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Effect of Prolonged Low Level Inclusion of Aflatoxin B_1 into Diet on Performance, Nutrient Digestibility, Histopathology and Blood Enzymes of Broiler Chickens

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Abstract: A feeding trail was conducted to evaluate the effects of diets contaminated with Aflatoxin B_1 (AFB_1) on the performance, digestibility of nutrients and blood enzymes of broiler chickens. In a randomized complete-block design with 4 blocks and 4 treatments, 112 day-old male broiler chickens were divided into 16 groups of 7 chicks each. Treatments consisted of 3 levels of AFB_1 in starter diet (0.4, 0.8 and 1.2 mg kg^{-1}) along with a control group (no AFB_1). Feed intake and daily weight gain were recorded weekly. On days 7, 14, 21 and 28, one bird from each replicate was weighed and humanly killed to collect blood samples and to weigh proventriculus, gizzard, duodenum, pancreas, heart, liver, spleen, bursa of fabricius and brain. From day 18, chromic oxide-marked feed was fed to the birds for 4 consecutive days. On day 21, feces collection was made every 6 h and a pooled sample was used for determination of nutrients digestibility. Feeding AFB_1 significantly decreased feed intake and body weight gain and increased relative weight of liver ($p<0.05$). Relative weight of brain decreased on day 7 and then showed a significant increase by day 28 ($p<0.05$). AFB_1 significantly ($P<0.05$) increased serum concentration of Aspartate aminotransferase (AST) and Alanin aminotransferase (ALT) and decreased serum concentration of Lactate Dehydrogenase (LDH). Feeding AFB_1 at 0.8 and 1.2 mg kg^{-1} diet, caused a significant reduction in AME content of the diet, organic matter, dry matter, calcium and phosphorus ($p<0.05$). Digestibility of fat and protein were not significantly affected by feeding AFB_1. A significant reduction in uric acid excretion was induced due to AFB_1 inclusion into the diet ($p<0.05$). This study demonstrated that feeding AFB_1 at the used levels can impair digestibility of nutrients, especially calcium and phosphorus. Furthermore, alongside with other negative effects, AFB_1 may have some adverse effects on the liver and brain of broilers.

Key words: Aflatoxin B_1, performance, nutrients digestibility, blood enzymes, broilers

INTRODUCTION

Aflatoxins are secondary fungal metabolites mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus* (Tedesco *et al*., 2004). Aflatoxin producing molds, can grow on different food, feed and waste materials and under a wide variety of environmental conditions including moisture, pH and temperature that enables them to have a wide spread distribution. There are more than 20 aflatoxin derivatives which among them, AFB_1 have the most toxic effects (Hussein and Brasel, 2001). Many different toxic effects for aflatoxins, including reduced performance, hepatic intoxication, adverse effects on carcass as well as egg shell quality, immunosuppression and carcinogenicity have been reported in poultry (Charmley *et al*., 1995). Increase in relative weights of liver, kidney, heart, proventriculus, gizzard, spleen and pancreas in broilers consuming aflatoxin contaminated diets, have been reported (Kubena *et al*., 1990). Also, it has been demonstrated that aflatoxins are able to alter concentrations of some blood enzymes (Kubena *et al*., 1990). Negative effects of aflatoxins in poultry production are both dose and time dependent (Leeson *et al*., 1995). There is increasing interest in investigating effects of long term-low level administration of mycotoxins in farm animal and poultry diets on t heir performances and productions (Dersjant-Li *et al*., 2003). On the other hand, in spite of abundant evidences showing reducing effects of aflatoxins on performance, there is very few even no

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report regarding effects of these toxins on the digestibility of nutrients. In the present study, the effect of prolonged low level inclusion of aflatoxin B_1 into diet on performance, nutrient digestibility, histopathology and blood enzymes of broiler chickens have been investigated.

MATERIALS AND METHODS

One hundred and twelve day-old male broiler chicks (Cobb 500) were divided into 16 groups and randomly assigned to each of 16 units of a four-floor battery cages. All birds had free access to feed and water during the experiment. Aflatoxin B_1 (AFB_1) for this experiment was produced using *Aspergillus parasiticus* PTCC 5286 (Organization of Scientific and Technological Research, Karaj, Iran), according to the method described by Shotwell *et al.* (1966). More than eighty percent of the aflatoxin produced by this strain is B_1 type (Rosa *et al.*, 2001). AFB_1 concentration was determined using HPLC according to AOAC (2000) method. Serum concentrations of Aspartate amino Transferase (AST), Alanine amino Transferase (ALT) and Lactate Dehydrogenase (LDH) were determined using commercial available kits (Darman Kave Research Laboratory, Isfahan, Iran). Starter and grower diets (Table 1) were formulated for 0-28 and 28-42 days of age, respectively, according to the NRC recommendations (NRC, 1994). Dietary treatments were consisted of three levels of AFB_1 in diet (0.4, 0.8 and 1.2 mg kg^{-1} diet) along with control diet (no added AFB_1). In order to reach intended AFB_1 concentrations in diets, an appropriate mixture of *Aspergillus parasiticus* culture on rice was replaced with rice flour in the basal diet. AFB_1 contaminated diets were fed from 0-28 days of age. From day 28-42 days of age, all groups were fed an AFB_1 free grower diet.

For measurement of nutrient digestibility, chromic oxide (Cr_2O_3) was used as an external marker. Chromic oxide-marked feed (3 g kg^{-1} diet) was fed from 18-21 days of age. On day 21, samples of excreta collected every 6 hours and kept in refrigerator (4°C). At the end of day 21, all four samples for each group were polled and a sample was taken and frozen (-20°C). Excreta samples were later weighed, thawed, oven dried (80°C for 48 h), weighed again, ground (0.5 mm screen) and used for analysis. Diets and excreta samples were analyzed for dry matter, organic matter, ether extract, protein (N) calcium and phosphorus according to the standard methods of the Association of Official Analytical Chemist (AOAC, 1980). Chromic oxide was analyzed according to the procedure described by Fenton and Fenton (1979). Gross energy of the diets and excreta were analyzed by bomb calorimeter

Table 1: Composition of the experimental diets

Ingredients (g $100g^{-1}$)	Starter (0-28 days of age)	Grower (28- 42 days of age)
Yellow corn	57.22	64.02
Rice flour	3.42	3.42
Soybean meal, 44%	34.54	29.43
Corn gluten meal	1.21	-
Dicalcium phosphate	1.48	1.04
Limestone	1.16	1.24
Vitamin premix[1]	0.25	0.25
Mineral premix[2]	0.25	0.25
Salt	0.34	0.32
DL-Methionine	0.13	0.03
Total	100.00	100.00
Calculated value		
Metabolic energy (MJ kg^{-1})	11.91	12.20
Crude protein (%)	20.50	18.20
Calcium (%)	0.91	0.82
Available phosphorus (%)	0.41	0.32
Arginine (%)	1.34	1.18
Lysine (%)	1.10	0.97
Methionine+cystine (%)	0.82	0.60
Sodium (%)	0.15	0.14

[1]Each kg of vitamin premix contained: Vitamin A, 3,600,000 IU; vitamin D_3, 800,000 IU; vitamin E, 7,200 IU; vitamin K_3, 800 mg; vitamin B_1, 720 mg; vitamin B_2, 2,640 mg; vitamin B_3, 4,000 mg; vitamin B_5, 12,000 mg; vitamin B_6, 1,200 mg; vitamin B_9, 400 mg; vitamin B_{12}, 6 mg; vitamin H_2, 40 mg; choline chloride, 200,000 mg, [2]Each kg of mineral premix contained: Mn, 40,000 mg; Fe, 20,000 mg; Zn, 40,000 mg; Cu, 4,000 mg; Se, 80 mg

(Parr Instruments Co. Moline, IL 61256). In order to correct protein digestibility for uric acid with urine origin, the amount of uric acid in the excreta was determined according to the method described by Marquardt (1983). In order to determine the AME of the diets, the following equation was used (Scott and Boldaji, 1997):

AME (mega joules per kilogram of diet) = GE_{diet} – [GEexcreta × ($Marker_{diet}$ / $Marker_{excreta}$)];

Where GE = Gross Energy; and Marker = concentration of chromic oxide.

Statistical analysis: The four treatments were allotted in a randomized complete-block design, with four blocks per treatment. Each quarter of the battery cage constituted a block. Within a block, each treatment was allocated to a pen of seven chicks. All data were analyzed using GLM procedure in the SAS software. Treatment means were compared by new Duncan multiple range test (SAS, 1990).

RESULTS AND DISCUSSION

Performance: Feed intake, weight gain and feed conversion data are presented in Table 2. Feeding AFB_1 at 1.2 mg kg^{-1} diet, caused a significant reduction in feed intake and weight gain for the period of 0-28 days of age ($p<0.05$). The differences among treatments for feed intake and weight gain during 28-42 days of age (the period in which the AFB_1 free diet was fed) were not significant. When considering all period of the experiment

Table 2: Performance of broiler chickens fed diets containing different levels of aflatoxin B_1

	Feed intake (g)			Body weight gain (g)			Feed to gain (g g^{-1})		
AFB_1[1] (mg kg^{-1})	0-28 d	28-42 d	0-42 d	0-28 d	28-42 d	0-42 d	0-28 d	28-42 d	0-42 d
0.0	1564[a]	1891	3455[a]	814[ab]	989	1803	1.92	1.92[ab]	1.91
0.4	1510[a]	1923	3432[a]	834[a]	952	1786	1.84	2.02[a]	1.93
0.8	1543[a]	1882	3424[a]	787[ab]	979	1766	1.96	1.93[ab]	1.94
1.2	1323[b]	1832	3155[b]	680[b]	992	1673	1.95	1.85[b]	1.89
±SEM	48.9	38.7	71.4	44.6	27.3	49.7	0.056	0.038	0.027

[1]AFB_1, Aflatoxin B_1; d, days of age, [a, b] In each column, means with different superscripts are significantly different ($p<0.05$)

Table 3: Relative organ weights (g per 100 g of body weight) of broiler chickens fed diets containing different levels of aflatoxin B_1

	1st week (day 7)		4th week (day 28)	
AFB_1[1] (mg kg^{-1})	Liver	Brain	Liver	Brain
0.0	3.45	1.02[a]	2.54[b]	0.25[b]
0.4	3.65	0.98[ab]	2.54[b]	0.28[ab]
0.8	3.68	0.87[ab]	3.20[a]	0.29[ab]
1.2	3.11	0.79[b]	3.65[a]	0.30[a]
±SEM	0.319	0.058	0.172	0.012

[1]AFB_1, Aflatoxin B_1, [a, b] In each column, means with different superscripts are significantly different ($p<0.05$)

Table 4: Serum ALT, AST and LDH concentrations in broiler chickens fed diets containing different levels of aflatoxin B_1

		Age			
Enzyme (U L^{-1})	AFB_1[1] (mg kg^{-1})	7 d	14 d	21 d	28 d
AST	0.0	162	145	142[b]	213
	0.4	151	143	145[ab]	202
	0.8	159	145	159[ab]	242
	1.2	169	146	179[a]	246
	±SEM	8.7	6.1	10.7	22.1
ALT	0.0	63	60	57[b]	55
	0.4	65	60	59[ab]	55
	0.8	68	59	61[ab]	60
	1.2	66	60	64[a]	61
	±SEM	3.0	1.2	1.6	2.3
LDH	0.0	1770[a]	1368	1514	1328[a]
	0.4	1647[ab]	1110	1388	986[b]
	0.8	1321[ab]	1289	1362	1241[ab]
	1.2	1142[b]	1074	1404	949[b]
	±SEM	161.4	165.9	140.9	99.1

[1]AFB_1, Aflatoxin B_1; d, days of age; ALT, Alanine amino transferase; AST, Aspartate amino transferase; LDH, Lactate Dehydrogenase, [a, b] In each column, means with different superscripts are significantly different ($p<0.05$)

(0-42 days of age), feed intake in 1.2 mg kg^{-1} AFB_1 group, was significantly less than those of other groups ($p<0.05$); however, this reduction in feed intake did not reduced the weight gain at this period. No significant difference was seen for feed conversion ratio during periods of 0-28 and 0-42 days of age. However, feeding AFB_1 at 1.2 mg kg^{-1} at 0-28 days of age, caused a significant improvement in feed conversion ratio during the next period of 28-42 days of age ($p<0.05$). The reduction in feed intake and weight gain due to feeding AFB_1 contaminated diets in this experiment are consistent with the result reported by Tedesco *et al.* (2004) but are not in agreement with Edrington *et al.* (1997) who reported AFB_1 did not influenced feed intake in broiler chickens when fed a mixture of aflatoxin (containing 79% AFB_1) at 4 mg kg^{-1} diet. Dersjant-Li *et al.* (2003) reviewed the impact of low concentrations of aflatoxins in poultry diets and suggested that the growth reduction due to aflatoxin contaminated diets can be related to reduction in both feed intake and feed efficiency. In this experiment, AFB_1 did not negatively influenced feed efficiency that is in agreement with the results of Edrington *et al.* (1997) but inconsistent with Rosa *et al.* (2001). Feed conversion ratio is an index of feed intake and body weight gain and usually wouldn't be considered alone.

Relative organ weights: Relative weights (g 100 g $^{-1}$ body weight) of liver and brain at 7 and 28 days of age are shown in Table 3. At 28 days of age, relative weight of liver and brain of the birds fed diet containing 1.2 mg AFB_1 kg^{-1}, were increased significantly ($p<0.05$). AFB_1 had no significant effect on liver relative weight at 7 days of age, but caused a significant increase in brain relative weight when fed at 1.2 mg kg^{-1} diet ($p<0.05$). Effects of different levels of AFB_1 in the diet on relative weights of proventriculus, gizzard, duodenum plus pancreas, heart, spleen and bursa of fabricius, were not significant. It seems that liver is the first influenced organ during aflatoxicosis (Leeson *et al.*, 1995). Increase in liver relative weight has also been reported by Kubena *et al.* (1993) during aflatoxicosis. This enhancement in liver weight is usually due to fat deposition in the liver (Leeson *et al.*, 1995). In this experiment, feeding AFB_1 contaminated diet at 1.2 mg kg^{-1}, initially decreased then increased ($p<0.05$) the brain relative weight (Table 3). Fewer data are available regarding effects of aflatoxins on the brain and nervous system of broiler chickens. In general, it has been suggested that aflatoxin is not able to cause nervous injury (Cole, 1986). Although more than 80% of aflatoxin produced by *Aspergillus parasiticus* is from B_1 type (Rosa *et al.*, 2001) it is not far from fact that other mycotoxins to be existed in fewer amounts in the culture mixture resulted from this strain. Cyclopiazonic acid is one of mycotoxins which is produced by many strains of *Aspergillus* and can cause nervous injury (Bryden, 1994). So, changes in the brain relative weight as seen in this experiment might be related to mycotoxins other than AFB_1 that may be present in *Aspergillus parasiticus* fermentation products. However, more

investigation needs to be done in order to better understanding of the effects of aflatoxins on broiler's nervous system.

Blood enzymes: Serum concentrations of ALT, AST and LDH for different treatment groups are shown in Table 4. AFB_1 in the diet at 1.2 mg kg^{-1}, caused a significant increase in serum concentrations of ALT and AST at 21 days of age ($p<0.05$). Serum concentrations of LDH at the end of 1st and 4th weeks significantly reduced by inclusion of AFB_1 at 1.2 mg kg^{-1} level into the diet ($p<0.05$). Dafalla *et al.* (1987) also reported an increase in serum concentration of AST and ALT due to AFB_1 administration into the diet. On the other hand, no changes were seen in serum concentrations of AST and ALT when AFB_1 contaminated diet fed to broilers by Edrington *et al.* (1997). Generally, AST and ALT are intracellular enzymes that do not belong to plasma; so, their appearance in serum indicates cell injury (Coles, 1974). Increase in serum concentrations of AST and ALT in this experiment, can be a result of hepatocytes injury. In this experiment, AFB_1 caused a significant decrease in serum concentration of LDH ($p<0.05$). Such a decrease has also been reported by Huff *et al.* (1986) while no significant changes in serum concentration of LDH was seen by Quist *et al.* (2000) when AFB_1 was added to the diets.

Digestibility of nutrients and energy utilization: Feeding AFB_1 contaminated diet at 0.8 and 1.2 mg kg^{-1} diet, caused a significant ($p<0.05$) reduction in digestibility of dry matter, organic matter, Ca and P (Table 5). However, digestibility of fat and protein (either corrected for excreted uric acid or not) were not significantly affected by feeding AFB_1 contaminated diets (Table 5). Also, there was a significant effect of AFB_1 on the AME of the diets (Table 5). Inclusion of AFB_1 at 0.8 and 1.2 mg kg^{-1} diet, caused a significant reduction in diet AME ($p<0.05$) when compared to the control group. AFB_1 with the highest inclusion rate (1.2 mg kg^{-1} diet), induced a significant ($p<0.05$) reduction in the excreted uric acid (data not shown). There are few data evaluating effects of aflatoxins on the digestibility of nutrients. In recent study, digestibility of dry matter, organic matter, calcium and phosphorus were reduced as a consequence of aflatoxicosis. Reduction in dry matter digestibility, in general, indicates that nutrients digestibility (including organic and inorganic) are affected. On the other hand, reduction in organic matter digestibility shows that, at least, the digestibility of one of the organic nutrients of the diet has been affected. In current study, digestibly of fat (ether extract) and crud protein were reduced

Table 5: Nutrients digestibility (21 days of age) in broiler chickens fed diets containing different levels of aflatoxin B_1

	AFB_1[1] (mg kg^{-1})				
Digestibility	0.0	0.4	0.8	1.2	±SEM
OM (%)	77.45[a]	78.02[a]	72.59[b]	73.47[b]	1.119
DM (%)	94.03[a]	94.39[a]	92.65[b]	92.51[b]	0.431
EE (%)	77.33	77.15	72.36	71.47	1.741
Protein[2] (%)	65.64[ab]	69.16[a]	61.43[b]	63.37[b]	1.640
Protein[3] (%)	87.67[ab]	88.86[a]	87.5[ab]	82.68[b]	1.544
Ca (%)	33.54[a]	22.45[b]	6.40[b]	8.41[b]	6.750
P (%)	73.31[a]	73.14[a]	66.42[b]	67.79[b]	1.360
AME (MJ kg^{-1})	13.63[a]	13.56[a]	12.56[b]	12.81[b]	0.203

[1]AFB_1, Aflatoxin B_1; OM, Organic Matter, DM, Dry Matter; EE, Ether Extract; AME, Apparent Metabolizable Energy, [2]Uncorrected for excreted uric acid, [3]Corrected for excreted uric acid, [a, b] In each row, means with different superscripts are significantly different ($p<0.05$)

numerically due to administration of AFB_1 into the diet; but this reduction was not statistically significant. Digestibility of starch and other carbohydrates were not determined in this experiment. It is therefore, possible that the digestibility of starch affected more than those of the digestibility of fat and protein during induced aflatoxicosis. The observed decrease in AME content of the diet due to administration of AFB_1 into the diet at 0.8 and 1.2 mg kg^{-1} (Table 5) is partially in agreement with the above hypothesis. Verma *et al.* (2002) fed broilers with diets containing aflatoxin at 0.0, 0.5, 1.0 and 2.0 mg kg^{-1} and reported a significant decrease in total protein efficiency at 2.0 mg kg^{-1} aflatoxin; while metabolizable energy content was reduced at both 1.0 and 2.0 mg kg^{-1} aflatoxin. These results together with results obtained in the current experiment show that energy utilization might be a more potentially sensitive factor to be affected during aflatoxicosis. The authors did not find any article describing effects of aflatoxins on calcium and phosphorous digestibility. Abdelhamid *et al.* (1990) fed aflatoxin contaminated diet to baladi rabbits and observed a significant reduction in bone ash and bone volume. Huff *et al.* (1986) also reported that feeding aflatoxin contaminated diet at levels more than 2.5 mg kg^{-1} to broiler chickens inhibited normal bone mineralization. Feeding aflatoxin contaminated diets caused a significant reduction in serum calcium and inorganic phosphorus levels in broilers (Jindal *et al.*, 1994) and laying hens (Kim *et al.*, 2003). Fernandez *et al.* (1995) showed that aflatoxicosis can increase prothrombin time in broilers as well as laying hens. Glahn *et al.* (1991) investigated the effects of aflatoxicosis on the metabolism of calcium, phosphorous and vitamin D in broiler chickens and reported a significant decrease in plasma 25-hydroxy vitamin D_3 and 1, 25-dihydroxy vitamin D_3 levels due to feeding aflatoxin contaminated diet (2 mg kg^{-1}). In the current study, feeding aflatoxin contaminated diets at 0.4, 0.8 and 1.2 mg kg^{-1}, respectively resulted in 3381 and

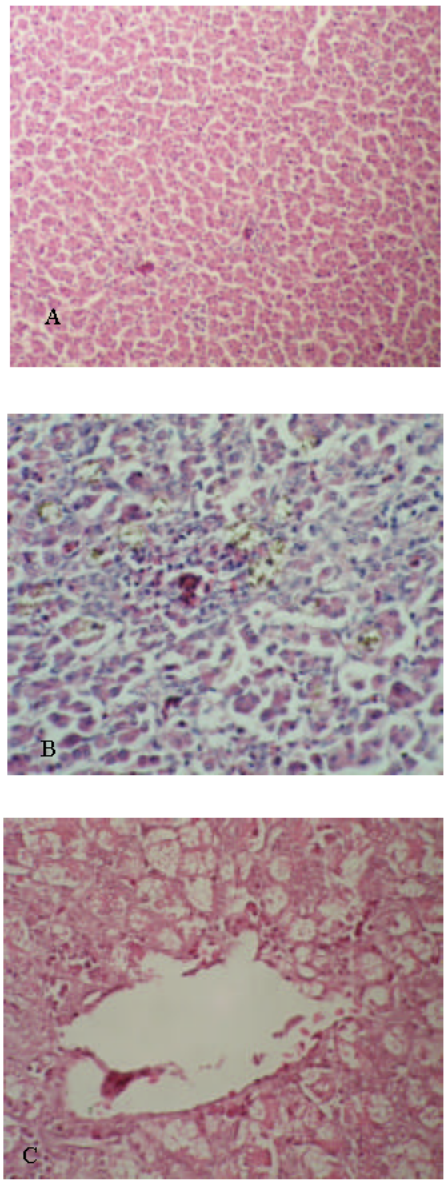

Fig. 1: Photomicrograph of hematoxylin and eosin stained liver sections: (A) section of normal liver in chickens fed aflatoxin B_1 free diet; (B) section of liver of the chickens fed aflatoxin B_1 contaminated diet that revealed moderate hepatocellular degeneration and necrosis with periportal hetrophil infiltration and bile retention; (C) liver section from chickens fed aflatoxin B_1 contaminated diet showing a sever hepatocellular degeneration and necrosi

75% reduction in calcium digestibility when compared to control group. Also phosphorus digestibility showed a 9.4 and 7.5% reduction due to feeding AFB_1 at 0.8 and 1.2 mg kg^{-1} of diet, respectively (Table 5). These results indicate that calcium digestibility is much more sensitive to aflatoxicosis than phosphorous digestibility. As mentioned before, liver and kidney are the first and the most severely affected organs during aflatoxicosis (Leeson *et al*., 1995). These two organs have a key role in synthesis of active vitamin D_3 (1, 25-dihydroxy vitamin D_3) and one of it's main function is calcium homeostasis. This form of vitamin D_3 (as a hormone) stimulates the synthesis of a specific gut cell protein (calbindin) that is responsible for calcium uptake. Activated vitamin D_3 also serves to induce the uptake of phosphate by the brush border of the small intestine due to an effect on the synthesis of a sodium-dependent membrane carrier for phosphate (Berdanier, 1998). So decrease in calcium and phosphorous digestibility as observed in this study is probably related to structural damage of liver and kidney and in turn, consecutive reduction in production of active vitamin D_3 during exposure to AFB_1.

Histopathology: Microscopic sections of heart, brain, spleen, bursa of fabricius, proventriculus, gizzard, duodenum and pancreas were unremarkable in all control and treatment groups. Sections of liver and kidney were also unremarkable in the chickens fed AFB_1 free (control) diet. Liver sections of the chickens fed diets containing AFB_1 showed moderate to sever hepatocellular degeneration (cell swelling and fatty changes) and necrosis with moderate periportal hetrophil infiltration and bile retention (Fig. 1). There was no significant relationship between hepatocellular changes and levels of AFB_1. In renal pathology, there was also mild hetrophil infiltration of glomeruli in chickens fed AFB_1 contaminated diet. Histopathological lesions in the liver and kidney of broiler chickens exposed to AFB_1 in this study are similar to those reported by Ledoux *et al*. (1998). The histopathological findings in the organs examined in this study indicate that liver is the primary and most severely affected organ during aflatoxicosis. The kidney is considered to be secondary target organ of aflatoxins. Although the effect of AFB_1 on the relative weight of the chick's brain was significant ($p<0.05$), no remarkable histopathological lesion was seen in brain sections of chickens feed AFB_1 contaminated diets.

CONCLUSION

Under the conditions of this study, it was concluded that feeding aflatoxin B_1 at the used levels can impair digestibility of nutrients, especially calcium and phosphorus. Furthermore, alongside with other negative effects, aflatoxin B_1 may have some adverse effects on the liver and brain of broilers.

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