The effects of aflatoxin B1 and silymarin-containing milk thistle seeds on ileal morphology and digestibility in broiler chickens

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Abstract

This study investigated the effects of aflatoxin B_1 (AFB1) and milk thistle seed (MTS) on some apparent ileal morphology and digestibility variables in the small intestines of broilers. A total of 216 Ross 308 male broiler chickens were allocated in a 3 3 factorial arrangement of the treatments with three concentration of AFB1 (0, 250, and 500 ppb) and three levels of MTS (0, 5, and 10 g kg^{-1}). On day 35, the birds that received diets with 500 ppb of AFB1 alone (main effect) showed significant decrease in apparent ileal digestibility [dry matter (DM; 72.46±0.27), calcium (Ca; 40.81±1.11), crude protein (CP; 29.42±1.89), apparent digestible energy (2653±58.82)], ileal morphology [villus length (VL; 822.5±7.47), villus width (VW; 90.16±2.17) and ratio of VL to crypt depth (VL/CD; 4.74±0.07)] in their ileum segments (P<0.01). However, the mean nitrogen (N; 61.39±0.48) and crypt depth (CD; 173.5±9.87), in the ileum were significantly greater for the birds that were fed with 500 ppb AFB1 alone in their diets when compared with the control (P<0.01). Also, thistle seeds can ameliorate the toxic effects of AFB1 on some ileal digestibility factors, that is, DM, N, Ca, and CP, in broiler chicks. Nevertheless, ileum morphology of VW and goblet cell numbers were not affected negatively by the AFB1 plus MTS in diets. The results of this study indicated that the use of MTS independently reduced the toxic effects of AFB1, facilitated the absorption of nutrients, and reduced the metabolic demands of the intestinal tract in broiler chickens.

Introduction

Aflatoxin B_1 (AFB1) is a secondary metabolite produced by *Aspergillus flavus* and *A. parasiticus*, and it has carcinogenic, mutagenic, hepatotoxic, and teratogenic effects.[1,2] Several diseases are associated with the human consumption of these toxins, including toxic hepatitis and even primary hepatocellular carcinomas.[1,2] Aflatoxin B_1 8,9-epoxide is the reactive form of the compound, and it binds to cellular macromolecules and causes periportal hepatic injury.[3] However, extrahepatic effects, namely, within the intestine, have not been studied thoroughly. Other researchers have documented the negative effects of AFB1 on total tract retention of energy, mean nitrogen (N), and amino acids in poultry.[4-7] It seems that AFB1 alone has a harmful effect on the metabolization of nutrients, a harmful effect on the intestine, or both, resulting in increased loss of endogenous nutrients, reduced digestibility of nutrients, or both.[8] From the aforementioned studies, it is difficult to discern a dose-effect relationship between AFB1 and histological changes in the gastrointestinal tract (GIT). AFB1 is widely believed to result in malabsorption syndrome regarding macronutrients and also to result in reduced activity of digestive enzymes.[9,10] Silymarin is a mixture of flavonoids extracted from milk thistle seed (MTS) (*Silybum marianum* L. Gaertn.), and it contains silybin, silydianin, and silychristin as the major fractions.[11] Silymarin acts in five different ways; as an antioxidant, absorber and regulator of the intracellular glutathione, as a stabilizer and regulator of cell membrane permeability that prevents the entering of hepatotoxic substances into hepatocytes, as the ribosomal ribonucleic acid (rRNA) synthesis promoter stimulating regeneration of the liver and an inhibitor of the transformation of liver stellate cells into myofibroblasts.[12] This suggests that silymarin may contribute to the prevention of aflatoxicosis-induced damage.[13-15] There has been no reports that have dealt with the effect of interactions of AFB1 combined with MTS on the ileal morphology and digestibility of broilers to date. Thus, this study was conducted to evaluate the effects of simultaneous supplementation of AFB1 and MTS on ileal morphology and digestibility in broiler chickens.

Materials and Methods

Plants collection

Milk thistle seeds were collected from Kashmar-Kohsorkh district (16.35° north latitude, 18.58° east longitude, about 1052 meters above sea level) in Khorasan-Razavi province, in the north-east of Iran, during autumn 2011 (Figure 1).

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Contaminating poultry feed

A. flavus was obtained from the Center of Scientific and Industrial Research Organization in Iran, PTCC NO: 5004 (IR111), and was cultured on potato dextrose agar (PDA) medium and used for *in vitro* studies. The AFB1 content in rice powder was analyzed by the method of Shotwell *et al.*[16] and measured on a thin layer chromatography (TLC) fluorometric densitometer (Camag-iII, Basel, Switzerland) on the TLC spots. The yield of AFB1 produced was 60 ppb gr^{-25} of sample from each flask.

Experimental design

Combinations of three levels of AFB1 (0, 250, and 500 ppb) with three levels of MTS (0, 5, and 10 g kg^{-1}) were incorporated into the basal diet (corn and soybean meal). A total of 216 one-day-old chicks (Ross 308) were allocated to nine treatments with four replicates based on a completely randomized design in a 3×3 factorial arrangement. There were nine experimental diets with four replicates of six birds in each replicate. All of the birds were fed a typical, commercial diet for the 35 days of the experiment. The birds were housed in wire cages with nipple waterers and 516 cm^2 of floor space per bird. Feed and water were provided ad libitum. A basal diet was formulated on

corn-soybean meal base for starter, grower, and finisher periods according to [National Research Council, NRC (1994)][17] the recommendations. The birds received the following diets with equal energy and protein levels: (T_1) control basal diet; (T_2) basal diet plus 250 ppb AFB_1; (T_3) basal diet plus 500 ppb AFB_1; (T_4) 5 g kg^{-1} of MTS; (T_5) 5 g kg^{-1} MTS plus 250 ppb AFB_1; (T_6) 5 g kg^{-1} MTS plus 500 ppb AFB_1; (T_7) 10 g kg^{-1} MTS; (T_8) 10 g kg^{-1} MTS plus 250 ppb AFB_1; and (T_9) 10g kg^{-1} MTS plus 500 ppb AFB_1. MTSs were acquired from the outskirts of the Birjand district in South Khorasan Province, Birjand, Iran. Also, all animals received humane care in compliance with the guidelines of animal science Dept. at Birjand University, Birjand, Iran.

Ileal digestibility

At the end of experiment (day 35), two birds per pen were sacrificed, and two-thirds from Meckel's diverticulum to the cecal junction was removed (about 10 cm), and the ileal digesta were flushed with distilled water. The ileal digesta was collected and stored at –20°C, freeze-dried, and ground with a mortar and pestle before the analyses. Feed, excreta, and ileal digesta were analyzed for determination of nutrient digestibility and retention. The dry matter (DM) content was determined on ground diets and freeze-dried ileal digesta and excreta by drying the samples at 100°C for 24 h. Titanium (Ti) was determined by the inductively coupled plasma atomic emission spectroscopy method (AOAC, 1995)[18] following nitric-perchloric acid wet ash digestion. Gross energy (N) determinations of feed and excreta samples were performed in a bomb calorimeter (Gallenkamp Autobomb, Loughborough, UK) with benzoic acid as a standard.[19] The apparent digestible energy (AMEn) (excreta) and AMEn (ileal digesta) of the diets were calculated using the index method (using Ti as the digestive marker) by using the formula of Meng and Slominski[20] as described by the NRC (1994).[17] The calcium (Ca) concentration of the feed and digesta were determined by Flame Atomic Absorption Spectrophotometer (A Analyst 100, Perkin-Elmer Inc., Waltham, MA, USA). The crude protein (CP) content (N 6.25) of the diet and individual samples of digesta were determined by the Kjeldahl method (AOAC, 1995).[18]

Ileal morphology

On day 35, two birds per pen were sacrificed by rupture of their carotid arteries and jugular veins. Then, two-thirds (about 5 cm) of the ileum was removed and flushed with distilled water. The mucosa was collected by scraping with a microscope slide and subsequently frozen in liquid nitrogen. A 3-cm section of the proximal ileum (Mecke's diverticulum to the cecal junction) was rinsed with 0.01 M phosphate buffered saline (PBS, pH 7.2) and placed in a 10% buffered, neutral formaldehyde (pH 7.2 to 7.4) solution. As a result, all samples were gradually dehydrated, sectioned at 6 mm thickness, and stained with hematoxylin and eosin. VL, CD, VW, and the thickness of the epithelium were measured at 100 magnification using computer software (Sigma Scan, Jandel Scientific, San Rafael, CA, USA). Also, the ratio of VL/CD was calculated (Figure 2). Two slides were made for each intestinal sample, and each slide from the ileum sample was stained with Periodic acid-Schiff (PAS) reagent (McManus, 1948).[21] The tissues were deparaffinized, hydrated, oxidized in periodic acid (6 g L^{-1}) for 5 min, rinsed in distilled water, and then placed in Coleman's Schiff's reagent (Polysciences, Inc.) for 30 min. After 15 min, the slides were rinsed in tap water, the tissues were counterstained in hematoxylin, rinsed, dehydrated, and mounted. The positively stained, PAS GCN were enumerated on six villi per sample, and the means were utilized for statistical analysis (Figure 2). Measurements of VL and VW were taken from the tip of the villus to the valley between the individual villi, and measurements for CD were taken from the valley between the individual villi to the basolateral membrane (AOAC, 1995).[18]

Statistical analysis

The data were statistically analyzed with the standard procedures of analysis of variance (ANOVA), using a 3 3 factorial with completely

Table 1. Effect of aflatoxin B1 (AFB1) and milk thistle seed (MTS) on apparent ileal digestibility in broilers at the end of the period (35 day).

AFB1 (ppb)	MTS (g kg^{-1})	Dry matter, %	Nitrogen, %	Apparent digestible energy, Kcal kg^{-1}	Calcium, %	Crude protein, %
Treatment						
0	0	77.36±2.12^{a}	56.33±1.97^{b}	2754±49.77	53.38±1.68^{a}	39.41±2.38^{a}
250	0	74.32±2.46^{ab}	57.42±1.83^{b}	2674±38.62	44.21±1.87^{b}	28.44±2.23^{bc}
500	0	72.39±2.34^{b}	69.52±2.18^{a}	2621±57.76	41.24±2.14^{c}	26.41±3.12^{c}
0	5	72.41±3.01^{b}	55.38±1.84^{b}	2762±67.64	53.31±1.68^{a}	41.46±3.75^{a}
250	5	73.29±2.57^{b}	54.36±2.39^{b}	2681±81.65	43.38±1.93^{a}	31.59±3.35^{b}
500	5	76.33±2.67^{ab}	55.51±1.83^{b}	2664±69.13	40.77±1.49^{a}	30.44±2.66^{b}
0	10	72.58±2.96^{b}	53.69±2.74^{b}	2809±84.77	51.33±2.25^{a}	38.51±2.96^{b}
250	10	72.35±2.65^{b}	54.76±1.67^{b}	2727±73.65	42.45±2.17^{a}	36.52±4.11^{b}
500	10	74.26±2.75^{b}	59.14±1.96^{b}	2675±77.71	40.42±1.69^{a}	31.43±3.68^{b}
Main effects						
0	-	75.98±0.27^{a}	55.13±0.48^{b}	2775±58.82^{a}	52.68±1.11^{a}	41.79±1.89^{a}
250	-	73.32±0.27^{b}	55.52±0.48^{ab}	2685±58.82^{b}	43.35±1.11^{b}	32.18±1.89^{b}
500	-	72.46±0.27^{b}	61.39±0.48^{a}	2653±58.82^{b}	40.81±1.11^{b}	29.42±1.89^{c}
-	0	73.12±0.27	61.11±0.48^{a}	2674±58.82	46.27±1.11	31.41±1.89^{b}
-	5	74.11±0.27	55.12±0.48^{b}	2702±58.82	45.82±1.11	34.51±1.89^{ab}
-	10	74.69±0.27	55.86±0.48^{b}	2737±58.82	44.73±1.11	37.49±1.89^{a}
Probabilities (P value)						
AFB1		0.01	0.01	0.05	0.01	0.01
MTS		Ns	0.01	Ns	Ns	0.05
AFB1 × MTS		0.05	0.05	Ns	0.05	0.05

$^{a-c}$Means within a column lacking a common superscript differ significantly (P<0.05 and P>0.05). Ns: not significant.

randomized design, as suggested by Macros software.[22] The data were compared with Tukey-Kramer *post hoc* test. Least squares means ± standard errors are reported and P≤0.05 and 0.01 indicates statistical significance. All of the care and procedures used in testing the birds in this experiment were conducted from 21 March to 24 May 2012 at University of Birjand (South Khorasan Province in 59° 13 east longitude and 32° 53 north latitude, East-Iran).

Results

The results of this study indicated that the interaction effects between AFB1 and MTS were significant for apparent ileal digestibility, that is, DM, N, Ca, and CP (P<0.05) (Table 1). In contrast, retention and digestibility of AMEn were unaffected by the combinations of AFB1 and MTS. The interaction effect from apparent N digestibility indicated that a quadratic increase occurred when the amount of AFB1 administered was increased from 250 (57.42±1.83 ppb) to 500 (69.52±2.18 ppb) (P<0.05). Also, different levels of AFB1 did not cause significant changes in the Ca and CP of diets that contained 5 or 10 g kg^{-1} of MTS (intraction effect) (Table 1). Feeding of 5 or 10 g kg^{-1} of MTS increased the CP (P<0.05) and decreased apparent N (P<0.01) digestibility (main effect). In contrast, apparent DM, AMEn, and Ca retention were unaffected by different levels of MTS alone. However, the average apparent ileal digestibility of CP and Ca that contained 5 and 10 g kg^{-1} of MTS alone were higher than different levels of AFB1 (250 and 500 ppb). Also, interaction and the main effect from ileal morphology indicated that there was a linear increase in CD and a linear decrease in VL when using diets contaminated with AFB1 compared to the control animals that were not fed the contaminated food (Table 2). Also, VL/CD ratio in the ileum was decreased significantly (P<0.05 and 0.01) at the end of study (day 35). In contrast, interaction from VW and GCN was unaffected by consumption of AFB1 plus MTS (Table 2). Also, for the broilers that were fed with the contaminated diet, a main effect was a decrease in VW (90.67±2.17 to 90.16±2.17) (P<0.01).

Discussion

The mechanism of action of MTS in apparent ileal digestibility on animals is not clearly understood. Currently, it seems that this plant can be referred to AFB1 absorbent on apparent ileal digestibility in broiler chicks. Diaz *et al.*[23] reported that low levels of AFB1 in the diet did not affect DM and N digestibility in birds. Verma *et al.*[24] reported a reduction in net protein utilization and AMEn when 1 to 2 mg kg^{-1} of AFB1 was fed to broiler chicks. The results of this study were in agreement with those of previous studies when the levels of AFB1 alone were increased from 250 to 500 ppb (main effect). Kermanshahi *et al.*[7] also noted differences in energy and protein utilization with low-level inclusion of AFB1 in the feed given to broiler chicks. In this report, feeding of 0.8 to 1.2 mg kg^{-1} of AFB1 reduced AMEn and apparent N retention.[7] Also, when apparent N retention was corrected for uric acid excretion, the differences were negated, suggesting a reduction in uric acid excretion and, plausibly, a reduction in amino acid digestibility.[25] Although the interactions between aflatoxin and MTSs are not clear, there are two possibilities, that is, first, MTSs may increase protein absorption by increasing its solubility in digesta and, as a result, by prolonging the transfer time in the small intestine and second, MTSs may provide better conditions for the action of ileal enzymes by acidification of the diet and the digestive fluids.[26] The effects of higher dosages of AFB1 in broilers on these variables are not known. Contrary to the observations in broilers, other authors noted a linear increase in the crypt depth in the distal jejunum with increasing levels of AFB1 in the diet, that is, 0, 0.6, 1.2, and 2.5 mg kg^{-1}, but they observed no effects of the toxin on villus height or the number of goblet cells.[27] From the recent studies of broilers by Kana *et al.*[28], Yunus *et al.*[27] and Kumar and Balachandran,[8] it appeared that the unit absorptive surface of the small intestine

Table 2. Effect of aflatoxin B1 (AFB1) and Milk thistle seed (MTS) on ileal morphology variables in broilers at the end of the period (35 day).

AFB1 (ppb)	MTS (g kg^{-1})	Villus length, µm	Villus width, µm	Crypt depth, µm	Ratio*	Goblet cell number**
Treatment						
0	0	844.4±8.22^{a}	93.23±3.34	147.3±18.34^{b}	5.74±0.13^{a}	10.93±1.23
250	0	824.6±8.17^{b}	90.24±3.62	153.5±18.68^{b}	5.37±0.12^{b}	11.92±1.12
500	0	819.6±8.51^{c}	89.61±4.11	177.3±19.66^{a}	4.62±0.14^{c}	14.45±1.38
0	5	841.5±9.12^{a}	93.18±5.75	146.3±19.58^{b}	5.76±0.14^{a}	11.54±1.62
250	5	825.4±9.28^{b}	90.34±5.95	150.3±14.55^{b}	5.51±0.14^{a}	11.35±1.18
500	5	822.3±9.61^{b}	88.44±4.47	169.6±15.71^{ab}	5.85±0.12^{a}	11.69±1.25
0	10	842.3±8.44^{a}	93.38±4.44	144.6±19.77^{b}	5.84±0.15^{a}	11.42±1.46
250	10	827.2±9.98^{b}	91.45±4.46	151.3±19.78^{b}	5.47±0.13^{a}	11.53±1.52
500	10	825.7±9.39^{b}	92.14±4.89	173.5±16.87^{ab}	4.75±0.17^{a}	11.73±1.22
Main effects						
0	-	842.7±7.47^{a}	93.26±2.17^{a}	146.1±9.87^{c}	5.74±0.07^{a}	11.31±0.43
250	-	825.7±7.47^{b}	90.67±2.17^{b}	151.7±9.87^{b}	5.45±0.07^{b}	11.62±0.43
500	-	822.5±7.47^{b}	90.16±2.17^{b}	173.5±9.87^{a}	4.74±0.07^{a}	12.62±0.43
-	0	829.5±7.47	91.13±2.17	159.3±9.87	5.24±0.07	12.44±0.43
-	5	829.7±7.47	90.65±2.17	155.4±9.87	5.37±0.07	11.53±0.43
-	10	831.7±7.47	92.32± 2.17	156.4±9.87	5.35±0.07	11.56±0.43
Probabilities (P value)						
AFB1		0.01	0.01	0.01	0.01	Ns
MTS		Ns	Ns	Ns	Ns	Ns
AFB1 × MTS		0.05	Ns	0.05	0.05	Ns

$^{a-c}$Means within a column lacking a common superscript differ significantly (P<0.05). *Ratio of villus length to crypt depth. **Numbers in area of epithelial cells. Ns: not significant.

Figure 1. Milk thistle plant collected from the outskirts of Kashmar-Kohsorkh district in Khorasan-Razavi province, Iran.

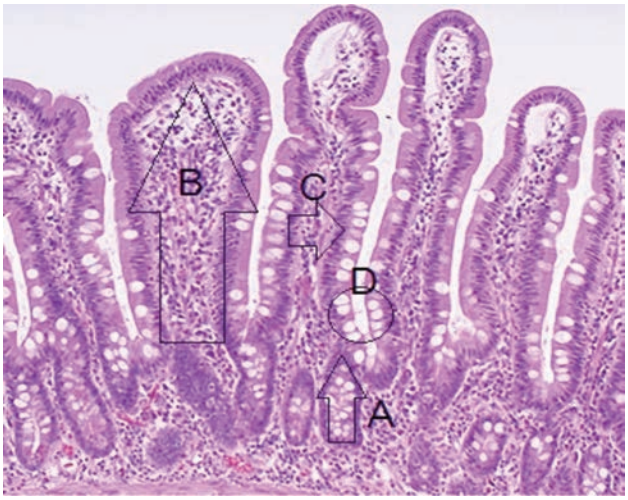

Figure 2. Ileal morphology variables measured at 100 X magnification: A) Crypt depth; B) Villus length; C) Villus width; D) Goblet cells.

deteriorated during chronic exposures to low levels of AFB1. Administration of AFB1 resulted in a reduction of T cells and alkaline phosphatase activity in the intestine.[29] Also, enterocytes and other ileal enzymes must differentiate during their time along the axis of the crypt-villus to fully express these digestive functions.[25-30] However, intestinal mucin production and secretion is a dynamic process that is continually degraded and renewed. It also has an effect on ileal morphology factors, especially villus length and the number of goblet cells.[31,32] Previous studies have not identified any positive effects of MTS on ileal digestibility and morphology. MTSs potentially are protective against intestinal diseases. However, the mechanisms of their action are not fully understood. Bean et al.[33] reported that silymarin has a good safety record, but some reports have indicated that it causes gastrointestinal disturbances and skin allergies.

Conclusions

In conclusion, these results suggest that MTSs might be used in chickens to prevent the effects of AFB1 in contaminated feed. This information provides a basis for further studies for the establishment of the mechanisms existing between MTS and protection against AFB1 toxicity. However, more research on this topic especially on the farm and field condition needs to be done to improve the safety and quality of poultry products.

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