

## Aflatoxin B<sub>1</sub> affects apoptosis and expression of death receptor and endoplasmic reticulum molecules in chicken spleen

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### ABSTRACT

**Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is a natural product of the *Aspergillus* genus of molds, which grow on several foodstuffs stored in hot moist conditions, and is among the most potent hepatocarcinogens and immunosuppression presently known. The latter was related to the up-regulated apoptosis of immune organs. However, the effect of expression of death receptor and endoplasmic reticulum molecules in AFB<sub>1</sub>-induced apoptosis of chicken splenocytes was largely unknown. The objective of this study was to investigate this unknown field. One hundred and forty four one-day-old chickens were randomly divided into control group (0 mg/kg AFB<sub>1</sub>) and AFB<sub>1</sub> group (0.6 mg/kg AFB<sub>1</sub>), respectively and fed with AFB<sub>1</sub> for 21 days. Histological observation demonstrated that AFB<sub>1</sub> caused slight congestion and lymphocytic depletion in the spleen. TUNEL and flow cytometry assays showed the excessive apoptosis of splenocytes provoked by AFB<sub>1</sub>. Moreover, quantitative real-time PCR analysis revealed that AFB<sub>1</sub> induced the elevated mRNA expression of Fas, FasL, TNF- $\alpha$ , TNF-R<sub>1</sub>, Caspase-3, Caspase-8, Caspase-10, Grp78 and Grp94 in the spleen. These findings suggested that AFB<sub>1</sub> could lead the excessive apoptosis and alter the expression of death receptor and endoplasmic reticulum molecules in chicken spleen.**

### INTRODUCTION

Aflatoxins were difuranocoumarin compounds, and included B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub>, M<sub>1</sub>, and M<sub>2</sub>, among which aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) showed highly hepatotoxic, genotoxic, immunotoxic and other adverse effects on humans and animals [1–7].

Secondary to the effect on liver, the immunosuppressive nature of AFB<sub>1</sub> is the best documented area of its toxicity [4]. AFB<sub>1</sub> inhibited the development of bursa of Fabricius [8], thymus [9], affected the weight and function of immune organs [10], decreased the percentages of T cell subsets, reduced the Ig-containing cell number [11] and the

counts of splenic plasma cells [12], depressed the mitosis of B cells [13] and immunoglobulin as well as antibody production [14, 15]. In addition, AFB<sub>1</sub> caused oxidative stress in lymphoid tissue [16, 17], cell cycle arrest [18], and mitochondria injury in the lymphoid organs [19].

Apoptosis is associated with the normal development and homeostasis of animal tissues, and also involves in the pathogenesis [20]. Available information revealed that AFB<sub>1</sub> caused excessive apoptosis of several poultry and mammal cells such as hepatocytes [21], thymocytes [19, 22], splenocytes [17], bursa of Fabricius cells [8], bronchial epithelial cells [23], jejunal mucosal cells [24], bone marrow cells [25], and renal

cells [26]. Furthermore, our early researches have shown the possible link between mitochondrial molecules and apoptosis of hepatocytes [21], thymocytes [19], and bursa of Fabricius cells provoked by AFB<sub>1</sub> [27]. However, some death receptor molecules may not be related to AFB<sub>1</sub>-induced excessive cell death in the bursal cells [28], and the endoplasmic reticulum molecules may not be connected with the thymocyte apoptosis caused by AFB<sub>1</sub> [19].

The spleen, as one of the peripheral immune organs, is the largest lymphoid organ of the body [29]. With a large number of T and B lymphocytes, it is the center of cellular and humoral immunity [30]. In peripheral lymphoid organs, apoptosis is linked with the proliferation and maturation of lymphocytes after antigen recognition [31]. However, the effect of death receptor and endoplasmic reticulum molecules on AFB<sub>1</sub>-induced splenic apoptosis remains practically unknown. Thus, we conducted this study in order to explore the alteration of death receptor and endoplasmic reticulum molecule expression in AFB<sub>1</sub>-induced apoptosis in splenocytes of chicken by histopathological observation, flow cytometry, immunohistochemistry and relative real-time fluorescent quantitative PCR (RT-qPCR) analysis. The outcomes from the present study could provide a reference for the further study of apoptosis mechanism caused by AFB<sub>1</sub> in human and other animals in the future.

## RESULTS

### Growth performance

The effects of dietary AFB<sub>1</sub> on growth performance of chickens are shown in Figure 1. Compared with the control group, consumption of the AFB<sub>1</sub> diet reduced body weight and caused poor feed

conversion rate at 14 and 21 days ( $p < 0.05$  or  $p < 0.01$ ). Meanwhile, feed intake in the AFB<sub>1</sub> group was not different from that in the control group ( $p > 0.05$ ).

### Absolute weight and relative weight of spleen

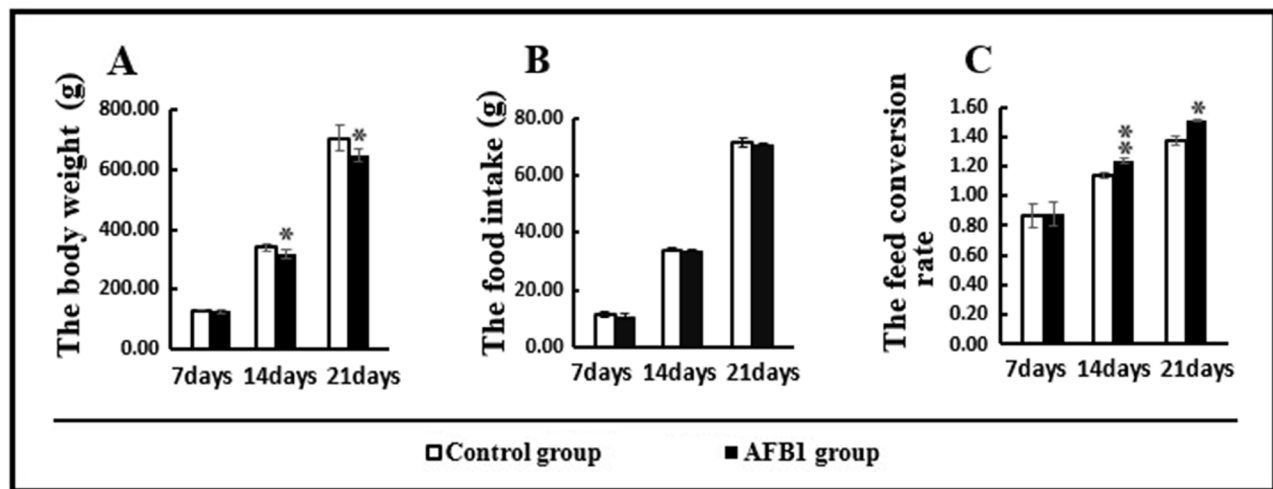
The absolute weight and relative weight of spleen in the AFB<sub>1</sub> group were significantly lower than those in the control group on day 14 and 21 ( $p < 0.05$  or  $p < 0.01$ ) (Figure 2).

### Histopathological observation

The parenchyma of chicken spleen was classified as white and red pulps. The former was subdivided into the splenic nodule, periarterial lymphoid tissue, and periellipsoidal lymphoid tissue. Compared with mammals, the chicken spleen had indistinct red pulp and white pulp, rich periellipsoidal lymphoid tissue, and few splenic nodules. The slight congestion was seen in some places of the red pulp, and the lymphocyte density was chiefly decreased in the white pulp in the AFB<sub>1</sub> group in comparison to the control group (Figure 3).

### Splenocyte apoptosis by TUNEL and flow cytometer analysis

The nuclei of TUNEL-positive cells were stained brown. Under microscope, more positive cells in the AFB<sub>1</sub> were observed than those in the control group during the experiment (Figure 4A–4D). Moreover, microscopic quantitative analysis also demonstrated the elevated number of positive cells in the AFB<sub>1</sub> group ( $p < 0.01$ ) when compared with the control group (Figure 4E). Flow cytometry assay showed the increased percentages of apoptotic splenocytes in the AFB<sub>1</sub> group ( $p < 0.05$  or  $p < 0.01$ ) during the experiment (Figure 4F–4H).



**Figure 1: Growth performance.** (A) body weight; (B) food intake; (C) feed conversion rate. Data are presented with the means ± standard deviation, \* $p < 0.05$  and \*\* $p < 0.01$ , compared with the control group.

## Expression levels of apoptosis associated genes by qRT-PCR

The results of expression levels of apoptosis associated genes by qRT-PCR are shown in the Figure 5. Compared with the control group, the expression of Fas, FasL, TNF- $\alpha$ , TNF-R<sub>1</sub>, Caspase-10, Caspase-8, Caspase-3, Grp78 and Grp94 mRNA in the AFB<sub>1</sub> group was significantly raised ( $p < 0.05$  or  $p < 0.01$ ).

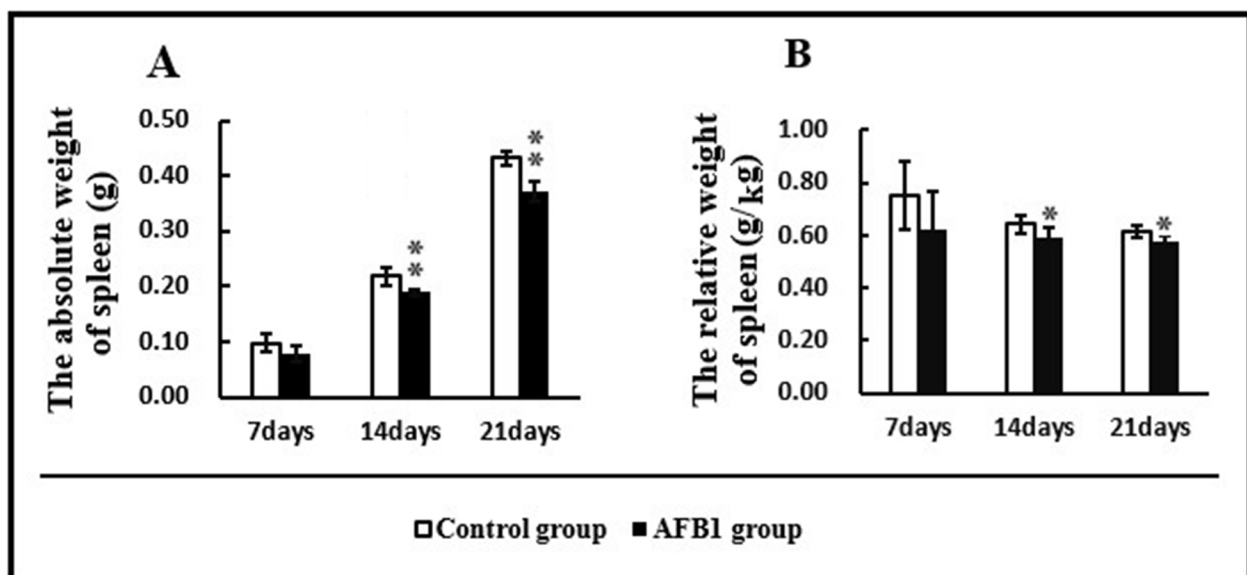
## DISCUSSION

Our present study revealed that AFB<sub>1</sub> significantly decreased body weight and affected feed conversion rate, suggesting that dietary AFB<sub>1</sub> (0.6 mg/kg) affected the growth performance of the chicken, similar to previous reports [4].

As the the largest secondary immune organ, spleen takes part in activating the immune response to antigens, and in screening foreign substances [29]. The splenic size and gross morphology vary duo to different species and distension, but, the splenic weight is crucial in its functional evaluation [29]. The relative weight of spleen keeps relatively stable irrespective of age [29]. This study demonstrated that AFB<sub>1</sub> could reduce the splenic absolute weight and relative weight along with lymphocytic depletion, similar to the report by Chen et al. [11], suggesting that this toxin had the detrimental effects on the development and immune function of spleen. Similarly, several reports also revealed that AFB<sub>1</sub> could decrease the relative weights of central immune organs of chicken [8, 28, 32]. However, contrary reports also existed. For instance, Ortatatli et al. [33] reported that no statistical difference was found in the relative weight of spleen between the aflatoxins-treated broiler chicks

and control ones. Peng et al. [34] demonstrated that aflatoxin-contaminated corn intake significantly increased the relative weight of chicken spleen. This discrepancy might partially be associated to the types of toxin because the aflatoxin-contaminated diet in Ortatatli and Peng's researches contained different kinds of mycotoxins including AFB<sub>1</sub> [33, 34].

Apoptosis has an important role in development, differentiation, proliferation and homeostasis of cell, tissue and organ [35]. AFB<sub>1</sub> directly or indirectly activated apoptotic process [36, 37], inducing apoptosis of several poultry and mammal cells [8, 9, 17, 19, 21–26]. The apoptotic cells could be evaluated by Flow cytometry and TUNEL assay [38, 39]. Our present research revealed an increased apoptosis in the AFB<sub>1</sub> group demonstrated by TUNEL and flow cytometry, suggesting that AFB<sub>1</sub> could lead excess apoptosis in the chickens' splenocytes, in line with previous researches in thymocytes [19, 22], bursa of Fabricius cells [27] and renal cells [26]. Lymphocytes are the main components within the lymphoid organs, and severe lymphocyte depletion in the lymphoid organs was due to apoptosis [40]. Therefore, it is tempting to speculate that the increased apoptosis of splenocytes provoked by AFB<sub>1</sub> might lead to the lymphocyte depletion, which may partly responsible for the declined splenic absolute and relative weights demonstrated in this study. Moreover, excessive apoptosis of lymphocytes was associated to immunosuppression in various circumstances [31]. Thus, our present results indicated that excessive apoptosis of spleen might cause immunosuppression in broilers exposed to AFB<sub>1</sub>. In addition, our present study revealed that the apoptotic percentage of the control group rose substantially by 21 days based on the flow cytometry assay, indicating that the normal apoptosis of the chicken's spleen showed

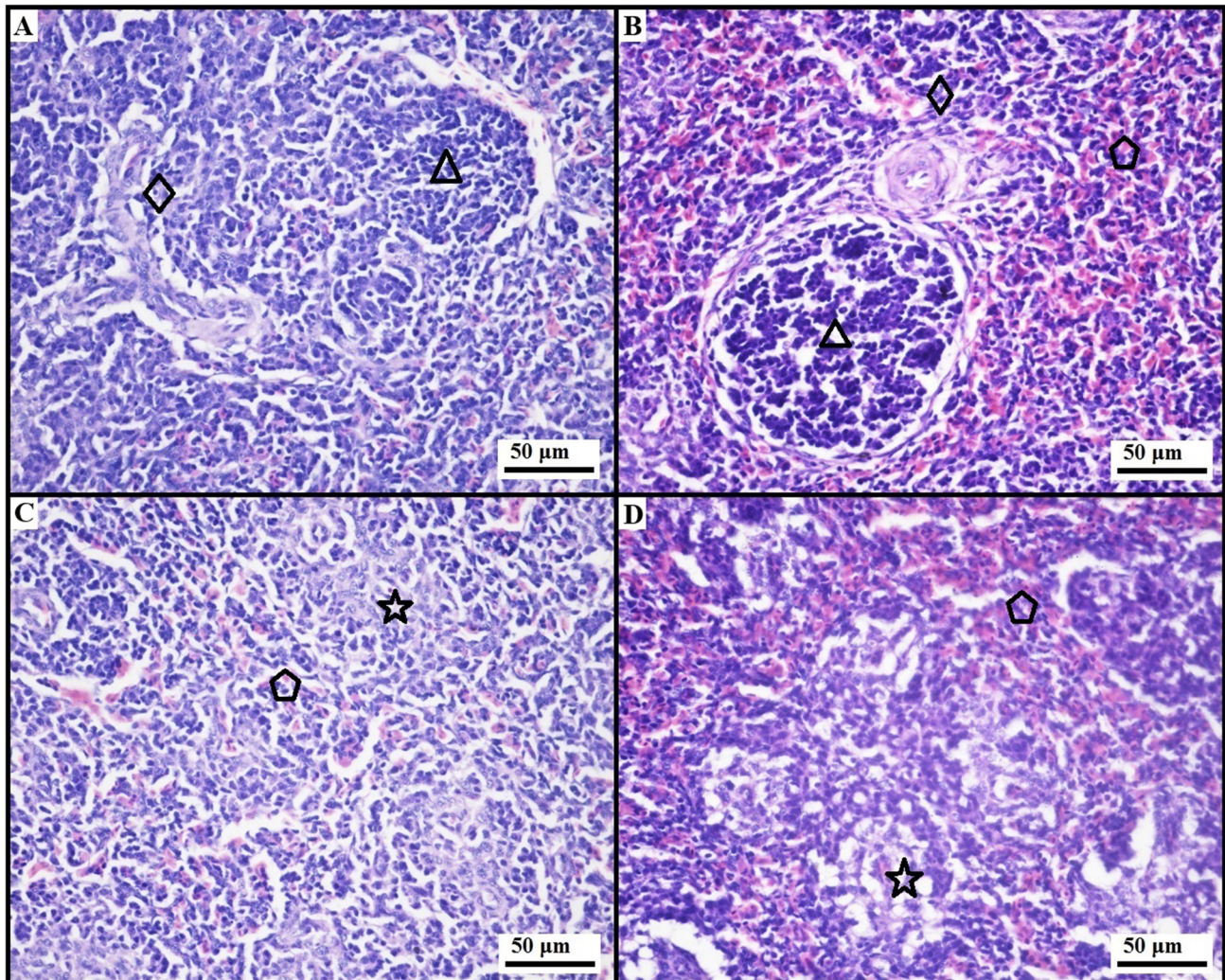


**Figure 2: Absolute and relative weights of spleen.** (A) absolute weight of spleen; (B) relative weight of spleen. Data are presented with the means  $\pm$  standard deviation ( $n = 6$ ),  $*p < 0.05$  and  $**p < 0.01$ , compared with the control group.

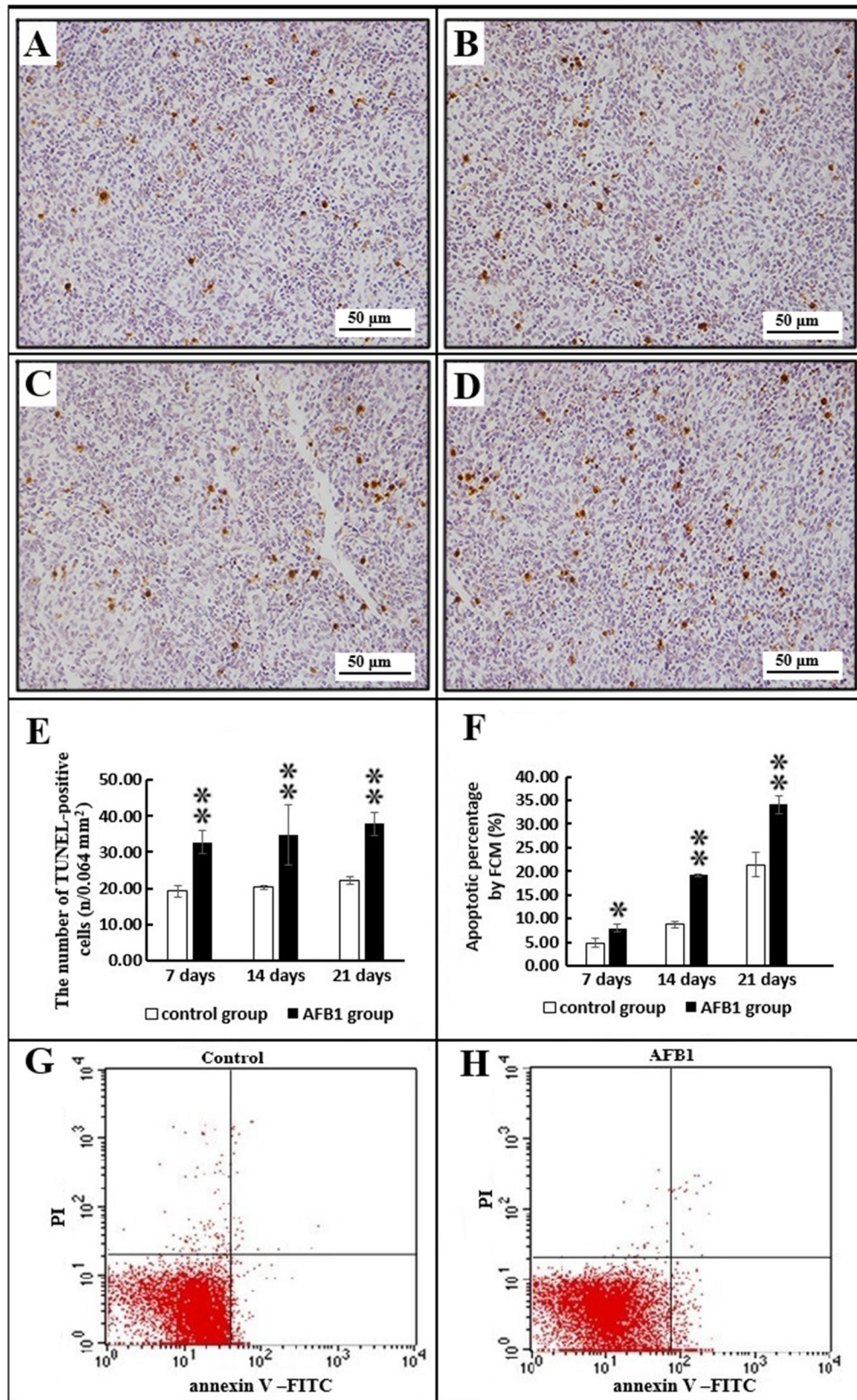
an increased changing pattern. Early researches also demonstrated that apoptosis of spleen was very obvious and presented development-related changes [41, 42]. Therefore, the present result that the apoptosis of AFB<sub>1</sub>-treated samples increased from 7 to 14 to 21 days may not be due to this increasing baseline, rather than from compounding effects of AFB<sub>1</sub>.

The signal pathways of apoptosis are complex and different under apoptosis induced factor stimulating. The death receptor and endoplasmic reticulum molecules are important molecules related to cell apoptosis, of which caspases are the final executioners [43]. To provide a reference for the further study of apoptosis mechanism caused by AFB<sub>1</sub>, we explored the alteration of Fas, FasL, TNF-R<sub>1</sub>, Caspase-3, Caspase-8, Caspase-10 Grp94 and Grp78 expression in AFB<sub>1</sub>-induced apoptosis in splenocytes of chicken.

After bound with FasL or TNF-R<sub>1</sub> and TNF-R<sub>2</sub>, respectively, Fas and TNF- $\alpha$  were activated, then Caspase-10 and Caspase-8 were recruited and these Caspases including Caspase-3 were activated, leading the cell apoptosis [44–47]. The present study demonstrated that AFB<sub>1</sub> diet led to the elevated expression of Fas, FasL, TNF-R<sub>1</sub>, Caspase-3, Caspase-8 and Caspase-10 mRNA expression in the spleen, which was consistent with earlier reports on the AFB<sub>1</sub>-induced apoptosis of thymocytes and hepatocytes [19, 21]. However, Yuan et al. reported that the mRNA expression of Fas, FasL, FADD, Caspase-8 and Caspase-10 in the bursa of Fabricius cells of the AFB<sub>1</sub>-treated chickens were not significant different from those of the control ones, suggesting that the excessive apoptosis of the bursal cells caused by AFB<sub>1</sub> was not attributed to death receptor molecules [28]. Therefore, the effect of molecules involved in the AFB<sub>1</sub>-caused apoptosis was



**Figure 3: Histological observaton of the chicken spleen at 21 days of age (HE. Staining, bar = 50  $\mu$ m). Note: (A) the control group showing normal structure of periarterial lymphoid tissue (◇) and splenic nodule (△); (B) the AFB<sub>1</sub> group showing slight congestion in the the red pulp (◊) and lymphocyte depletion in the periarterial lymphoid tissue (◇) and splenic nodule (△); (C) the control group showing normal structure of periellipsoidal lymphoid tissue (☆) and red pulp (◊); (D) the AFB<sub>1</sub> group showing slight congestion in the red pulp (◊) and the lymphocyte depletion in the periellipsoidal lymphoid tissue (☆).**



**Figure 4: The splenocyte apoptosis by TUNEL immunohistochemistry and flow cytometry analysis.** Note: (A–D) TUNEL-positive cells in the control group (A) at 14 days, and AFB<sub>1</sub> groups (B–D) at 7, 14 and 21 days of age (TUNEL assay, Scale bar: 50 μm). (E) The numbers of TUNEL-positive cells (microscopic quantitative analysis). Data are presented with the means ± standard deviation (*n* = 6). (F) Apoptotic percentages of splenocytes by flow cytometry assay. Data are presented with the means ± standard deviation (*n* = 6). (G–H) Scattergram of apoptotic splenocytes obtained by flow cytometry assay in the control group (G) and AFB<sub>1</sub> group (H) at 21 days of age.

different and complicated, and may vary depending on different tissues.

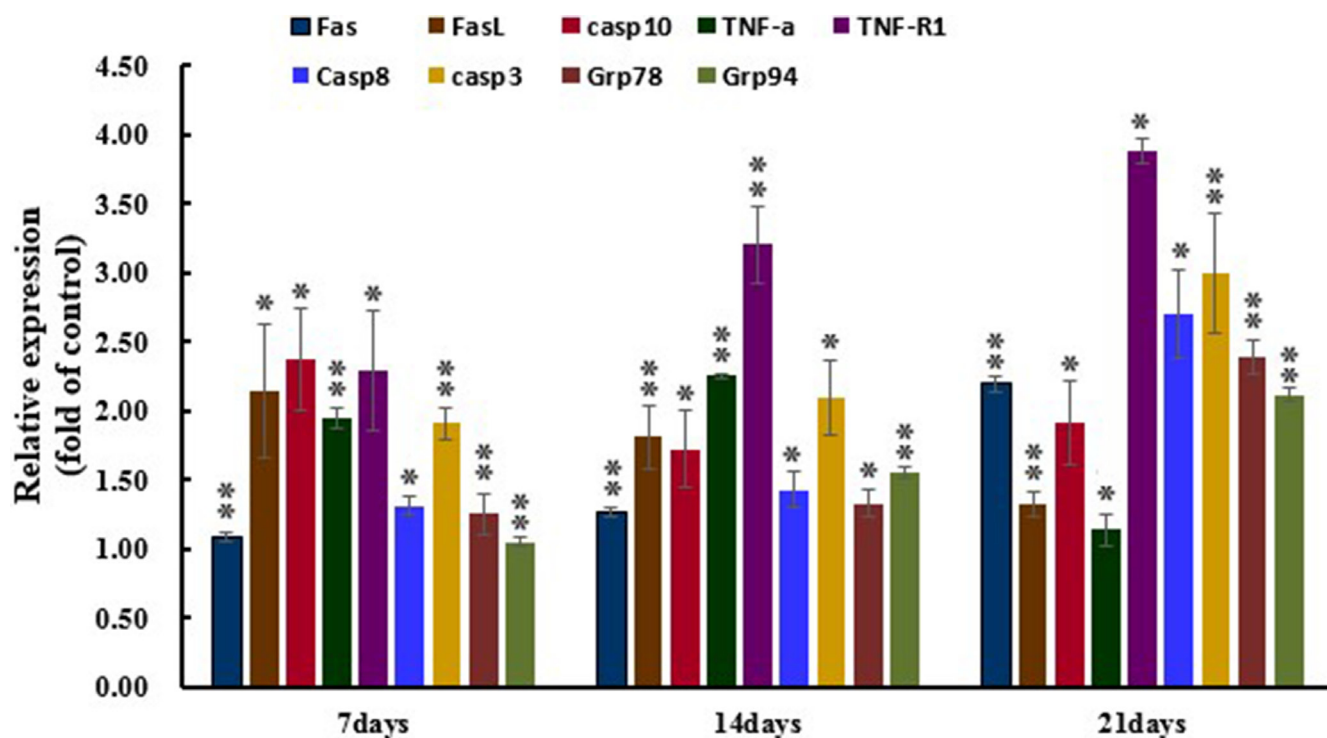
The endoplasmic reticulum (ER) pathway is initiated by ER stress which causes the accumulation of misfolded or unfolded proteins in the ER [48, 49]. This accumulation activates the expression of Grp94 and Grp78 and enhance the protein folding machinery [49, 50]. If the unfolded protein reaction is unable to control the unfolded and misfolded protein levels in the ER, the apoptotic signaling provoked by ER is triggered by activating Caspase-12, and Caspase-3, and ultimately induces cell death [43, 49]. Our present result demonstrated that AFB<sub>1</sub> could lead the elevated expression of Grp78 and Grp94 mRNA. This is consistent to previous report in the bursa of Fabricius cells [28]. However, contradictory result showed that the expression of Grp94 and Grp78 mRNA were not significant different between the AFB<sub>1</sub>-treated group and control group of thymocytes, suggesting that the ER molecules may not involve in the AFB<sub>1</sub>-induced apoptosis of thymocytes [19]. This is also confirmed that the effect of molecules related to the AFB<sub>1</sub>-induced apoptosis may be different duo to various cell types.

Our study demonstrated that dietary AFB<sub>1</sub> (0.6 mg/kg) could cause the decline in the absolute weight and relative weight of chickens' spleen along with mild congestion and lymphocytic depletion, and induce splenocyte apoptosis accompanied by the up-regulation of Fas, FasL, TNF- $\alpha$ , TNF-R<sub>1</sub>, Caspase-3, Caspase-8, Caspase-10, Grp78 and Grp94 mRNA expression.

## MATERIALS AND METHODS

### Animals and diets

The animal protocols used in this work and all procedures of the experiment were performed in compliance with the laws and guidelines of Sichuan Agricultural University Animal Care and Use Committee (Approval No: 2012-024). One hundred and forty four one-day-old healthy Cobb male chickens were purchased from a commercial rearing farm (Wenjiang poultry farm, Sichuan province, China), and randomly divided into two equal groups, namely control group (0 mg/kg AFB<sub>1</sub>) and AFB<sub>1</sub> group (0.6 mg/kg AFB<sub>1</sub>). All of the chickens were put into cages with three replicates per group and 24 birds per replicate. The basal diet, namely the control diet, was formulated according to National Research Council (NRC, 1994) [51] and Chinese Feeding Standard of Chicken (NY/T33-2004). AFB<sub>1</sub> was purchased from Sigma-Aldrich (USA, A6636). The AFB<sub>1</sub>-contaminated diet was made, similarly to the method described by Kaoud [52]. Briefly, 27 mg AFB<sub>1</sub> farinose solid was dissolved into 30 mL methanol completely, and then the 30 mL mixture was mixed into 45 kg corn-soybean basal diet to formulate AFB<sub>1</sub> diet of experimental groups containing 0.6mg/kg AFB<sub>1</sub>. The equivalent methanol was added into corn-soybean basal diet to formulate the control diet. Then the methanol of diets was evaporated at 98°F (37°C). The AFB<sub>1</sub> concentrations were analyzed by HPLC



**Figure 5: Expression levels of apoptosis associated gens by qRT-PCR.** Note: Data are presented with the means  $\pm$  standard deviation ( $n = 6$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the control group.

(Waters, Milford, MA, USA) with fluorescence detection (Waters, Model 2475, Milford, MA, USA), and the AFB<sub>1</sub> concentration was determined as < 0.001 mg/kg and 0.601 mg/kg in the control diet and AFB<sub>1</sub>-contaminated diet, respectively. Chickens were fed in cages with electrically heated units and provided with water as well as aforementioned diet *ad libitum* for 21 days.

### Growth performance

Body weight, feed intake, and feed conversion rate per cage were recorded weekly from 7 to 21 days of age.

### The absolute and relative weights of spleen

At 7, 14, and 21 days of age, six chickens in each group were randomly chosen, weighted, and humanely euthanized. And spleens were removed, and weighted. The relative weight of spleen was calculated by the following formula:

Relative weight of spleen = absolute weight of spleen (g)/body weight (kg)

### Histopathological observation

At 7, 14, and 21 days of age, the splenic tissue samples from six chickens in each group were collected and fixed in 4% paraformaldehyde (PFA), and then were dehydrated and embedded in paraffin wax. Blocks were cut into 5 μm sections with a microtome (Leica, Germany, RM2135) for haematoxylin and eosin (HE.) staining and TUNEL assay. The histological structure of the tissues was observed under light microscope and photographed with a digital camera (Nikon DS-Ri1, Japan).

### Apoptosis detection by TUNEL assay

TUNEL assay was performed with an apoptosis detection Kit (MK1020 Boster, Wuhan, China) according to the manufacturer's instructions. Briefly, tissue sections were dewaxed with 100% xylene, and rehydrated in successive changes of 100%, 95%, 85% and 75% ethanol. After endogenous peroxidase activity was quenched for 10 min in 3% H<sub>2</sub>O<sub>2</sub> with distilled water at 37°C, the sections were incubated with proteinase K diluted 1:200 in TBS at 37°C for 5–10 min in a humidified chamber. A labeling mixture containing digoxin-dUTP in TdT (Terminal deoxynucleotidyl Transferase) enzyme buffer was added to the sections and incubated at 37°C for 2 h. After three successive washings with TBS for 2 min, sections were covered with anti-digoxin-biotin conjugate diluted 1:100 in blocking reagent and incubated for 30 min at 37°C. The tissues were then incubated for 1 h at 37°C with strept avidin-biotin-complex (SABC) diluted 1:100 in TBS. Labeling was visualized with 3'3'-diaminobenzidine. The sections were then counterstained with haematoxylin. For the negative control, representative sections were

processed in the same way but incubation with TdT enzyme buffer was omitted.

The number of TUNEL-positive cells in the spleen was evaluated by Image-Pro Plus5.1 (USA) image analysis software. Briefly, photographs of TUNEL staining were taken with a digital microscope camera system (Nikon DS-Ri1, Japan). For each section, five fields of 0.064 mm<sup>2</sup> (corresponding to five fields at 400 × magnification) were analyzed. By selecting “colour-chosen target” in the option bar of the morphologic analysis system, all TUNEL-positive cells in the field were marked in colour. Then, “calculating” in the option bar was selected to automatically calculate the number of TUNEL-positive cells. Results were expressed as the average of TUNEL-positive cells per 0.064 mm<sup>2</sup> area.

### Apoptosis detection by flow cytometry

At 7, 14, and 21 days of the experiment, six chickens in each group were euthanized, and spleens were sampled to determine the percentage of apoptotic cells by flow cytometer, using the method by Chen et al. [53]. Briefly, the dissected spleens were thereupon homogenized to form a cell suspension and filtered, and then the cells were washed and resuspended in phosphate buffer at a concentration of 1 × 10<sup>6</sup> cells/mL. 5 μL Annexin V-Fluorescein isothiocyanate (V-FITC) and 5 μL propidium iodide (PI) were added into 100 μL cell suspension, and incubated at 25°C for 15 min in the dark. 400 μL 1 × Annexin binding buffer was added to the mixture, and then the apoptotic cells were assayed by flow cytometer (BD FACSCalibur) within 1 h. The annexin V-FITC Kit was purchase from BD Pharmingen (USA, 556547).

### Expression levels of apoptotic regulator mRNAs by quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) assay was carried out as reported by Chen et al. [22]. Briefly, the spleens from six chickens in each groups at 7, 14, and 21 days of the experiment were obtained and stored in liquid nitrogen, respectively. Adding liquid nitrogen, the samples were crushed with pestle to homogenize until powdery, respectively. Total RNA was extracted from the powdery of samples using RNAiso Plus (9108/9109, Takara, Otsu, Japan). The mRNA was then reverse transcribed into cDNA using PrimScript™ RT reagent Kit with gDNA Eraser (RR047A, Takara, Otsu, Japan). The cDNA was used as a template for qRT-PCR analysis.

For qRT-PCR reactions, 25 μL mixtures were made by using SYBR® Premix Ex Taq™ II (DRR820A, Takara, Otsu, Japan), containing 12.5 μL Tli RNaseH Plus, 1.0 μL of forward and 1.0 μL of reverse primer, 8.5 μL RNAase-free water and 2 μL cDNA. Reaction conditions were set to 3 min at 95°C (first segment, one cycle), 10 s at 95°C and 30 s at T<sub>m</sub> of a specific primer pair (second segment,

**Table 1: Primer sequence for apoptotic genes**

Gene symbol	Ref Seq	Forward primers	Reverse primers
Caspase-3	NM_204725	TGGCCCTCTTGAAGTCAAAG	TCCACTGTCTGCTTCAATACC
Caspase-8	NM_204592	GTCTCCGTTTCAAGTATCTGCT	TCTCAATGAAAACGTCCGGC
Caspase-10	XM_421936	CTGGGGGCTCCAAAAGTCC	AAAGGGGGACAAAAGCCAACA
Fas	NM_001199487	TCCACCTGCTCCTCGTCATT	GTGCAGTGTGTGTGGGAACT
FasL	NM_001031559	GGCATTACAGTACCGTGACCA	CCGGAAGAGCACATTGGAGT
Grp78	NM_205491	GGTGTGCTTGATGTGTGTCC	GCTGATTGTCAGAAGCTGTGG
Grp94	NM_204289	TGACCTGGATGCAAAGGTGGA	TTAAACCCACACCATCCCTCAAC
TNF- $\alpha$	AY765397	TCAGACCAGATGGGAAGGGA	ACTGGGCGGTCATAGAACAG
TNF-R1	NM_001030779	CCTGTCTGTCTCCCTGTCC	GGTGCATGGGGTCTTTTCTA
$\beta$ -actin	L08165	TGCTGTGTTCCCATCTATCG	TTGGTGACAATACCGTGTCA

44 cycles) followed by 10 s at 95°C, and 72°C for 10 s (dissociation curve segment) using Thermal Cycler (C1000, BIO RAD, CA, USA). The mRNA expression of Fas, FasL, TNF- $\alpha$ , TNF-R<sub>1</sub>, Caspase-10, Caspase-8, Caspase-3, Grp78 and Grp94 was analyzed.  $\beta$ -actin was used as an internal control gene. Sequence of primers was obtained from GenBank of NCBI. Primers were designed with Primer 5, and synthesized by BGI Tech (Shenzhen, China) (Table 1). The qRT-PCR data were analyzed and fold change in expressions were calculated using the 2<sup>- $\Delta$ ACT</sup> method [54].

### Statistical analysis

The significance of difference between two groups was analyzed by variance analysis, and the results were expressed by mean  $\pm$  standard deviation. The analyses were performed using the independent sample test of SPSS 20.0 software (IBM Corp, Armonk, NY, USA) for windows. Statistical significant differences were considered at  $p < 0.05$  and markedly significant differences were considered at  $p < 0.01$ .

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### CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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