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Effect of milk thistle on the immune system, intestinal related variables, appearance and mortality of broilers contaminated with Aflatoxin B_1

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ABSTRACT

Background & Aim: Aflatoxin is the most important fungus that contaminates food and feed and can enter the food chain of humans. The aim of this study was to investigate the ability of thistle seeds to reduce the adverse effects of aflatoxin B_1 on the immune system, variables related to the intestine, appearance and mortality of the broiler chickens.
Experimental: In this experiment, a total of 216 Ross 308 broilerchickens in a completely randomized factorial design 3×3 with nine treatments,fourreplications, and six chickens unit were grown on the ground for 35 days. Treatments, included three levels of aflatoxin (0, 250, and 500 ppb) and three levels of thistle (0, 0.5 and 1%). Influenza and Newcastle diseases titers in two steps (25 and 34 days), variables related to the intestine at the end of the experiment along with the weekly appearance and mortality of broiler chickens were evaluated.
Results & Discussion: Analysis of influenza and Newcastle diseases results in the day 34 showed a reduction in titers of them in birds receiving diets contaminated with 500 ppb aflatoxin. The length of the ileum and the total intestine at the end of the experimental period (day35) indicated decrease in the group of birds received diets containing 500 ppb aflatoxin. Chicks receiving diet containing 500 ppb of aflatoxin had the most of feather abnormalities, aggressive behavior and minimal body size compared with other treatments. Mortality due to aflatoxin consumption in various experimental treatments was not significant.
Recommended applications/industries: Probably phitosomes of *Silibummarianum* seeds reduce the mortality rate, nervous and aggressive behavior and increase the immunity of broilers against Newcastle and influenza diseases in aflatoxin exposure time.

1. Introduction

Aflatoxins (AF), potent mycotoxins produced by *Aspergillusflavus* and *Aspergillusparasiticus*, are the major concern in human and animal nutrition. Even though 18 different aflatoxins have been identified, aflatoxins B_1, B_2, G_1, and G_2 have been detected as natural contaminants of feed and food (Tedesco *et al*., 2004). Several researchers have recently focused on the

inhibition of aflatoxin biotransformation into its 8,9-epoxide constituents through interaction with cytochrome P450 enzymes using oltipraz (Kuilman*et al*., 2000) or natural compounds (Kim *et al*., 2000; Lee *et al*., 2001). *Silibummarianum* seeds (SMS) have been used from about 2000 years ago as a natural treatment for the liver and biliary duct disorders. Silymarin presents a pharmacologically effective substance containing four main constituents: silybin (50 –60%), isosilybin (5%), silychristin (20%) and silydianin (10%)(Zahid&Durrani, 2007).Silymarin acts in four 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 RNA synthesis promoter simulating regeneration of the liver, and an inhibitor of the transformation of liver stellate cells into myofibroblasts. These processes are responsible for the deposition of collagen fibers in the liver. Furthermore, absorption of free radicals is considered to be one of the key mechanisms securing the liver (Fraschini*et al*., 2002). Kalorey*et al*. (2005) reported that milk thistle improved feed intake in the presence of aflatoxin B_1. The intestine length has been reported to decrease after three weeks of dietary exposure to AFB_1 at levels as low as 0.02 (Kana *et al*., 2010), and 0.7 mg/kg (Yunus*et al*., 2011). As the width of muscularis tends to be relatively constant, the density of intestine could be a good indication of the unit absorptive area. On this variable, the effects of higher AFB_1 dosage in broilers are unknown. Contrary to these reports, no histo-pathological changes in duodenum, jejunum, cecum, and ileum could be mentioned by Ledoux et al.(1999)when male broilers were exposed to 4 mg AFB_1/kg diet for three weeks. Generally, the immunotoxic dose of AFB_1 is considered as less than the dose required eliciting a reduction in bird performance. Though several contradictory reports are available, the threshold dose of AFB_1 may be generalized to be 0.4 and 1 mg/kg for the negative effects on cell mediated and humoral immunity, respectively. However, the question regarding the susceptibility of modern broiler regarding immunotoxicity remains yet to be answered. Furthermore, there is evidence regarding the biphasic nature of the effects of AFB_1 on humoral immunity (Yunus*et al*., 2011). In a later study, these authors reported non-significantly higher titers against influenza and Newcastle diseases in birds fed 0.1 to 0.8 mg/kg AFB_1 rations as compared to the birds fed a control ration (Giambrone*et al*., 1985a). The underlying mechanisms for this temporary increase in humoral immune response are not known. The present study was conducted to investigate the effect of milk thistle on the feed intake, immune performance of broiler chicks against Newcastle and Influenza diseases (NDV and IDV), total length of the intestine, appearance and mortality in broiler chickens contaminated with Aflatoxin B_1.

2. Materials and Methods

2.1. Production of aflatoxin on rice

Aspergillusflavus obtained from the Center of Scientific and Industrial Research Organization in Iran, PTCC NO: 5004 (IR111), and proliferate on PDA (Potato Dextrose Agar) medium and used for *in vitro* studies. Aflatoxin B_1 was produced by the fermentation process on the rice. Aflatoxin analyses were performed by TLC (Thin Layer Chromatography). The yield of aflatoxin B_1 produced per 25 g sample was 60 ppm.

2.2. Chickens and diets

The study included 216, Ross-308 broiler chicks (One-day-old at the beginning of the study). They were divided randomly into nine treatment groups, each of which had four replications of six broiler chicks. 36 steel cages (40 × 65 × 98 cm) were used to accommodate six chicks per m^2. Continuous lighting was provided during the experimental period. The room temperature was gradually decreased from 32°C on day 0 to 25°C on the day 14, and remained constant thereafter. The chicks were allowed *ad libitum* access to feed and water. The study lasted for 35 days and consisted of: the control, and groups received for five weeks the feed contained: 2) 250 ppb of AFB_1, 3) 500 ppb of AFB_1, 4) 0.5% SMS, 5) 0.5%SMS with 250 ppb AFB_1, 6) 0.5% SMS with 500 ppb of AFB_1, 7) 1.0%SMS, 8) 1.0%SMS with 250 ppb AFB_1, 9) 1.0%SMS with 500 ppb AFB_1. Treatment was administered daily by gavage to assure the correct dose administration.The experiment was approved by the animal welfare committee of the Agriculture Faculty of Birjand University, Iran.

2.3. Data collection

2.3.1. Performance and Intestinal length. Chickens in each week were examined for the aggressive behavior, disarray wings, lethargy and mortality. At the end of the experimental period (day 35) different parts of the intestine, including the ileum, duodenum plus jejunum and total of intestinal length per cm were measured and recorded.

2.3.2. Evaluation of humoral immune response. Two broilers from each replicate of treatments were randomly selected to challenge with sheep red blood cell (SRBC). Blood samples for antibody analysis were taken by puncture of the brachial vein. Serum antibody titers against Newcastle Disease Virus (NDV) and Influenza Disease Virus (IDV) were measured by the hemagglutination inhibition test (HI). HI antibodies were converted into log to further evaluation. In this experiment, the titer of NDV and IDV was evaluated in two stages. At the first injection on the day 20, two birds per each group were injected 0.4 ml of SRBC (8%) in the right wing vein. Five days later, the blood samples were taken from the wing vein. On day 27, 0.8 ml of SRBC (8%) was injected into the muscle of the breast. Blood samples were taken seven days after the injection (34 days-old of broiler chicks).

2.4. Statistical analysis

The data were subjected to ANOVA as a factorial completely randomized design using the GLM procedure of SAS (SAS Inst., 2001). The model contained the effects of mortality, intestinal length, a titer of Newcastle and influenza diseases virus. The data were compared with Turkey Kramer post-hoc test. Least squares means ± standard error are reported and $p \leq 0.05$ indicating statistical significance.

3. Results and Discussion

3.1. Appearance

In the first and second weeks of the experiment, there were not seen any changes in the appearance of the birds such as feathers, confusion, lethargy, neurological signs and mortality in the broiler chicks received different treatments.

In the third week of the experiment, the chicken consumed 500 ppb of Aflatoxin B_1 were showing the highest level of aggressive behavior, disarray wings and lowest body size compared to other experimental treatments. At the end of the period (day35), these signs were shown in poultry received the 250 ppb of Aflatoxin. Similar symptoms not observed in the treatments which received the different levels of Silibummarianum seeds (SMS), alone and plus with (AFB_1), compared with the control group.

3.2. Mortality and intestinal length

In this experiment, there wasno significant differencefor the mortality rate. But the highest and the lowest mortality rate was related to the broiler fed 500 ppb of aflatoxin B_1 and the birds received 1.0% of (SMS) alone, respectively. Tedesco et al. (2004) were observed no mortality due to aflatoxin ingestion. A result from the present study is in agreement with (Miazzo*et al*., 2000 and 2005) that showed that AFB_1 affected the mortality of chickens. The lower intestine length was seen in the broilers received 500 ppb of aflatoxin B_1 (135.25 cm) ($p \leq 0.05$). There were no significant changes in total intestine length in broilers received different levels of aflatoxin B_1 (Table 1). However this difference in broilers received different levels of aflatoxin B_1 was significant compared with other experimental treatments ($p \leq 0.05$). Other researchers reported that the whole intestine length has been decreased after three weeks of exposure to AFB_1 at levels of 0.02 ppm (Kana *et al*., 2010) and 0.7 ppm (Yunus*et al*., 2011). In the present study, the minimum and maximum length of ileum and duodenum plus jejunum was observed in the chickens received 500 ppb of Aflatoxin B_1 and 1.0% of milk thistle seeds alone, respectively (Table 1).

3.3. Humoralimmunity

3.3.1. The Newcastle disease Virus (NDV) Titer. Results of statistical analysis of NDV titer on the day 25 revealed a significant reduction ($p \leq 0.05$) in birds received the contaminated diet with 500 ppb of Aflatoxin (6.02), compared with the control group (8.86) (Table 2). The NDV titer has not significantly altered in the broiler received 250 ppb of Aflatoxin (7.38) compared with control group and the broilers received different levels of SMS alone. The lowest amount of HI titer for NDV was belonged to the broilers received 500 ppb of Aflatoxin (6.02) (Yunus*et al*., 2011). Feeding broilers with different levels of SMS did not alter NDV titer compare the control group (8.86). The results of NDV titer on day 34 of the experiment did not show any significant decrease in the group of broilers received 250 and 500 ppb of

Aflatoxin (7.45 and 6.10, respectively), compared with the control group (10.23) and the broilers received different levels of SMS. There were not significant changes in the titer of NDV in the broilers received different levels of Aflatoxin B_1. The lowest amount of NDV titer was seen in the birds received the diet contaminated with 500 ppb of Aflatoxin (6.10). However, broilers fed different levels of SMS alone, did not show significant changes in the NDV titer compared to control group (10.23). Some authors reported non-significant higher titers against influenza and Newcastle disease in the birds fed 0.1 to 0.8 mg of AFB_1 Per kg rations compared to the birds fed a control ration (Giambrone*et al*., 1985a). The underlying mechanisms for this temporary increase in humoral immune responses are unknown. These results suggest that treatment with silymarin can be effective in combating with the negative effects of AFB_1 intoxication on NDV titer in broiler chicks.

3.3.2. The titer of influenza disease virus (IDV). IDV titer at the day 25 of the experiment did not show any significant changes in experimental groups, compared with the control (4.42), (Table 2). The lowest amount of the IDV titer (2.13) was seen in the birds received diets contaminated with 500 ppb of Aflatoxin (3.12). On the day 34, a significant reduction ($P<0.05$) of IDV titer was seen in the birds received the contaminated diet with 500 ppb of Aflatoxin (2.89), compared with the control group (4.78). The titer of IDV in chickens received diets containing 250 ppb of Aflatoxin (3.99) did not show any significant differences with the broilers received diet of control group and the broilers fed different levels of SMS.

The lowest amount of the IDV titer was seen in the birds received the diet contaminated with 500 ppb of Aflatoxin alone. The diets containing about 0.5 and 1.0 percent of SMS, did not show a significant change in the titer of IDV compared to control group. Also, we did not see any significant increasement in the titer of IDV in groups received 250 ppb of aflatoxin plus 0.5 percent of milk thistle seeds (4.49), compared with the broiler received 250 ppb of aflatoxin (3.99) alone.

Giambroneet al. (1985a,b) in two separate experiments on Hubbard broilers, indicated a non-significant increasing trend in ND titer along with an increase in the AFB_1 content of ration from zero to 0.5 mg Per kg. In the other experiment, higher IDV and NDV titers were shown in the birds fed 0.1 mg, and 0.2 mg of AFB_1 per kg diet, respectively (Yunus*et al*., 2011). These results suggest that treatment with silymarin may be effective on counteracting the negative effects of AFB_1 intoxication on IDV titer in broiler chicks.

4. Conclusions

Treatment with milk thistle seed decreases the toxic severity of AFB_1 in broilers. These findings suggest that silymarin might be used in chickens to prevent the effects of AFB_1 in contaminated feed. This information provides a basis for further studies for the establishment of the mechanisms existing between silymarin and protection against AFB_1 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.

Table 1. Effect of aflatoxin B_1 (AFB1) and milk thistle seeds on mortality and intestinal length

Aflatoxin(ppb)	Extract (%)	Mortality	Total of Intestinal length	Duodenum plus jejunum	Ileum
0	0	0.5±0.28	151.35±2.28 ^{a}	90.25±2.04 ^{ab}	61.5±1.25 ^{a}
250	0	1±0.4	145±1.91 ^{ab}	85±1.23 ^{ab}	60.11±1.21 ^{ab}
500	0	1.5±0.64	135.25±1.46 ^{b}	80.25±1.48 ^{b}	55.25±1.42 ^{b}
0	0.5	0.25±0.25	151±4.43 ^{a}	90.75±1.52 ^{a}	60.25±1.34 ^{ab}
250	0.5	0.75±0.47	150.25±2.10 ^{a}	89.75±1.03 ^{ab}	60.5±1.52 ^{ab}
500	0.5	0.5±0.28	148.75±4.60 ^{a}	87.25±1.41 ^{ab}	61.5±1.44 ^{a}
0	1.0	0±0	157±1.40 ^{a}	93.21±2.53 ^{a}	64.11±1.92 ^{a}
250	1.0	0.5±0.28	153±1.64 ^{a}	91.5±2.01 ^{a}	62.53±1.87 ^{a}
500	1.0	0.75±0.25	148.75±2.32 ^{a}	87.24±1.09 ^{ab}	61.75±1.55 ^{a}

$^{(a,b)}$Means ±standard error within a row with a common superscript differs significantly ($p \leq 0.05$).

Table 2. Effect of experimental treatments on the titers of Newcastle and influenza disease virus

Treatment		Titers of (NDV), (Log 2)		Titers of (IDV), (Log 2)	
Aflatoxin (ppb)	*Silybummarianum*(%)	Step 1	Step 2	Step 1	Step 2
		Day25	Day34	Day25	Day34
0	0	8.86±0.35 [a]	10.23±0.75 [a]	4.42±0.87	4.78±0.43 [a]
250	0	7.38±0.67 [ab]	7.45±0.79 [bc]	3.43±0.41	3.99±0.08 [ab]
500	0	6.02±0.28 [b]	6.10±0.11 [c]	3.12±0.37	2.89±0.08 [b]
0	0.5	8.70±0.86 [a]	9.96±0.64 [a]	4.28±0.19	4.82±0.24 [a]
250	0.5	8.46±0.18 [a]	9.50±0.24 [ab]	4.43±0.19	4.49±0.39 [a]
500	0.5	8.23±0.15 [a]	9.30±0.45 [ab]	3.51±0.47	4.32±0.40 [a]
0	1.0	8.55±0.34 [a]	10.36±0.29 [a]	4.48±0.24	4.35±0.19 [a]
250	1.0	8.33±0.41 [a]	9.1±0.03 [ab]	3.97±0.05	4.33±0.20 [a]
500	1.0	7.47±0.12 [ab]	8.21±0.10 [abc]	3.24±0.11	4.07±0.38 [ab]

[(a,b)]Means ±standard error within a row with a common superscript differs significantly ($p \leq 0.05$).

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