoptosis were evaluated. Results showed that the proliferation rate of splenic lymphocytes was decreased by FB₁ treatments. The activity of lactate dehydrogenase (LDH) was increased by FB₁ treatments in a dose-dependent manner, implying the induction of cell damage. Consistently, the ultrastructure of splenic lymphocytes showed that FB₁ at all the tested concentrations caused cell structure alterations, including nuclear vacuolation, mitochondrial swelling and mitochondrial crest fracture. Besides, immunosuppressive effects of FB₁ 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 FB₁-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 FB₁ 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 FB₁ 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 FB₁ concentration. The increasing FB₁ concentration could decrease IL-12 concentration and increase Caspase-3 mRNA expression. Altogether, this study reported that FB₁ 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 B₁, chicken, splenic lymphocytes, immunotoxicity, apoptosis
INTRODUCTION

Mycotoxin fumonisins (FBs) are the secondary metabolites produced by Fusarium verticillioides and Fusarium proliferatum (1). Among the fumonisin homologs, fumonisin B1 (FB1) is the most prevalent and abundant mycotoxin contamination in stale corn. It is reported that FB1 has great potential health hazards to humans and animals (2, 3). FB1 can lead to intestinal damage (4, 5), neurotoxicity (6, 7) and various cancers (8–10). Moreover, in chickens, FB1 exposure lead to reduced performance, nutrient digestibility, immune function, and increased diarrhea as well as mortality (11–14).

The underlying cellular mechanisms of FB1-induced toxicity include the induction of oxidative stress, apoptosis and immunotoxicity (15, 16). The immunotoxicity of FB1 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 FB1 reduced basal rate of splenic lymphocyte proliferation in female mice (22). In chickens, although Todorova et al. showed that FB1 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 FB1 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 FB1.

MATERIALS AND METHODS

Materials

FB1 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 (BcL-2), P53, BcL-2 associated X (Bax), BcL-2 antagonist/killer 1 (Bak-1), and cysteinyl aspartate specific proteinase-3 (Casapse-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 resuspended 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 FB1 according to previous studies (24–26), in which 2.5–50 µg/mL FB1 suppressed the proliferation of chicken primary cells, such as splenocytes and peripheral lymphocytes. The FB1 was dissolved in deionized water to obtain a 40 mg/mL concentration solution. Then, various dilutions of the 40 mg/mL FB1 solution were added to cell cultures with final concentrations of 0, 2.5, 5, 10, 20 and 40 µg/mL FB1. For MTT assay, splenic lymphocytes were cultured in 96-well microplates (1 × 10^4 cells/mL) under 5% CO2 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 FB1 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% CO2 at 42°C, and treated with 0, 2.5, 5, 10, 20, and 40 µg/mL FB1 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 FB1, 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 MK3, USA). Proliferation rate (%) = (OD of cells treated with FB1–OD of cells without FB1 treatment)/OD of cells without FB1 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 overnight with 0.5% aqueous uranyl acetate. Specimens were dehydrated, 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 overnight with 0.5% aqueous uranyl acetate. Specimens were covered with a layer of monoclonal antibody, then incubated at room temperature for 30 min at the end of which 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.

**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 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.

**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, 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−ΔΔCq 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.

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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 $P < 0.05$.

**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
TABLE 2 | Effects of different concentrations of FB₁ on the inflammatory cytokines.

| FB₁ 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<sup>a</sup> | 123.37 ± 14.24<sup>a</sup> | 38.35 ± 1.63 | 140.24 ± 37.59<sup>a</sup> | 84.73 ± 2.84<sup>a</sup> |
| 2.5                       | 95.25 ± 0.64<sup>b</sup> | 116.77 ± 2.20<sup>a</sup> | 36.02 ± 4.39 | 123.52 ± 20.52<sup>a</sup> | 73.48 ± 7.15<sup>b</sup> |
| 5                         | 87.04 ± 4.62<sup>c</sup> | 105.31 ± 3.59<sup>a</sup> | 37.30 ± 5.58 | 92.17 ± 8.43<sup>d</sup> | 77.35 ± 3.13<sup>c</sup> |
| 10                        | 51.96 ± 10.16<sup>d</sup> | 93.91 ± 4.21<sup>c</sup> | 37.58 ± 2.87 | 91.10 ± 6.61<sup>c</sup> | 73.67 ± 2.05<sup>d</sup> |
| 20                        | 37.76 ± 5.12<sup>e</sup> | 79.60 ± 4.12<sup>d</sup> | 35.23 ± 5.73 | 76.39 ± 3.01<sup>c</sup> | 72.66 ± 5.30<sup>e</sup> |
| 40                        | 34.54 ± 0.82<sup>e</sup> | 62.47 ± 2.83<sup>a</sup> | 37.02 ± 1.03 | 62.01 ± 2.43<sup>c</sup> | 65.05 ± 2.44<sup>e</sup> |

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

FIGURE 4 | Effects of different concentrations of FB₁ 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).
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 $\mu$g/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 $\mu$g/mL FB$_1$-treated cells ($P < 0.05$). Furthermore, the concentration of IL-12 was also significantly reduced by 5, 10, 20, and 40 $\mu$g/mL FB$_1$ ($P < 0.05$). Compared to 5 and 10 $\mu$g/mL FB$_1$, 40 $\mu$g/mL FB$_1$ treatment led to a much lower IL-12 concentration ($P < 0.05$). Besides, the concentration of IFN-$\gamma$ was significantly reduced by the FB$_1$ at all the tested concentrations ($P < 0.05$), and 40 $\mu$g/mL FB$_1$ induced the lowest IFN-$\gamma$ 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 3). 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 $\mu$g/mL FB$_1$ treatments, 10, 20, and 40 $\mu$g/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 $\mu$g/mL FB$_1$ compared to the cells treated with 0, 2.5 and 10 $\mu$g/mL FB$_1$ ($P < 0.05$). And 40 $\mu$g/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 $\mu$g/mL FB$_1$ treatment group ($P < 0.05$). Besides, the expression of Bax was significantly up-regulated by 10 and 40 $\mu$g/mL FB$_1$ compared to other groups ($P < 0.05$). In addition, 10, 20 and 40 $\mu$g/mL FB$_1$ significantly elevated the expression of Bak-1 ($P < 0.05$). And 40 $\mu$g/mL FB$_1$ induced the highest expression of Bak-1 among groups ($P < 0.05$). Furthermore, 10 and 40 $\mu$g/mL FB$_1$ significantly up-regulated the Caspase-3 expression compared to the non-treated cells ($P < 0.05$) (Table 3).

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 ± 0.06a | 1.01 ± 0.12b | 1.03 ± 0.03bc | 1.01 ± 0.08c | 1.03 ± 0.18c |
| 2.5                          | 0.96 ± 0.04d | 1.09 ± 0.11d | 0.98 ± 0.04c | 1.09 ± 0.12c | 1.18 ± 0.31c |
| 5                            | 0.86 ± 0.07b | 1.05 ± 0.10b | 1.09 ± 0.04d | 1.27 ± 0.18c | 1.36 ± 0.29b |
| 10                           | 0.91 ± 0.09c | 1.14 ± 0.08c | 1.17 ± 0.07a | 1.19 ± 0.22b | 1.65 ± 0.21c |
| 20                           | 0.66 ± 0.10d | 1.01 ± 0.02d | 1.07 ± 0.02c | 1.31 ± 0.03c | 1.52 ± 0.45b |
| 40                           | 0.54 ± 0.02d | 1.24 ± 0.13a | 1.22 ± 0.04a | 1.54 ± 0.10a | 1.70 ± 0.57a |

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 ± 0.13a   | 281.75 ± 10.88a | 3.45 ± 0.17a | 15.07 ± 1.19a | 1.58 ± 0.15ac |
| 2.5                          | 2.39 ± 0.04b   | 336.34 ± 29.56b | 3.74 ± 0.19a | 13.98 ± 0.98b | 1.70 ± 0.14b  |
| 5                            | 2.21 ± 0.23b   | 379.58 ± 28.42b | 3.91 ± 0.14d | 14.29 ± 1.09b | 1.51 ± 0.12c  |
| 10                           | 2.36 ± 0.09b   | 340.76 ± 30.42b | 4.23 ± 0.20b | 14.55 ± 0.96b | 1.72 ± 0.14b  |
| 20                           | 2.36 ± 0.22b   | 469.84 ± 17.63b | 4.11 ± 0.19bc | 17.48 ± 0.40b | 1.48 ± 0.06c  |
| 40                           | 2.16 ± 0.23b   | 445.88 ± 55.15a | 5.12 ± 0.29b | 17.52 ± 1.57a | 1.94 ± 0.13a  |

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 ($P < 0.05$).
Effects of Different Concentrations of FB₁ 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 FB₁ 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 FB₁ (P > 0.05). Moreover, the concentration of P53 was significantly elevated by FB₁ 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 FB₁-treated groups than that of the 2.5 and 5 µg/mL FB₁-treated groups (P < 0.05). FB₁ at all the tested concentrations also significantly increased the Bax concentration (P < 0.05), and 40 µg/mL FB₁ induced the highest Bax concentration among the groups. Besides, the Bak-1 concentration in the 20 and 40 µg/mL FB₁-treated groups was much higher than that of the other groups (P < 0.05). Furthermore, 40 µg/mL FB₁ 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 FB₁ 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. Regression analysis also showed reciprocal relationship between IL-12 concentration, Caspase-3 mRNA expression and increasing FB₁ concentration. The increasing FB₁ concentration could decrease IL-12 concentration and increase Caspase-3 expression. Proliferation rate tended to decrease with increasing FB₁ concentration (P = 0.06).

DISCUSSION

Fusarium mycotoxins such as FB₁ 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 FB₁ on splenic lymphocytes. We found the proliferation rate of splenic lymphocytes was significantly decreased by treatments of FB₁ at 2.5, 5, 10, 20, and 40 µg/mL. Similarly, Johnson and Sharma also reported that FB₁ 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, FB₁ significantly increased the LDH activity in a dose-dependent manner. Regression analysis also showed that the LDH activity was increased by increasing FB₁ concentrations. Consistent with this result, the ultrastructure of FB₁-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 FB₁ and deoxynivalenol.

The immunotoxicity of FB₁ 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 reduced by FB₁ 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 FB₁. Regression analysis further demonstrated that the concentrations of IL-2, IL-4, IL-12, and IFN-γ were reduced by increasing FB₁ concentrations. Studies demonstrated that splenocytes from female mice exposed to FB₁ had a reduced expression of IL-2 mRNA (22). In vitro treatment of swine lymphocytes with FB₁ 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 FB₁ in a dose dependent manner (34). The mRNA concentration of IFN-γ in macrophages was also reduced by supplementation of FB₁ in chickens (35). Similar with our results, Bhandari et al. reported that the IL-6 concentration in the spleen of FB₁-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 FB₁ direct cytotoxicity effects but also be due to apoptosis of lymphocytes (37). Therefore, the pro-apoptotic role of FB₁ in splenic lymphocytes was further investigated. Previously, studies indicated that FB₁ 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 FB₁ 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

| Biochemical indices | Equation | R² | P-value |
|---------------------|----------|----|---------|
| Proliferation Rate (%) | y = 50.47x + 42.28 | 0.74 | 0.06 |
| IL-2 (ng/L) | y = 0.10x² - 0.71x + 108.49 | 0.96 | 0.04 |
| IL-4 (ng/L) | y = 0.04x² - 2.87x + 121.02 | 0.99 | 0.01 |
| IL-12 (ng/L) | y = 143.20x + 68.64 | 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 = -0.03x² + 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 = -1.37x + 1.70 | 0.84 | 0.03 |
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 FB1 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 FB1 concentrations. These results indicated that FB1 can induce splenic lymphocytes apoptosis by the Bcl-2 family-mediated mitochondrial pathway.

In conclusion, data in this study imply that FB1 strongly suppressed the chicken splenic lymphocytes proliferation and caused cell damage, especially the impairment of structure of mitochondria. The immunosuppressive effect of FB1 was also found by the decreased concentrations of inflammatory cytokines. Moreover, FB1 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 FB1 on chicken and provide new targets for regulating the FB1-related subclinical symptoms with possible unfavorable economic outcome.

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**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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