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Original Article

Effects of aflatoxin B_1 on the cell cycle distribution of splenocytes in chickens

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Abstract: The purpose of the present study was to evaluate effects of aflatoxin B_1 (AFB_1) on the cell cycle and proliferation of splenic cells in chickens. A total of 144 one-day-old Cobb male chickens were randomly divided into 2 equal groups of 72 each and were fed on diets as follows: a control diet and a 0.6 mg/kg AFB_1 diet for 21 days. The AFB_1 diet reduced body weight, absolute weight and relative weight of the spleen in broilers. Histopathological lesions in AFB_1 groups were characterized as slight congestion in red pulp and lymphocytic depletion in white pulp. Compared with the control group, the expression levels of ataxia–telangiectasia mutated (ATM), cyclin E_1, cyclin-dependent kinases 6 (CDK6), CDK2, p53, p21 and cyclin B_3 mRNA were significantly increased, while the mRNA expression levels of cyclin D_1, cdc2 (CDK1), p16, p15 were significantly decreased in the AFB_1 groups. Significantly decreased proliferating cell nuclear antigen (PCNA) expression and arrested G_0G_1 phases of the cell cycle were also seen in the AFB_1 groups. In conclusion, dietary AFB_1 could induce cell cycle blockage at G_0G_1 phase and impair the immune function of the spleen. Cyclin D_1/CDK6 complex, which inhibits the activin/nodal signaling pathway, might play a significant role in the cell cycle arrest induced by AFB_1. (DOI: 10.1293/tox.2018-0015; J Toxicol Pathol 2019; 32: 27–36)

Key words: AFB_1, G_0G_1 phase, cell cycle arrest, mechanism, spleen, chicken

Introduction

Aflatoxin B_1 (AFB_1) is a type of mycotoxin that is classified as a Group I carcinogen to humans by the International Agency for Research on Cancer and is a mycotoxins produced typically during storage of crops[1].It is commonly encountered and has potent carcinogenic, genotoxic, immunotoxic and other adverse effects in many animal species including poultry[2, 3]. An active intermediate products of AFB_1, AFB_1-exo-8, 9-epoxide, can bind with DNA to form the predominant trans-8, 9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB_1 (AFB_1-N7-Gua) adduct, which causes DNA lesions[4]. Aflatoxin exposure is one of the multple of human hepatocellular carcinoma (HCC) development[5].

Many researchers have reported that aflatoxin could affect many biological characteristics of intestine[6-8], cecal tonsil[9], thymus[10, 11], spleen[12, 13], bursa of Fabricius[11, 14]and kidney[15] morphology in poultry, such as by causing histological lesions, oxidative stress, apoptosis, changes in T-cell subsets, and changes in cellular and humoral immune function[16, 17].

AFB_1-8, 9-exo-epoxide can affect every period of the cell cycle with its potent biological activity[18, 19]. The researches has demonstrated that AFB_1 caused chick jejunum cells to be arrested at G_2/M phase[8, 20], renal cells to be arrested at G_0/G_1 phase[21], increased percentages of chick thymocytes in the G_2/M[3] phase, and dose dependent accumulation in S phase *in vitro* in human cell lines[22]. AFB_1 can impact a variety of cell cycle regulation gene, protein and related enzyme activities including p53 gene mutations and p16 gene methylation in tissue[23], affecting activities of CDK4, CDK6 and Rb[19, 24, 25], and reducing the amount of

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PCNA-positive cells[8, 26]. Bressac *et al.*[27] and Hollestein *et al.*[28] have demonstrated that dietary intake of aflatoxins was closely related to p53 gene mutations.

The spleen is the most important organ for antibacterial and antifungal immune reactivity[29]. AFB_1 could decrease the number of CD4+ and CD8+ T cells in the spleens of mice[30] and induce biomolecular oxidative damage and decrease cell proliferation of spleen mononuclear cells in rats[31–33]. Cell cycle arrest of splenocytes in chickens fed aflatoxin-contaminated corn has been reported[34], but the exact molecular mechanism of cell cycle arrest in the spleen of chickens induced by AFB_1 diet has not been elucidated. The results could help us to understand the molecule mechanism for the spleen immunosuppression attributable to AFB_1.

Materials and Methods

Animals and diets

The experiment was performed with male Cobb chickens weighing 45 ± 5 g that were purchased from a commercial rearing farm (Wenjiang poultry farm, Chengdu, Sichuan Province, China).

A total of 144 one-day-old chickens were randomly divided into 2 groups, namely control group (0 mg/kg AFB_1) and AFB_1 group (0.6 mg/kg AFB_1). All of the chickens were put into cages with three replicates per group and 24 birds per replicate. AFB_1 was purchased from A6636, Sigma-Aldrich, St.Louis, MO, USA. Nutritional requirements were adequate according to National Research Council (National Research Council, 1994)[35]. The AFB_1-contamin diet was produced by method similar to described by Kaoud[36]. In short, 27 mg AFB_1 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_1 diet containing 0.6 mg/kg AFB_1. Equivalent methanol was mixed into corn-soybean basal diet to produce a control diet. Then, methanol in diets was evaporated at 98°F (37°C). The concentrations of AFB_1 were analyzed by HPLC (Waters, Milford, MA, USA) with fluorescence detection 2475 Fluorescence Detector (Waters, Milford, MA, USA) and were determined to be <0.001 mg/kg in the control diet and 0.601 mg/kg in the AFB_1 diet. Chickens were housed in cages with electrically heated units and provided with water as well as the diets *ad libitum* for 21 days.

Body weight and absolute and relative weight of spleen

At 7, 14, and 21 days of age, six chickens in each group were weighed and euthanized. The spleen was dissected from each chick immediately and weighed with electronic balance after removing the surrounding fat and connective tissue. The following formula was used to calculate the relative weight:

Relative weight (g/kg) = organ weight (g) / fasting weight of chick (kg)

Pathological observation

After weighing, spleens were fixed in 4% paraformaldehyde for 24 h and routinely processed in dehydration, transparent disposal, paraffin embedded and sectioned at 5 μm. Slides were stained with hematoxylin and eosin Y (H.E). Paraffin sections were also collected to perform immunohistochemistry. The histological structures of the tissues were observed and photographed with a digital camera (DS-Ri1, Nikon Instech Co., Ltd., Tokyo, Japan).

Cell cycle of the spleen by flow cytometry method

At 7, 14, and 21 days of age, six chickens in each group were euthanized, and the spleen was dissected from each animal an immediately minced with surgical scissors. The cell suspension was filtered through a 300-mesh nylon mesh. Then, the cells were washed and suspended in phosphate buffer at a concentration of 1×10^6 cells/mL (PBS, pH 7.2–7.4). 1 mL suspension was transferred to a 500 μL culture tube and centrifuged at 200× g for 5 min at 4°C. The supernatant was separated and discarded. Then, propidium iodide (5 μL, 51-66211E, BD Pharmingen, San Diego, CA, USA) was added into 100 μL cell suspension and incubated for 30 min at 4°C in a dark room. Finally, 500 μL PBS was added to each tube, and cells were analyzed by flow cytometry (FACSCalibur, BD, Franklin Lake, NJ, USA). The results were analyzed using the ModFit LT for Mac V3.0 computer program.

proliferating index (PI) = $(S + G_2M) / (G_0G_1 + S + G_2M)$

PCNA detection by the immunohistochemical method

The method of immunohistochemistry was applied according to the report by Fang *et al.*[37] Spleen paraffin sections were dewaxed in xylene, rehydrated through a graded series of ethanol solutions and washed three times in distilled water and PBS (0.1 M, PH 7.2-7.4). Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 in methanol for 15 min. Following a wash with PBS, the sections were exposed to normal 10% goat sera for 30 min at 37°C to block nonspecific antibody binding. In a humidified chamber, 10 μg/mL primary antibodies rabbit anti-PCNA (bs-0754R, Wuhan Boster Bio-Engineering Limited Company, Wuhan, China) was applied sections for 20 h at 4°C. After three washings in PBS, the slices were exposed to 1% biotinylated goat anti-rabbit/mouse IgG secondary antibody (SA1030, Wuhan Boster Bio-engineering Limited Company, Wuhan, China) for 1 h at 37°C, and then incubated with streptavidin-biotin complex (SABC; Wuhan Boster Bio-engineering Limited Company, Wuhan, China) for 30 min at 37°C. Slides were visualized with 3,3'-diaminobenzidin. The slices were monitored microscopically and stopped by immersion in distilled water, as soon as brown staining was visible. Slices were lightly counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and cover slipped. For negative control purposes, representative sections were processed in the same way by replacing primary antibodies in PBS. The stained sections were photographed with a digi-

tal camera (DS-Ri1, Nikon Instech Co., Ltd., Tokyo, Japan). Within a range of 1,000 times lens, 6 fields of view were randomly selected for each slice, and photos were taken for determination of integral optical density (IOD) value. The results were analyzed using Image-Pro Plus 6.0 software.

Quantitative real-time PCR

In order to comprehend the molecular mechanism of cell cycle arrest induced by AFB_1, we continued our research to detect the mRNA expression of cell cyclins by quantitative real-time PCR (qRT-PCR). The spleens from six chickens in each group were remove at 7, 14, and 21 days of age and stored in liquid nitrogen. The spleens were crushed with pestle to homogenize them until they powdery, respectively. As previously described[38], total RNA was extracted from the powder of the spleens using RNAiso Plus (9108/9109, Takara, Kusatsu, Japan). RNA concentrations and purity were checked by NanoDrop™ One (Thermo Fisher Scientific, Waltham, MA, USA). Next, cDNA was synthesized using a PrimScript RT reagent Kit (RR047A, Takara, Kusatsu, Japan) according to the manufacturer's protocol. The cDNA product was used as a template for qRT-PCR analysis. Sequences for target genes were obtained from the NCBI database. Oligonucleotide primers were designed using Primer 5 software and synthesized at Takara (Dalian, China; Table 1). All qRT-PCR reactions were performed using the SYBR® Premix Ex TaqT II system (DRR820A, Takara, Kusatsu, Japan) and a C1000 Touch (Bio-Rad Laboratories, Hercules, CA, USA). Chicken β-actin was used as an internal reference housekeeping gene. All data output from the qRT-PCR experiments were analyzed using the $2^{-\Delta\Delta CT}$ method[39].

Statistical analysis

The significance of differences between two groups was analyzed by variance analysis, and results were expressed as mean ± standard deviation (X ± SD). The analysis was performed using the independent sample test in the IBM SPSS Statistics for Windows, Version 20.0 software (IBM Corp, Armonk, NY, USA). Difference were considered statistically significant at $p<0.05$ and markedly significant was considered at $p<0.01$.

Results

Body weight and absolute weight and relative weight of spleen

The effects of dietary AFB_1 on body weight and absolute weight and relative weight of the spleen of chickens are shown in Fig. 1. No significant differences were observed in body weight or absolute weight and relative weight in the AFB_1 group at 7 days of age. Compared with the control group, the AFB_1 diet reduced body weight at 14 and 21 days of age ($p<0.05$). Meanwhile, the absolute and relative weight of the spleen in the AFB_1 group were significantly lower than those in the control group at 14 and 21 days of age ($p<0.05$ or $p<0.01$).

Table 1. Sequence of Primers Used in qRT-PCR

Gene symbol	Accession number	Primer	Primer sequence (5'-3')	Product size	Tm (°C)
ATM	NM001162400.1	Forward	TTGCCACACTCTTTCCATGT	110 bp	60
		Reverse	CCCACTGCATATTCCTCCAT		
P53	NM205264.1	Forward	ACCTGCACTTACTCCCCGGT	127 bp	59
		Reverse	TCTTATAGACGGCCACGGCG		
P21	AF513031.1	Forward	TCCCTGCCCTGTACTGTCTAA	123 bp	60
		Reverse	GCGTGGGCTCTTCCTATACAT		
PCNA	AB053163.1	Forward	GATGTTCCTCTCGTTGTGGAG	104 bp	60
		Reverse	CAGTGCAGTTAAGAGCCTTCC		
P16	NM_204434.1	Forward	GCGTTTGGAGAAGTGAGACAG	120 bp	59
		Reverse	GAATACAATCAGCCCGGTTAAG		
Cyclin D_1	U40844.1	Forward	CTGCTGCTGGTGAATAAGCTG	108 bp	60
		Reverse	GATCTGTTTGGTGTCCTCTGC		
CDK6	NM_001007892.2	Forward	AGCAGCCCAGAAGAGATGATT	132 bp	60
		Reverse	GAGAAATACGCACAAACCCTGT		
Cyclin E	NM_001031358.1	Forward	GCCACCACAAAGCAGTAAGAA	100 bp	60
		Reverse	AACCAGCAGAACAGGCACTT		
CDK2	EF182713.1	Forward	CTGGTCTTTGAGTTCCTGCAC	179 bp	60
		Reverse	GCGTTGATGAGGAGGTTCTG		
P15	NM204433.1	Forward	GTTGCGGTGGTACATCAAGC	113 bp	60
		Reverse	TGCGCTTCTCGTACATCCTT		
Cyclin B_3	NM205239.2	Forward	ATCACCAACGCTCACAAGAAC	171 bp	59
		Reverse	AGGCTCCACAGGAACATCTG		
cdc2 (CDK1)	NM.205314.1	Forward	TCTGCTCTGTATTCCACTCCTG	144 bp	60
		Reverse	ATTGTTGGGTGTCCCTAAAGC		
β-actin	L08165	Forward	TGCTGTGTTCCCATCTATCG	178 bp	62
		Reverse	TTGGTGACAATACCGTCTTCA		

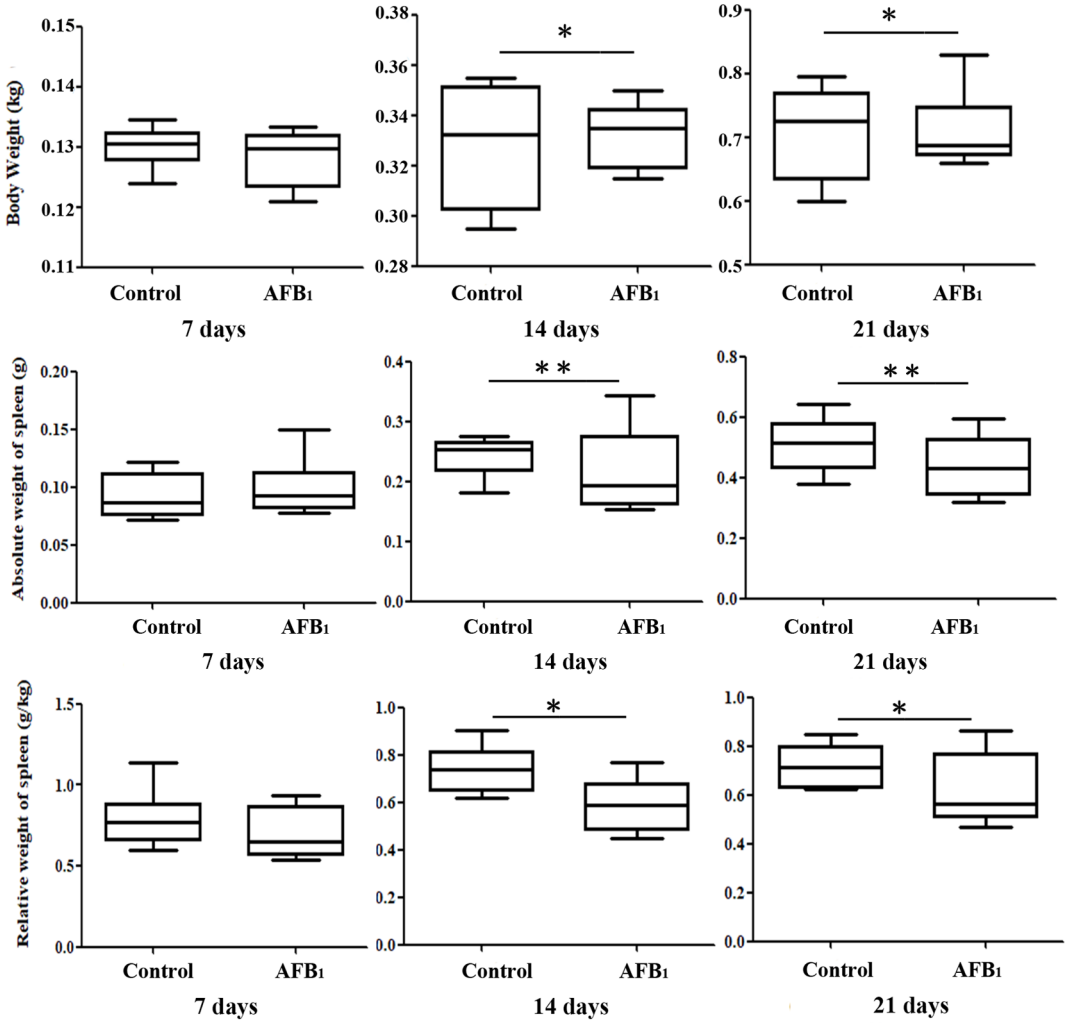

Fig. 1. The effects of dietary AFB_1 on body weight and absolute weight and relative weight of the spleen. Notes: Data are presented with the means ± standard deviation (n = 6). *p<0.05 compared with control group, **p<0.01 compared with control group.

Pathological observation on spleen

In the AFB_1 group, lymphocyte density was mainly reduced in the white pulp, and loose arrangement of histocytes was observed when compared with those in the control group (Fig. 2). The main lesions of spleen were statistically analysed (Table 2). The results showed that AFB_1 treatment led to spleen congestion in the red pulp in 1/6, 2/6 and 3/6 chickens at 7, 14, and 21 days of age, respectively, and loosely lined histocytes within the white pulp caused by a reduction in lymphocyte density in 6/6 chickens at 7–21 days of age.

Table 2. Incidence of Major Lesions in Spleen (n=6)

Pathological lesions	Time	Control group	AFB_1 group
Congestion in red pulp	7 days	0/6	1/6
	14 days	0/6	2/6
	21 days	1/6	3/6
Blank within the white pulp	7 days	1/6	6/6
	14 days	1/6	6/6
	21 days	0/6	6/6

Cell cycle phase of splenocytes

The percentages of cells in the G_2M phase in the AFB_1 group were significantly lower than those in the control group at 14 and 21 days of age ($p<0.05$). The percentages of cells in S phase in the AFB_1 group were significantly lower than those in the control group at 7 and 21 days of age ($p<0.05$ or $p<0.01$). Furthermore, changes of the G_0G_1 phase were obvious. The percentage of cells in the G_0G_1 phase in the AFB_1 group was markedly increased at 21 days of age, when compared with the control group ($p<0.01$). The proliferating index (PI) value was significantly decreased in the AFB_1 group at 14 and 21 days of age ($p<0.05$) when compared with the control group (Fig. 3). Histograms obtained by cytometer analysis show that the cell peaks of S and G_2M phases are obviously lower and that the cell peaks of G_0G_1 phase are higher in the AFB_1 group than those in the control group, especially at 14 and 21 days of age.

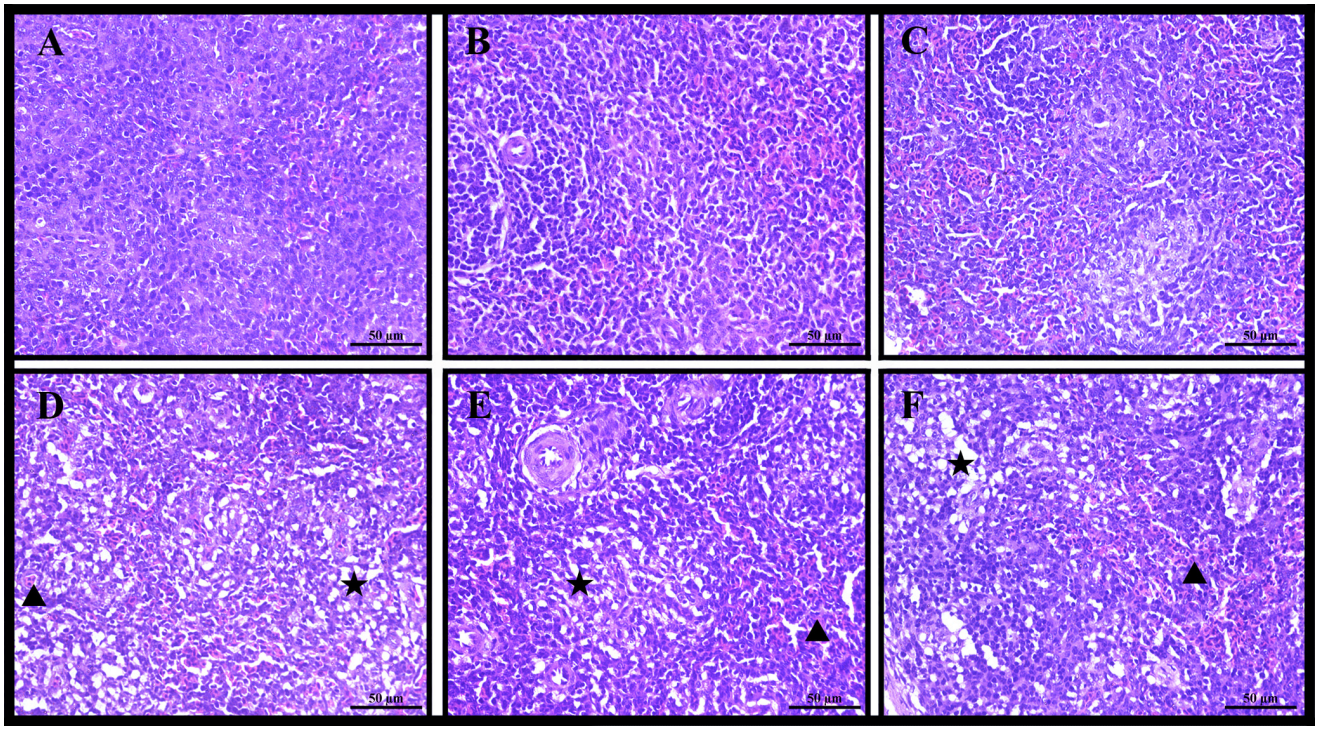

Fig. 2. Photomicrographs of hematoxylin and eosin stained chicken spleen. Notes: (A) the control group at 7 days of age; (B) the control group at 14 days of age; (C) the control group at 21 days of age; (D) the AFB_1 group at 7 days of age; (E) the AFB_1 group at 14 days of age; (F) the AFB_1 group at 21 days of age; (Triangles) congestion in the red pulp; (Stars) lymphocytic depletion in white pulp. Bars = 50 μm.

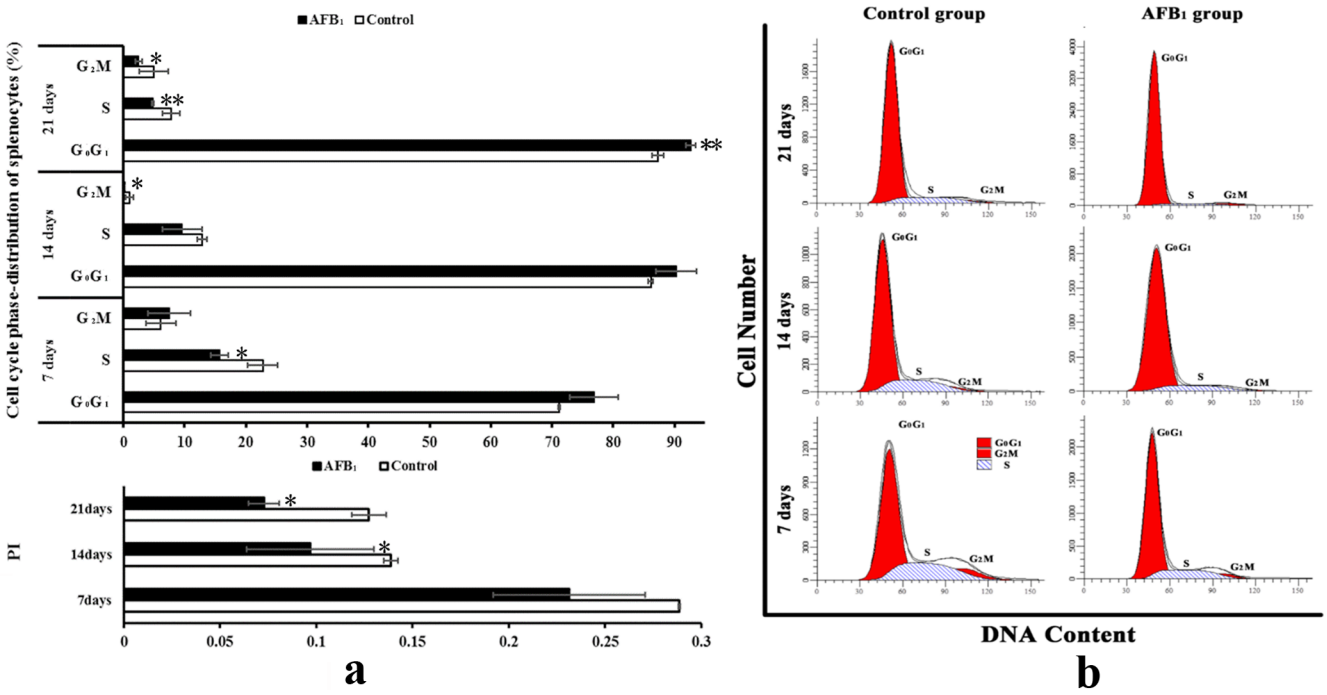

Fig. 3. Cell cycle phase distribution of splenic cell. a: Effect of AFB_1 on cell cycle phase distribution of spleen in chicken (%). b: Histogram of splenocyte cell cycle by flow cytometry. Notes: Data are presented with the means ± standard deviation (n = 6). *p<0.05 compared with control group, **p<0.01 compared with control group.

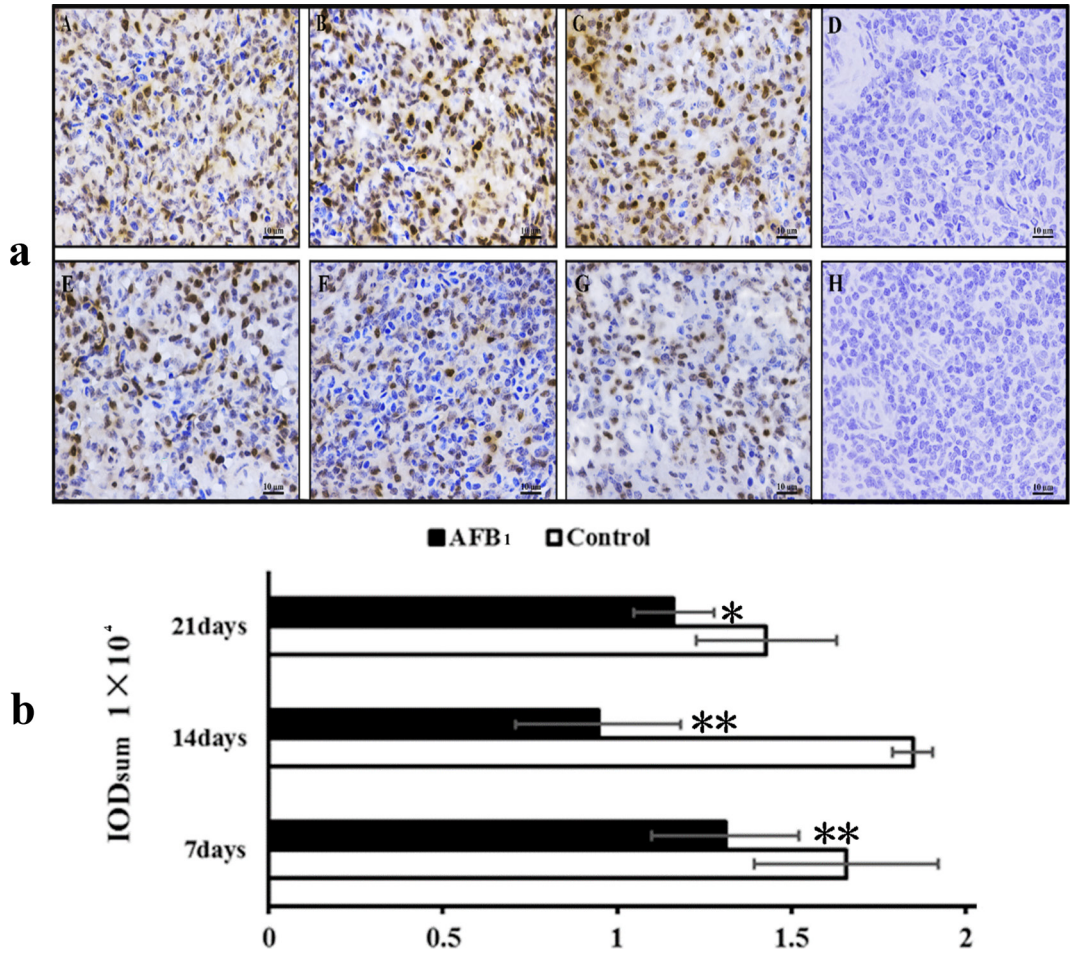

Fig. 4. Immunohistochemistry of the spleen. a: Expression of PCNA protein by immunohistochemistry in the spleen. b: Integrated optical density (IOD sum) of PCNA protein expression in the spleen. Notes: A, B, and C showing the control group at 7, 14, and 21 days respectively; E, F, and H showing the AFB_1 group at 7, 14, and 21 days respectively; D and H showing negative control in the control group and the AFB_1 group respectively. Bars = 10 μm. Data are presented with the mean ± standard deviation (n = 10). * means $p<0.05$ compared with the control group, ** means $p<0.01$ compared with the control group.

PCNA expression by immunohistochemical method

Brownish-yellow staining indicated the expression of PCNA expression. Control sections showed a negative reaction, as shown in Fig. 4. The PCNA protein expression in the spleen of the AFB_1 group was obviously lower than that in the control group. PCNA expression was slightly increased at 21 days compared to 14 days treatment. When compared with the control group, the Integrated optical density (IOD sum) of PCNA protein expression in the AFB_1 group was significantly decreased at 7, 14, and 21 days of age ($p<0.05$ or $p<0.01$).

The mRNA expression of cell cyclins by qRT-PCR

At 7 days of AFB_1 treatment, the mRNA expression levels of ATM, CDK2, cdc2, p53, p21, cyclin E_1, and cyclin B_3 were obviously increased ($p<0.05$ or $p<0.01$) compared with the control group, and the mRNA expression levels of cdk6 were increased. Furthermore, the mRNA expression levels of cyclin D_1 in the AFB_1 group were obviously decreased ($p<0.05$), whereas the mRNA expression levels of P15, P16, and PCNA showed no obvious changes ($P>0.05$).

At 14 and 21 days of AFB_1 treatment, the mRNA expression levels of ATM, CDK2, cdc2, CDK6, p53, p21, cyclin E_1, and cyclin B_3 were obviously increased ($p<0.05$ or $p<0.01$). Furthermore, the mRNA expression levels of cyclin D_1, P15, P16, and PCNA in the AFB_1 group were obviously decreased ($p<0.05$ or $p<0.01$; Fig. 5).

Discussion

Compared with the control group, the AFB_1 diet reduced chicken weight at 14 and 21 days, indicating that AFB_1 diet inhibited chicken growth performance. In the present study, the relative weight of the spleen was used to judge the development status and degree of pathologic change in spleen. At 14 and 21 days of age, the relative weight of the spleen in AFB_1 group was significantly lower than that in the control group, which was consistent with the results of Chen *et al.*[12] and Quist *et al.*[40] However, it

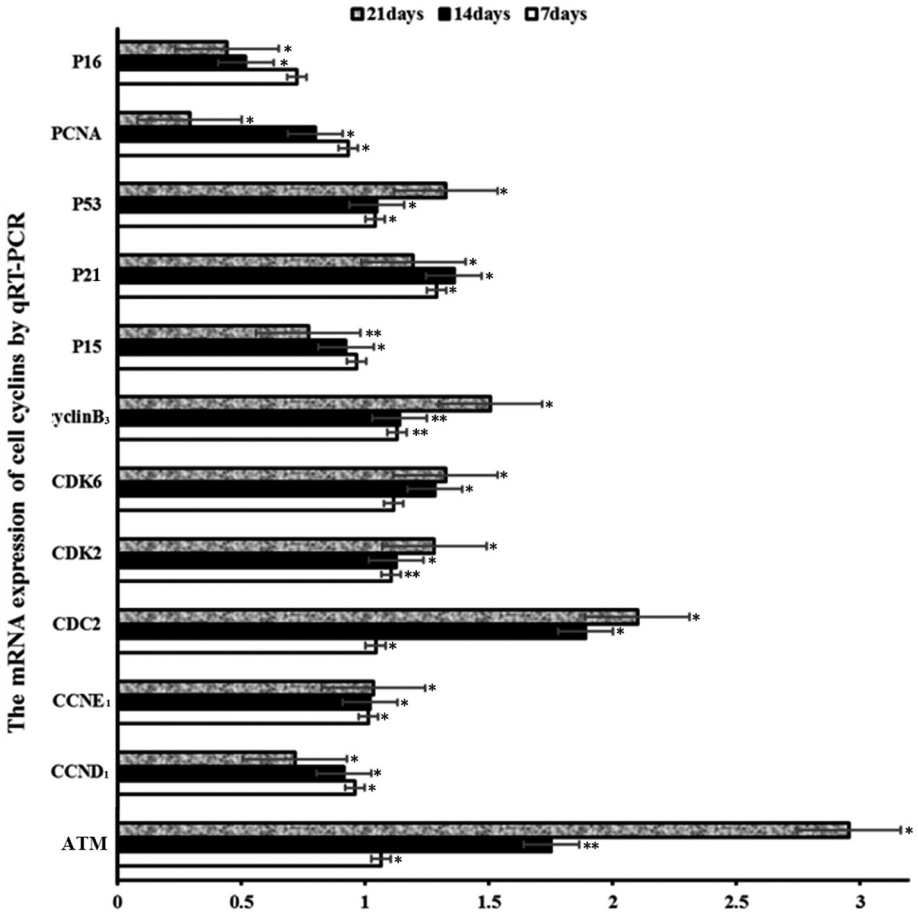

Fig. 5. The levels of the ATM, cyclinB_3, cyclinD_1, CyclinE_1, cdc2, CDK6, CDK2, p53, p21, p15, p16, and PCNA mRNA expression in the spleen. Notes: Data are presented with the means ± standard deviation (n = 6). *p<0.05 compared with control group, **p<0.01 compared with control group.

was not consistent the results of Peng *et al.*[34] Who showed obvious congestion red pulp of the spleen in chicks exposed to corn with containing of AFB_1 and AFB_2. In this study, the results of histopathological observation suggested that the decreased weight and relative weight of the spleen might be due to lymphocytes depletion, as a decreased number of lymphocytes and loosely lined histocytes in the lymphatic nodules and periarterial lymphatic sheath were observed. The results of histopathological observation were also similar to the results of other researchers[12, 34]. Numerous studies and reports have documented that aflatoxin could inhibit the development of thymus[10, 11, 14, 41]. The loosely lined histocytes within the white pulp were suspected of resulting from the decreased number of lymphocytes.

In the present study the S phase and G_2M phase were obviously decreased, and the G_0G_1 phase was markedly increased at 21 days of age. These results indicated that 0.6 mg/kg AFB_1 could induce G_0G_1 phase arrest in chickens splenocytes, which was in line with previous research[34]. However, feeding with AFB-contaminated could lead to G_2M and G_0G_1 phase blockage in the spleens of chickens[14]. Zhang *et al.*[8] demonstrated that AFB_1 caused jejunal cells to be arrested at G_2/M phase. Scott *et al.*[3] reported that AFB_1 treatment increased the percentages of chick thymocytes in the G_2/M. Ricordy *et al.*[22] showed that human cell lines exposed AFB_1 toxin for 24 h caused dose-dependent accumulation in S phase *in vitro*. Differences in animal species, viscera, cell type and mycotoxin type may cause different characteristics of cell cycle arrest.

Several reports have already suggested cellular and humoral immune function changes induced by AFB_1 exposure [6, 7, 9–14, 41] and AFB_1-induced cell arrest, which can be impacted by a variety of cell cycle regulation genes. However, the exact molecular mechanism of AFB_1-induced cell cycle arrest in the spleen of chickens has not been elucidated so far. The experimental results from this study could enrich the knowledge concerning the molecular mechanism of AFB_1-induce immunosuppression. Further studies are needed on protein levels.

The active intermediate products of AFB_1 causes DNA lesions and DNA damage (including double-strand breaks), which can activate ATM[42]. ATM initiates cell cycle arrest, DNA repair, or apoptosis by phosphorylating downstream targets. When ATM phosphorylates p53[42], which is a tumosuppressor gene integrating numerous signals to control cell life and death, the p53 protein can directly stimulate

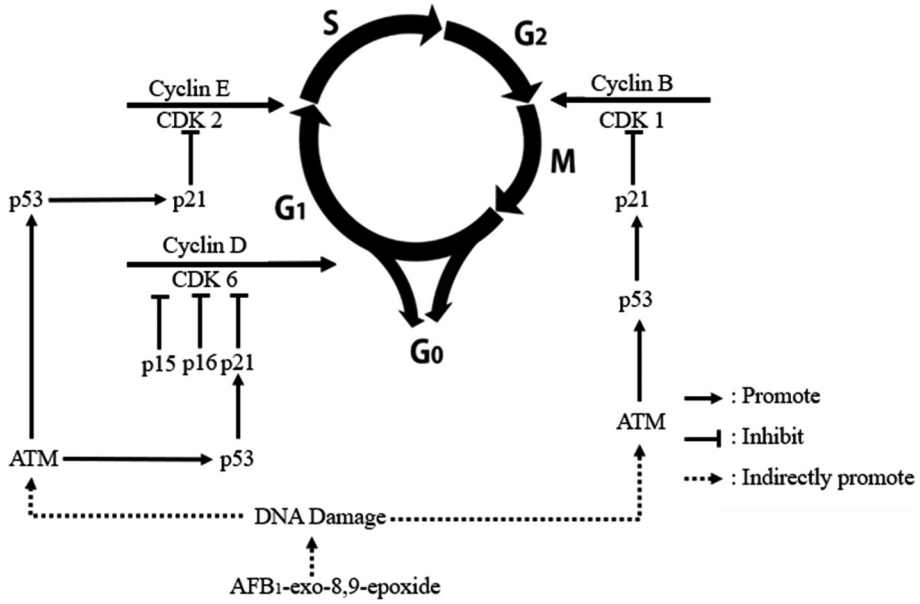

Fig. 6. Schematic diagram of the proposed the molecular mechanism of AFB_1 arrest cell cycle in chicken spleen.

the expression of p21WAF1/CIP1, which is an inhibitor of cyclin-dependent kinases (CDKs) and p21WAF1/CIP1, and inhibit the G_1 to S and G_2 to M[43]. Besides, p53 can indirectly sequester cyclin B_1/CDK1 complexes and help to maintain a G_2 block[44]. The ATM(ATR)/CHK2(CHK1)–p53/MDM2-p21 pathway is the dominant checkpoint response to DNA damage in mammalian cells traversing through the G_1[45]. In this study, our results showed that AFB_1 caused an increase in ATM, p53, and p21 mRNA expression. Therefore, we speculated that dietary AFB_1 could induce G_1 arrest by activating ATM(ATR)/CHK2(CHK1)–p53/MDM2-p21 pathway. Meanwhile, $p21^{WAF1}$ complexed with CDKs can inhibit their kinase activity[43]. pl5 inhibits cyclin D/CDK6 without causing the dissociation of this complex[46]. The P16 family inhibits cell growth by inhibiting the activities of CDK6 and CDK4 kinases[24]. P21WAF1 boun to PCNA can directly inhibit DNA synthesis. Progression through the G_1 is mediated by cyclin D/CDK6[46], and CDK2/cyclin E were thought to exclusively promote the G_1S transition[47], and the cyclin B/CDK1 kinase is a G_2 checkpoint[45]. Our results showed that AFB_1 caused an increase in CDK2, CDK6, cyclin E, and cyclin B_3 mRNA expression and a decrease in cyclin D_1, p15, p16, PCNA, and CDK1 mRNA expression as well as PCNA proteins, indicating that AFB_1 induced G_0G_1 phase arrest via ATM-p53-p21-cyclin D/CDK6 route.

Conclusions

In summary, 0.6 mg/kg AFB_1 in the diet inhibited development of the chicken's spleen by causing G_0G_1 cell cycle arrest. The mRNA expression levels of ATM, p53, p21, and CDK6 were all increased, while the mRNA expression of cyclin D_1 was decreased, suggesting that cyclin D_1 mRNA expression may play an important role during G_0G_1 cell cycle arrest of splenocytes induced by AFB_1. The results and above mention discussion suggest that G_0G_1 phase arrest of splenocytes could be induced via activation ATM-p53-p21-cyclin D/CDK6 route, and the proposed mechanisms are shown in Fig. 6.

Disclosure of Potential Conflicts of Interest: The authors declare that they have no conflict of interest.

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