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SHORT COMMUNICATION

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Inhibitory effects of dietary aflatoxin B_1 on cytokines expression and T-cell subsets in the cecal tonsil of broiler chickens

Chunyu Liu[1†], Min Jiang[1†], Jing Fang[1,2], Xi Peng[1,2], and Hengmin Cui[1,2]

[1] *Key Laboratory of Animal Diseases and Environmental Hazards of Sichuan Province, Chengdu, Sichuan, China.* [2] *Sichuan Agricultural University, College of Veterinary Medicine, Chengdu, Sichuan, China.*

[†] *These authors contributed equally to this work*

Abstract

Aflatoxin B_1 (AFB_1) is the most toxic form among the mycotoxins. Cytokines are important mediators of the immune system. T-cell subsets play a crucial role in cell-mediated immunity. The aim of present study was to evaluate the effects of dietary AFB_1 on the cytokines expression and T-cell subsets in the cecal tonsil of broiler chickens throughout a 21-day experimental period. One hundred and fifty six one-day-old broiler chickens were randomly divided into control group (0 mg AFB_1/kg feed) and AFB_1 group (0.6 mg pure AFB_1/kg feed). At 7, 14 and 21 days of age, the levels of seven cytokines (IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ and TNF-α) mRNA expression as well as the proportions of T-cell subsets (CD3+, CD3+CD4+, CD3+CD8+) by qRT-PCR and flow cytometry methods were assessed in the cecal tonsils. The levels of the seven cytokines mRNA expression and the percentages of T-cell subsets significantly decreased at 14 and 21 days of age in the AFB_1 group compared with the control group. However, the CD4+/CD8+ ratio was not significantly changed. These results demonstrate that 0.6 mg/kg AFB_1 dietary exposure reduced the levels of cytokines mRNA expression and the percentages of T-cell subsets in the cecal tonsils of broiler chickens, suggesting that the cell-mediated immunity of cecal tonsils might be impaired in broilers.

Additional key words: aflatoxin B_1; cell-mediated immunity; cecal tonsil; broiler chicken

Abbreviations used: AFB_1 (aflatoxin B_1); FCM (flow cytometry); qRT-PCR (quantitative real-time PCR); IL (interleukin); IFN-γ (interferon gamma); TNF-α (tumor necrosis factor alpha).

Authors' contributions: Conceived and designed the experiments: CL, MJ, JF and XP. Performed the experiments, analyzed the data and wrote the paper: CL and MJ. Contributed reagents/materials/analysis tools: HC.

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Correspondence should be addressed to Jing Fang: fangjing4109@163.com, or Xi Peng: pengxi197313@163.com (shared corresponding authors).

Introduction

Aflatoxin B_1 (AFB_1) is a strong mutagenic, carcinogenic and teratogenic compound (Mohamed & Metwally, 2009) and can be found in foods and fodder in the world as a common contaminant. The inhibitory effects of AFB_1 on cytokines expression and T-cell subsets have been demonstrated in various studies. The dietary AFB_1 can induce inhibitory effects on interleukin-1 (IL-1), interleukin-2 (IL-2) and interleukin-6 (IL-6) production in rats' splenocytes (Hinton *et al.*, 2003). There is also a decrease in contents of serum IL-2 and interferon gamma (IFN-γ) in broilers exposed to AFB_1 (Chen *et al.*, 2013a). However, Meissonnier *et al.* (2008) found linear increases in IFN-γ, IL-6 and interleukin-10 (IL-10) mRNA with increase in dietary levels of AFB_1 in pigs. In addition, the increase of IFN-γ, IL-6 and tumor necrosis factor alpha (TNF-α) mRNA (proinflammatory cytokines) has been observed in chickens exposed to AFB_1 (Li *et al.*, 2014). The percentage of splenic CD8+ T-cells in rats shows dose-dependent decreases with dietary AFB_1 increasing (Qian *et al.*, 2014). Intoxicated mice with AFB_1 also exhibit significant decreases in the percentage of CD4+ T-cells in the spleen and in the number of CD3+ T-cells in the intestinal T-cells (Hatori *et al.*, 1991; Tomková *et al.*, 2002). Chen *et al.* (2014)

observed reduced percentages of CD3+, CD3+CD4+ and CD3+CD8+ T-cells in the spleen of broilers administered AFB_1. Contrary to these reports, Qian *et al.* (2014) reported a significant increase in the percentages of CD3+ and CD8+ T-cells in the spleen when rats are exposed to AFB_1. These findings indicate that the effects of AFB_1 on the cytokines and T-cell subsets are not always similar in different settings.

In poultry, the cecal tonsil is the largest lymphoid organ of the gut-associated lymphoid tissue (Deng *et al.*, 2012). This organ is involved in both antibody production and cell-mediated immune functions and plays an important role in intestinal mucosal immunity (Olah *et al.*, 1984; Humberd *et al.*, 2007). However, there are no systematic reports regarding the effect of dietary AFB_1 on cytokines and T-cell subsets in the cecal tonsil of poultry. The purpose of this paper was to investigate the effects of dietary AFB_1 at different time points on the cytokines mRNA expression and T-cell subsets in the cecal tonsil of broiler chickens by quantitative real-time PCR (qRT-PCR) and flow cytometry (FCM) analyses in order to provide helpful information for future studies in immunosuppressive effects induced by AFB_1 in poultry.

Material and methods

One hundred and fifty six 1-day-old healthy male broiler chickens were divided into control group (0 mg AFB_1/kg feed) and AFB_1 group (0.6 mg pure AFB_1/kg feed). Table 1 shows the control diet which was formulated according to National Research Council (NRC) (Shini & Kaiser, 2009) and Chinese Feeding Standard of Chicken (NY/T33-2004) recommendations. Broilers were housed in cages with electrically heated units and were provided with water as well as aforementioned diets (Table 1) *ad libitum* for 21 days. At 7, 14 and 21 days of age during the experiment, the cecal tonsils of six birds in each group were taken to determine CD3+, CD3+CD4+, CD3+CD8+ T-cell percentages by FCM as described by Liu *et al.* (2012) and the levels of IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ and TNF-α mRNA expression by qRT-PCR analysis according to the method reported by Chen *et al.* (2013b). 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). Statistical analyses were performed using the Mann-Whitney U test, a non-parametric test for two independent samples, by SPSS software for Mac v.20.0 (IBM Corp, Armonk, NY, USA). A value of $p< 0.05$ was considered significant.

Table 1. Composition of the basal diet.

	Content (%)
Composition	
Corn	51.95
Soybean	39.50
Rapeseed oil	4.10
DL-Metionine	0.20
Calcium hydrogen phosphate	1.85
Calcium carbonate	1.30
Sodium chloride	0.40
Trace element premix[a]	0.50
Choline	0.17
Multivitamins[b]	0.03
Total	100
Nutrients	
Crude protein (CP)	21.50
Methionine (Met)	0.50
Calcium (Ca)	1.00
All phosphorus (P)	0.70
Methionine + Cystine (Met + Cys)	0.84
Lysine (Lys)	1.15
Threonine (Thr)	0.83
Metabolizable energy (ME) (MJ/kg)	12.52

[a] Trace element premix (mg/kg): $FeSO_4 \cdot 7H_2O$, 530; $CuSO_4 \cdot 5H_2O$, 30; $MnSO_4 \cdot H_2O$, 400; $ZnSO_4 \cdot 7H_2O$, 470; KI, 18; $NaSeO_3$, 0.3. [b] Multivitamins: vitamin A, 13500 IU/kg; vitamin D, 3000 IU/kg; vitamin E, 24 IU/kg; vitamin K_3, 3 mg/kg; pantothenic acid, 15 mg/kg; folic acid, 1.05 mg/kg; nicotinamide, 30 mg/kg; biotin, 0.14 mg/kg.

Results and discussion

Table 2 shows that the expression levels of all cytokines in the AFB_1 group significantly decreased ($p<0.01$) at 14 and 21 days in comparison with those of the control group. Furthermore, Table 2 and Figure 1 revealed that the percentages of CD3+, CD3+CD4+ and CD3+CD8+ T-cells significantly decreased ($p<0.05$ or $p<0.01$) in the AFB_1 group at 14 and 21 days in comparison to the control group. These results indicate that both cytokines mRNA expression and T-cell subsets population in the cecal tonsil were reduced when broilers were fed with 0.6 mg/kg dietary AFB_1. There is a close relationship between the thymus and differentiation of T lymphocytes (Kidd *et al.*, 1996). Thymus is the place where T-cells are activated and differentiated to CD4 or CD8 T-cells, and then mature T-cells migrate from thymus to the peripheral blood and secondary immune organs (Erf *et al.*, 1998). According to the report of Guo *et al.* (2012), AFB_1 induces suppression of thymus development. Therefore, the decrease of T-cell subsets of the cecal tonsils observed in this study could be simultaneously associated to the suppressed development of thymus. Moreover, it had been confirmed that AFB_1 could cause depressed synthesis

Table 2. Levels of cytokines mRNA expression and the percentages of T-cell subsets and the CD4+/CD8+ ratios in the cecal tonsil. Values are median (quartile range, QR), n = 6.

Item	Groups	7 days	14 days	21 days
Cytokines				
IL-2	Control group	1.03 (0.94-1.22)	1.00 (0.92-1.08)	1.06 (1.00-1.08)
	AFB_1 group	0.94 (0.88-0.98)	0.73 (0.71-0.77)**	0.56 (0.53-0.65)**
IL-4	Control group	1.13 (1.06-1.19)	1.03 (0.92-1.08)	1.00 (0.98-1.03)
	AFB_1 group	1.07 (1.04-1.11)	0.83 (0.78-0.85)**	0.56 (0.54-0.62)**
IL-6	Control group	1.06 (0.88-1.20)	0.95 (0.87-1.23)	1.12 (0.88-1.20)
	AFB_1 group	1.13 (1.02-1.23)	0.91 (0.86-0.97)	0.77 (0.69-0.79)**
IL-10	Control group	1.02 (0.91-1.29)	0.90 (0.86-1.13)	1.07 (1.00-1.16)
	AFB_1 group	0.97 (0.95-1.01)	0.86 (0.77-0.90)	0.64 (0.59-0.66)**
IL-17	Control group	0.95 (0.86-1.06)	1.04 (0.89-1.10)	0.98 (0.94-1.10)
	AFB_1 group	1.06 (1.04-1.10)	0.78 (0.73-0.86)**	0.56 (0.53-0.63)**
IFN-γ	Control group	1.00 (0.92-1.03)	1.17 (0.98-1.21)	0.99 (0.86-1.20)
	AFB_1 group	0.71 (0.68-0.77) **	0.88 (0.87-0.91) **	0.62 (0.60-0.66) **
TNF-α	Control group	1.05 (0.85-1.20)	1.15 (1.07-1.24)	0.96 (0.92-1.13)
	AFB_1 group	0.84 (0.82-0.88)	0.66 (0.63-0.70)**	0.62 (0.59-0.65)**
T-cell subsets				
CD3+ (%)	Control group	67.24 (61.44-71.20)	72.94 (62.96-77.71)	77.06 (72.02-83.00)
	AFB_1 group	60.13 (56.25-64.54)	58.90 (56.43-68.03)*	65.31 (64.47-69.35)*
CD3+CD4+(%)	Control group	34.35 (29.58-37.17)	41.33 (38.42-45.61)	48.04 (43.45-50.15)
	AFB_1 group	26.59 (23.65-29.41)*	33.77 (28.10-36.19)**	31.64 (27.53-38.61)**
CD3+CD8+(%)	Control group	26.92 (18.07-30.10)	31.58 (28.15-36.05)	32.59 (30.11-36.53)
	AFB_1 group	21.40 (19.84-24.09)	26.40 (24.44-28.35)*	28.14 (23.94-28.86)**
CD4+/CD8+	Control group	1.33 (1.21-1.59)	1.33 (1.08-1.59)	1.42 (1.30-1.56)
	AFB_1 group	1.16 (1.02-1.42)	1.29 (1.00-1.50)	1.17 (1.12-1.35)

Compared with the control: * $p < 0.05$, ** $p < 0.01$.

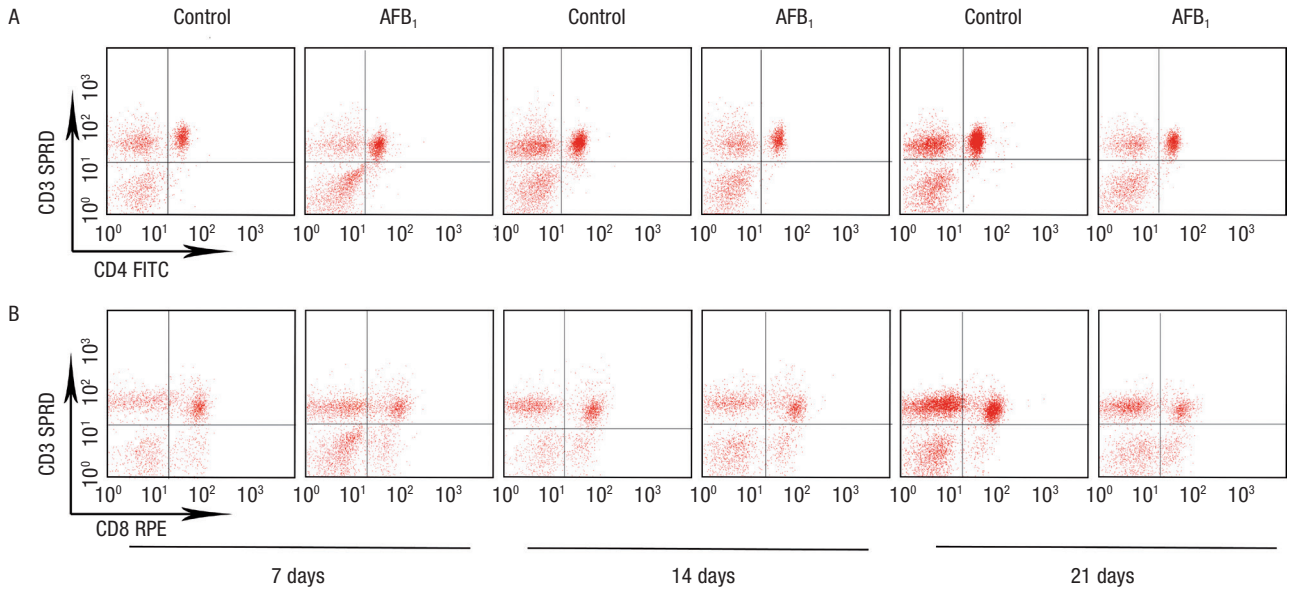

Figure 1. The quadrantal diagram of CD3+, CD3+CD4+ and CD3+CD8+ T-cell percentages in the cecal tonsil of the control group and AFB_1 group at 7, 14 and 21 days of age, respectively. Panel A: CD3+CD4+ T-cell in the upper right, and CD3+ T-cell in the upper right and upper left. Panel B: CD3+CD8+ T-cell in the upper right.

of DNA and RNA (Erf *et al.*, 1998), which may also contribute to the reduced percentages of T-cell subsets. In addition, it is known that the differentiation of T-cells is closely related to the cytokines (Huang *et al.*, 2014) and many cytokines are secreted by T-cells (Scott, 1993).

The CD4+/CD8+ ratio can be used to provide a convenient standard for changes in cellular immune status during disease, nutritional stress, and auto-immune problems (Kantrow *et al.*, 1997). In the present study, the CD4+/CD8+ ratio in the AFB_1 group showed a decreased tendency compared with the control group, but without apparent difference ($p > 0.05$) (Table 2) in the AFB_1 group during the experiment. These results indicate that the relative proportion of T-cell subpopulations was maintained in the two groups during the experiment.

Our results (Table 2) show that the levels of cytokines mRNA expression in the AFB_1 group decreased progressively from 7 to 21 days of age, and the values (except for IL-6 and IL-10) in the AFB_1 group were lower at 14 ($p<0.01$) and 21 ($p<0.01$) days of age than those in the control group. In addition, the percentages of CD3+, CD3+CD4+, CD3+CD8+ T-cells showed a significant decline already by 14 ($p<0.05$ or $p<0.01$) and 21 ($p<0.05$ or $p<0.01$) days of age (Table 2). Finally, the CD4+/CD8+ ratio fell during the experiment, but not in statistically significant manner (Table 2). These results suggest that AFB_1-induced reduction of cytokines mRNA expression and the percentages of T-cell subsets in the cecal tonsil differed with progression of the exposure. The mechanism for this is worth of further study.

It is concluded that the dietary exposure to 0.6 mg/kg of AFB_1 reduced the levels of cytokines (IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α and IFN-γ) mRNA expression and the percentages of T-cell subsets (CD3+, CD3+CD4+ and CD3+CD8+) in the cecal tonsils of broiler chicken.

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