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Biochemical and Histopathological Study of Aflatoxicosis on Ross 308 Broiler Chicks

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ABSTRACT Totally, one hundred and sixty 1-day-old Ross 308 broiler chicks were fed with a diet containing 0, 0.5, 1.0, and 2.0 mg of aflatoxin B_1(AFB_1)/kg of feed for 21 days. Body weight was lower for the AFB_1-treated broilers than for the control group. At 14 and 21 DPF, the broilers fed with 2.0 mg of AFB_1/kg of feed weighed significantly lower than those of the other groups ($p<0.05$). Relative liver weights increased significantly in a dose-dependent manner, and relative spleen weights were significantly high in the chicks fed with 2.0 mg of AFB_1/kg of feed at 21 DPF ($p<0.001$). Biochemical analyses showed that total protein and albumin levels decreased significantly at 7 and 14 DPF for the chicks of the group fed with 2.0 of mg AFB_1/kg of feed, compared with those fed with 0.5 and 1.0 mg of AFB_1/kg of feed ($p<0.05$). AST and ALT levels increased significantly at 14 and 21 DPF ($p<0.05$), and the AST levels, particularly, increased dose-dependently ($p<0.05$). Histopathological analyses showed that the liver tissues of the AFB_1-treated chicks showed significant lesions, including hemorrhage, hepatocyte necrosis, inflammatory cell infiltration, and fatty degeneration. The severity of both hepatocyte necrosis and inflammatory cell infiltration appeared to increase dose- and time-dependently. Similarly, hepatic fibrosis increased dose-dependently ($p<0.05$). The results of this study could improve our understanding of parameters used for evaluating aflatoxicosis in poultry.

(Key words: aflatoxin B_1, broiler performance, dose dependently effect, liver fibrosis)

INTRODUCTION

Aflatoxins are the most intensively researched group of mycotoxins, and the effects of aflatoxins on broiler productivity have been previously reviewed (Kensler et al., 2011; Yunus et al., 2011; Monson et al., 2015).

Previous studies have shown that aflatoxins have a variety of negative effects, including slower growth (Magnoli et al., 2011), carcinogenic effects, immunosuppression, and increased susceptibility to disease (Rawal et al., 2010). Among the known aflatoxins, aflatoxin B_1 (AFB_1) is the most potent hepatotoxin (Wogan et al., 1992; Rawal et al., 2010) and is classified as a Group I carcinogen by the International Agency for Research on Cancer (2012).

Resistance to aflatoxin in chickens is higher than in other animals and susceptibility varies with breed, species, age, dose and length of exposure (Monson et al., 2015).

Despite numerous prior studies, the dietary concentrations and length of exposure to AFB_1 have greatly varied between studies, making it difficult to define a time-and dose-dependent effect on such parameters as weight gain, effects on the liver, and changes in blood chemistry.

This study designed to establish a relationship between the dosage and length of exposure to purified AFB_1 on the growth, biochemical parameters, and liver histopathology.

In broiler chicks, the results of which will provide parameter for the toxic effect provoked by AFB_1.

MATERIALS AND METHODS

1. Animals and Experimental Design

Mixed-gender broiler chickens (Ross 308) at 1d of age were obtained from a commercial hatchery (Join Co., Ltd, Korea). They were housed in an isolator (Threeshine Inc., Daejeon, Korea) which was equipped with an electrically heated, negative pressure, forced ventilation unit as well as a feeder and water trough for each cage. The brooding temperature was set at 33~35℃ for day 0 then decreased gradually to 23~21℃ until day 21 and was maintained there until the end of the experiment. The temperature and relative humidity were mo-

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nitored every 3 hours. Every effort was made to achieve and maintain the optimum temperature and relative humidity according to the Ross Broiler Management Handbook (2014). The light regime began with 24 hours a day for 2 days, then decreased by 30 minutes every other day until it reached 20 hours of light per day, which was maintained until the end of the experiment.

The AFB_1-contaminated diets were prepared according to the method described by Kaoud (2013). Briefly, crystalline AFB_1 (Cayman chemical, MI, USA) was dissolved in methanol (1 mg AFB_1/mL in methanol) and subsequently added to a commercial crumble diet (AT-bioco., Ltd, Ochang, Korea), which was formulated to meet the nutrient requirements of broilers from 1 to 21 days of age (crude protein: above 22.0 %, metabolizable energy (ME): 3,100 kcal/kg). The methanol was then evaporated at room temperature, and the AFB_1-treated diet was refrigerated until needed. The control group was fed the same commercial diet without the AFB_1. The four diets contained the following: Control (0 mg AFB_1), 0.5 mg, 1.0 mg and 2.0 mg AFB_1 per kg of feed. All chicks were provided *ad libitum* access to the diet and water throughout the study.

All experimental procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at Bansuk LTC (permission no.: 2016-007).

2. Blood Sampling, Relative Weight of Liver and Spleen

Broiler chicks were randomly selected from each treatment group at 7 (n=10), 14 (n=10) and 21 days (n=20) post-feeding (DPF) and weighed. Blood samples were collected by heart puncture or from the wing veins. After necropsy, the livers and spleens were removed and weighed immediately. The relative weights of the livers and spleens were calculated per gram of body weight.

3. Serum Biochemistry Analysis

The blood samples were allowed to coagulate at room temperature, centrifuged, and the sera collected. All parameters were evaluated using an automatic analyzer (Hitachi 7020 automatic analyzer, Tokyo). The examined parameters included glucose, albumin, total protein (TP), globulin, cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine, calcium, and phosphorus.

4. Histopathological Examination of Liver

For the histopathological analysis (n=5 per group), the left lobe of the liver was immediately fixed in a 10% neutral buffered formalin solution followed by routine processing for paraffin wax embedding. The liver tissues were cut into 5-μm-thick sections. After being deparaffinized, sections were stained with Harris modified hematoxylin and eosin solution (Sigma-Aldrich).

5. Picro-Sirius Red Staining

Paraffin-embedded liver tissues were cut into 5-μm thick sections. Deparaffinized sections were stained for 60 min with Picro-Sirius red solution (Sigma-Aldrich) and then rinsed three times with 0.5% acetic acid. Sections were dehydrated with absolute alcohol. Fibrosis fibers were quantified in Sirius Red-stained sections of the liver using a ProgRes C5 digital camera (Olympus DP72) attached to a light microscope (Olympus BX53/U-LH 100HG, Olympus Corp., Tokyo, Japan) using at least three birds per group (three areas/section), and semi-quantified using Image J software (NIH, Bethesda, MD, USA). We measured the light polarized Sirius Red area and divided by the total area [(light polarized area/total area) × 100], and the results are shown as means±standard error of the mean (SEM).

6. Statistical Analysis

Data are presented as means±SEM. Data were subjected to one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls *post hoc* test for multiple comparisons. In all cases, p values <0.05 were used to indicate statistical significances.

RESULTS

1. Body Weight, Relative Liver and Spleen Weight

The initial average body weight per chick was 39.6±0.2 g. The body weights of the broiler chicks showed no significant differences among the treatment groups on day 7 of AFB_1 feeding (Table 1). On 14 and 21 DPF in the 2.0 mg AFB_1 group, the body weights were significantly lower than that of the other treatment groups ($p<0.01$, $p<0.001$, respectively) (Table 1). Although no significant difference occurred among the control, 0.5 and 1.0 mg AFB_1/kg groups, the body weight of the AFB_1-fed broilers was generally lower than their counter parts fed the control diet (Table 1).

The relative liver weights began to be affected by AFB_1 at 14 days DPF, showing a significant increase in the group fed 2.0 mg/kg of feed (Table 2). On 21 DPF, the relative liver weights were significantly increased in a dose-dependent manner (Table 2).

The relative spleen weights increased significantly in response to 2.0 mg AFB_1/kg of feed at 21 days DPF ($p<0.001$) when compared to those fed the control diet, 0.5 and 1.0 mg AFB_1/kg of feed (Table 2).

2. Effects of Aflatoxin B_1 on Serum Biochemical Parameters during Exposure Time

Fig. 1 shows the effects of dietary AFB_1 on serum biochemical parameters.

Serum total protein (TP) levels were significantly decreased on 7 and 14 DPF in the group treated with 2.0 mg AFB_1/kg of feed compared with the other treatment groups ($p<0.05$), but the change was not significant at 21 DPF (Fig. 1A).

Albumin levels were significantly decreased in the group treated with 2.0 mg AFB_1/kg of feed compared with the other treatment groups at 7 DPF ($p<0.05$) and compared to the control group at 14 DPF ($p<0.01$) (Fig. 1B).

AST and ALT levels were not significantly changed at 7 and 14 DPF, but significant changes appeared at 21 DPF. AST levels were significantly increased in the 1.0 and 2.0 mg AFB_1/kg of feed groups compared with the control group at

Table 1. The body weights on broilers (g)

Period (n)	Cont.	T1	T2*	T3
0 day (10)	39.4± 0.37	39.8± 0.39	39.7± 0.36	39.7± 0.37
7 days (10)	209.4± 2.79	192.2± 4.46	210.0± 6.74	206.7± 4.05
14 days (10)	552.5± 9.04[a]	523.9±11.02[a]	526.3±10.69[a]	474.6±14.76[b]
21 days (20)	935.8±22.23[a]	922.8±20.82[a]	918.8±22.69[a]	774.3±25.35[b]

Values are means±SEM.
[ab] Means with different superscripts in each row differ significantly ($p<0.05$).
* n is 19 on 21 days post-feeding (DPF). Cont.: basal feed (without AFB_1), T1: Aflatoxin B_1 0.5 mg/kg of feed, T2: Aflatoxin B_1 1.0 mg/kg of feed, T3: Aflatoxin B_1 2.0 mg/kg of feed.

Table 2. Relative liver and relative spleen weight on broilers

Period (n)	Relative liver weight (%)				Relative spleen weight (%)			
	Cont.	T1	T2*	T3	Cont.	T1	T2*	T3
7 days (10)	3.73±0.06[a]	3.59±0.07[a]	4.03±0.12[b]	3.46±0.10[a]	0.70±0.06	0.65±0.06	0.61±0.05	0.67±0.06
14 days (10)	3.08±0.10[a]	3.35±0.12[a]	3.19±0.12[a]	3.66±0.10[b]	0.74±0.05	0.57±0.04	0.65±0.04	0.74±0.09
21 days (20)	2.38±0.06[a]	2.72±0.12[b]	3.07±0.10[c]	4.12±0.15[d]	0.60±0.03[a]	0.60±0.04[a]	0.63±0.03[a]	0.93±0.06[b]

Values are means±SEM.
Relative liver weight is liver weight (g) as a % of BW; Relative spleen weight is spleen weight (mg) as a % of BW.
[a~d] Means with different superscripts in each row differ significantly ($p<0.05$).
* n is 19 on 21 days post-feeding (DPF). Cont.: basal feed (without AFB_1), T1: Aflatoxin B_1 0.5 mg/kg of feed, T2: Aflatoxin B_1 1.0 mg/kg of feed, T3: Aflatoxin B_1 2.0 mg/kg of feed.

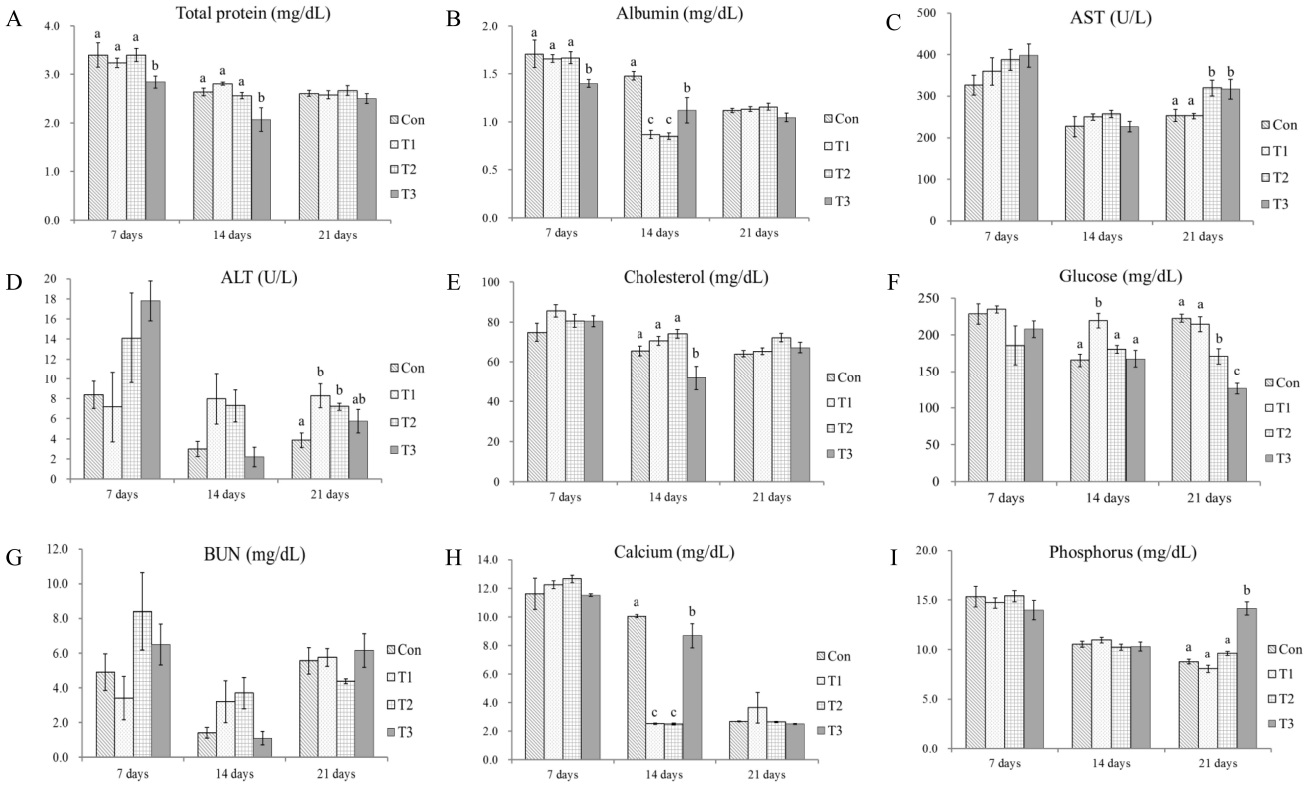

Fig. 1. Serum biochemistry analysis of the broiler chicks given different levels of aflatoxin B_1 exposure at each time point.
Cont: basal feed (without AFB_1), T1: Aflatoxin B_1 0.5 mg/kg of feed, T2: Aflatoxin B_1 1.0 mg/kg of feed, T3: Aflatoxin B_1 2.0 mg/kg of feed.
$^{a\sim d}$ Means with different superscripts are significantly different ($p<0.05$).

21 DPF ($p<0.05$) (Fig. 1C). ALT levels in all of the AFB_1-contained feed groups were higher than the control group and significantly increased in the groups treated with 0.5 mg ($p<0.01$) and 1.0 mg AFB_1/kg of feed ($p<0.05$) (Fig. 1D).

Serum cholesterol levels were significantly decreased in the chicks treated with 2.0 mg AFB_1/kg of feed compared with other groups at 14 DPF ($p<0.05$) (Fig. 1E).

Serum glucose levels showed changes at 14 DPF in the groups treated with 0.5 mg AFB_1/kg of feed compared with the other treatment groups ($p<0.01$, $p<0.001$). At 21 DPF, glucose levels in the AFB_1-treated groups decreased in a dose-dependent manner ($p<0.001$) compared with the control and 0.5 mg AFB_1/kg groups (Fig. 1F).

BUN levels did not show any significant changes (Fig. 1G).

Serum calcium levels were significantly decreased in the groups treated with 0.5, 1.0 ($p<0.001$) and 2.0 mg AFB_1/kg of feed ($p<0.05$) compared to the control group at 14 DPF (Fig. 1H).

Serum phosphorus levels were significantly increased in the group treated with 2.0 mg AFB_1/kg of feed compared with the other groups at 21 DPF ($p<0.001$) (Fig. 1I).

3. Histological Examination of the Liver

There were no visible liver lesions in the control group birds. Livers from the birds consuming AFB_1-containing diets, however, showed significant lesions, such as hemorrhage, hepatocyte necrosis, inflammatory cell infiltration, and fatty degeneration. Hepatocyte necrosis and inflammatory cell infiltration both appeared to increase in severity in a dose- and time-dependent manner (Fig. 2). Fig. 2 shows the histopathological changes from aflatoxin B_1-contaminated feed at each time point. To evaluate fibrosis, Sirius red staining was performed on the liver sections at 21 DPF (Fig. 3). The control group had a normal distribution of collagen (Fig. 3A), whereas those treated with AFB_1 demonstrated collagen deposition in a dose-dependent manner ($p<0.05$) (Fig. 3E). Table 3 sum

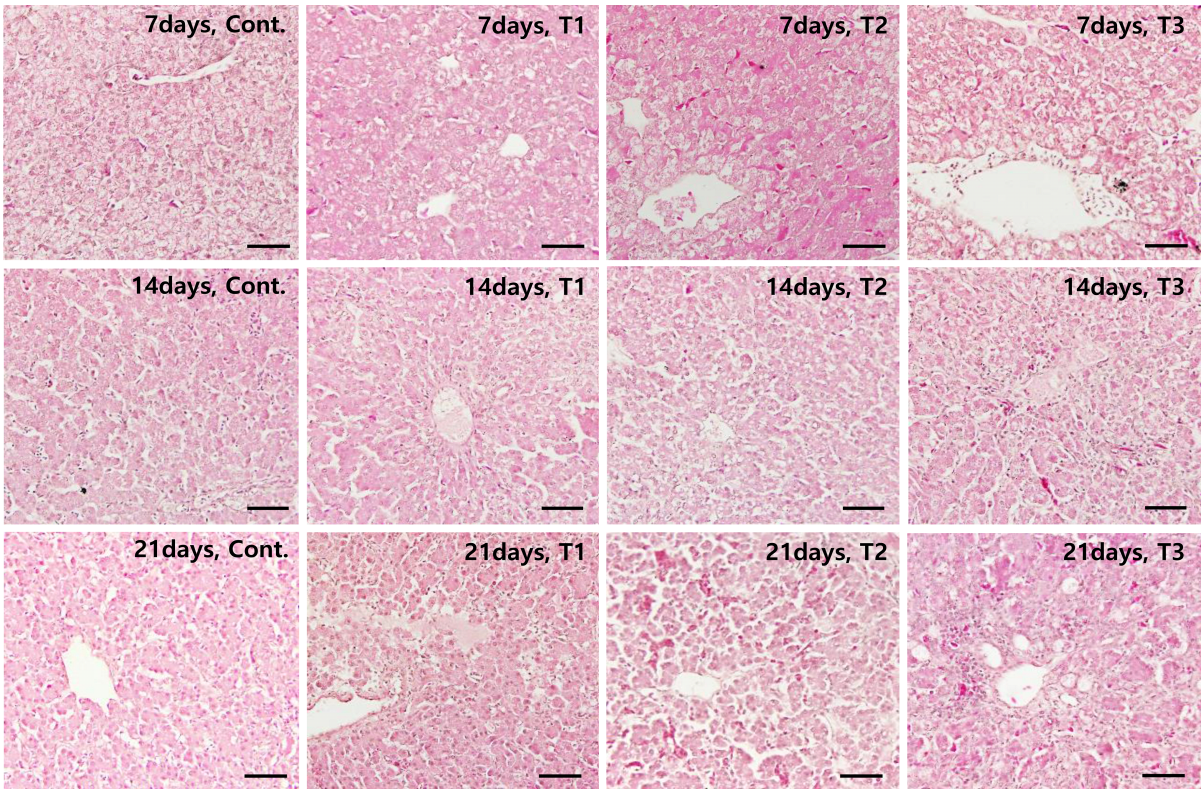

Fig. 2. Histopathological changes in the liver of broiler chicks given different levels of aflatoxin B_1 exposure at each time point. Cont: basal feed (without AFB_1), T1: Aflatoxin B_1 0.5 mg/kg of feed, T2: Aflatoxin B_1 1.0 mg/kg of feed, T3: Aflatoxin B_1 2.0 mg/kg of feed. Scalebar=100 μm.

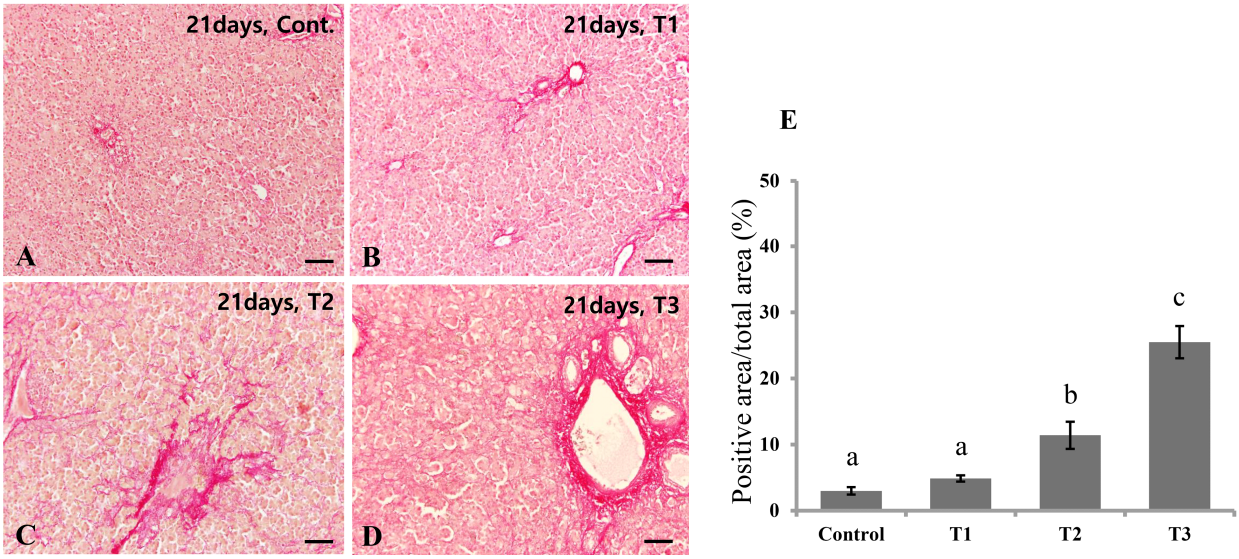

Fig. 3. Fibrillar collagen deposition was evaluated by Sirius red staining. AFB_1-exposed broilers demonstrated a dose-dependent increase in fibrosis at 21 DPF.
A: basal feed (without AFB1), B: T1 (AFB_1 0.5 mg/kg of feed), C: T2 (Aflatoxin B_1 1.0 mg/kg of feed), D: T3 (Aflatoxin B_1 2.0 mg/kg of feed), E: Quantification analysis of fibrosis. Scalebar=100 μm.
$^{a\sim d}$ Means with different superscripts are significantly different ($p<0.05$).

Table 3. Hepatic histology of liver damage in aflatoxin-exposed chickens

Group (days)		Parameters			
		Hemo-rrhage	Hepatocyte necrosis	Inflammatory cell infiltration	Fatty degeneration
7	Cont.	−	−	−	−
	T1	−	+	+	+
	T2	+	+	+	+
	T3	+	++	+	+
14	Cont.	−	−	−	−
	T1	+	+	+	+
	T2	+	+	+	++
	T3	++	++	+	+
21	Cont.	−	−	−	+
	T1	+	+	+	+
	T2	++	++	++	++
	T3	++	+++	+++	++

Grades are follows: - absent, + trace (1~25%). ++ weak (26~50%), +++ moderate (50~75%). Cont: basal feed (without AFB_1), T1: Aflatoxin B_1 0.5 mg/kg of feed, T2: Aflatoxin B_1 1.0 mg/kg of feed, T3: Aflatoxin B_1 2.0 mg/kg of feed.

marizes the results of the histopathological analysis at each time point.

DISCUSSION

Many studies have shown that AFB_1 exposure can lead to a reduction in weight gain in broiler chicks in a dose-and time-dependent manner (Valdivia et al., 2001; Tedesco et al., 2004; Zhao et al., 2010; Peng et al., 2014b; Fowler et al., 2015). Diaz et al (2008) proposed a biphasic nature (hormesis) of aflatoxins on the broiler's weight gain, i.e. improvement at low doses (0.625 mg/kg and 1.25 mg/kg) and reduction at high doses (2.5 mg/kg and 5.0 mg/kg).

The results of the present study do not support this biphasic proposal of Diaz et al. (2008). Although at low doses (0.5 and 1.0 mg AFB_1/kg of feed) there was no significant reduction, the body weights also did not increase compared with those in the control group until the end of the experiment (21 DPF). As exposure periods to aflatoxin B_1 increased, body weight gain in the group fed 2.0 mg AFB_1/kg of feed significantly decreased in a linear pattern beginning at 14 DPF.

Huff et al. (1986) reported that the relative liver weights decreased initially, but in our study, the relative liver weights significantly increased in the groups fed AFB_1 at 1.0 and 2.0 mg/kg of feed beginning at 7 DPF. The relative liver weights also increased in a dose-dependent manner at 21 DPF (Fowler et al., 2015).

Similar to the results of previous studies, we found that the weights of the spleens were significantly increased during aflatoxicosis at 2 mg AFB_1/kg of feed at 21 DPF (Huff et al., 1986; Peng et al., 2014a; Fowler et al., 2015).

At the cellular level, dietary AFB_1 induced histopathological liver damage, including focal hepatocyte necrosis, hemorrhage, inflammatory cell infiltration, fibrosis, and nodular regeneration (Huff et al., 1986; Pandey et al., 2007; Tessari et al., 2010) in a dose- and time- dependent manner. The dose-dependent increase in the relative liver weights was similar to what was seen in the amount of liver fibrosis observed at 21 DPF.

At 21 DPF, the control group showed a slight amount of fatty degeneration, which is thought to be due to the rapid growth of the broiler. Although there was an increase in cellular fatty deposition, the gross liver color was difficult to distinguish (data not shown).

Serum biochemical and hematological alterations are also good tools for diagnosing chronic aflatoxicosis (Oğuz et al., 2000), because the detrimental effects on these values (Keceri et al., 1998) are apparent prior to the manifestation of clinical symptoms. The serum biochemical parameters and the effects caused by AFB_1, however, has remained inconclusive.

Studies looking at the effects of AFB_1 on serum chemistry have shown that serum cholesterol and total serum protein (TP) both decrease in birds fed a diet with 0.3 mg AFB_1/kg of feed (Raju et al., 2000). Previous studies have also shown a decrease in the total serum protein and albumin levels at 1.0 mg AFB_1/kg of feed, and a decrease in serum glucose, Ca^{++}, and in organic *P* levels was reported in Ross-308 broiler chicks fed 2.0 mg AFB_1/kg of feed at 21 days (Zhao et al., 2010).

We agree that aflatoxicosis negatively affects serum levels of total protein, albumin, and cholesterol (Huff et al., 1986; Zhao et al., 2010; Chen et al., 2014). In our experiments, the levels of TP, albumin, and cholesterol decreased significantly in the AFB_1-fed group compared with the control group, but these changes varied with dose and duration. A significant decrease in serum cholesterol was seen at 14 DPF in the group fed 2.0 mg AFB_1/kg of feed. A decrease in total protein was seen at 7 and 14 DPF in the group fed 2.0 mg AFB_1/kg of feed. Albumin levels were lower at 7 DPF in the group fed 2.0 mg AFB_1/kg and lower at 14 DPF in all of the AFB_1-fed groups compared with the control group. Serum glucose levels in the group fed 0.5 mg AFB_1/kg of feed were significantly higher compared with the other groups at 14 DPF, and levels at 21 DPF were significantly and dose-dependently reduced.

Calcium levels were significantly reduced in all of the AFB_1-fed groups compared with the control at 14 DPF, but there was no change between the experimental groups at 21 DPF.

The inorganic P levels were significantly increased in the group fed 2.0 mg AFB_1/kg of feed at 21 DPF, contrary to Zhao's results (2010).

In the previous studies (Raju et al., 2000; Zhao et al., 2010), dietary aflatoxin showed no effect on serum AST or ALT levels. Yunus et al. (2011) reported that it was not possible to draw a dose-effect relationship for either AST or ALT levels. Our experiments, however, showed a significant dose-dependent increasement at 21 DPF.

Although the AST and ALT changes varied according to the AFB_1 dose and exposure time (Tessari et al., 2010; Fowler et al., 2015; Hussain et al., 2016), we have shown that along with histological evaluation, serum values of AST, ALT, TP, glucose, and albumin may all serve as marker for chronic aflatoxicosis in poultry.

The data presented here indicate that both the dose of aflatoxin and the length of exposure influence the biochemical and histological response in broilers.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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