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RESEARCH ARTICLE

Clinicopathological Effects of Prolonged Intoxication of Aflatoxin B1 in Broiler Chicken

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A B S T R A C T

In this study pure Aflatoxin B1 (AFB1) was given to broiler birds at the rate of 50, 100, 200, 400 and 800µg/kg feed for 28 days. Birds of experimental groups intoxicated with 50, 100 and 200µg/kg feed did not show any clinical and behavior alteration. Birds of experimental groups intoxicated with 400 and 800µg/kg showed depression, ruffled feathers and had decreased feed intake, increased water consumption with soft to watery feces. Birds fed 800µg/kg AFB1 for 28 days also exhibited nervous signs (torticollis). Mortality occurred in all treatment groups with the exception of those given 50 and 100µg/kg AFB1 for 28 days. Clinical signs and mortality percentages score increased with the increase in AFB1 levels. Dose related significantly decreased in relative organs weight were recorded in all groups with the exception of birds given 50 and 100µg/kg AFB1 for 28 days. Significantly increased scores of gross lesions were recorded with increasing dietary levels of AFB1. The present study concluded that, AFB1 at level of 50µg/kg feed did not show any behavioral, gross and pathological effect on broilers when fed continuously for 28 day. In other groups pathological changes were seen in dose related manner. Severe changes were seen in higher dose levels.

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INTRODUCTION

Mycotoxins are produced by some toxigenic fungi under favorable environmental conditions (Ahmad *et al*., 2012; Lee and Ryu, 2015; Elzupir *et al*., 2015). Mycotoxins are second most important issue faced by the world poultry industry after high feed prices. Aflatoxins (AF) are detected in finished poultry feeds and its ingredients throughout this globe (Binder *et al*., 2007; Saleemi *et al*., 2012; Ahangarkani *et al*., 2014; Greco *et al*., 2015; Zainudin and Perumal, 2015). Presence of aflatoxins in poultry feed and consumption of AF-contaminated feed and feed stuff by the birds leads to retarded growth, decreased feed intake, high mortality, lower egg laying capacity, and discarding of carcass (Hassan *et al*., 2012b; Khan *et al*., 2014; Nazir *et al*., 2014; Bilal *et al*., 2014; Valtchev *et al*., 2015).

Aflatoxins are of four types B1, B2, G1 and G2. AFB1 is a known carcinogen. In the published literature, immunotoxicity in birds and mammals of AFB1 is proven (Mehrzad *et al*., 2015; Ben Salah-Abbes *et al*., 2015) and even it harmfully distresses the immune system of developing embryos (Qureshi *et al*., 1998) which has been also reported in recent findings by Zahoor-ul-Hassan *et al*. (2012). Moreover, in embryos hatched from eggs collected from aflatoxins exposed hens also showed severe DNA damage in B- and T-lymphocytes (Zhang *et al*., 2015). In the developing countries, the same food chain is shared by the animal and human population which may result in provision of poor quality grains to the human population. Keeping in view the grains quality, the present study was planned and executed with the objectives to determine clinicopathological effects of prolonged aflatoxins intoxication in broiler chicks.

MATERIALS AND METHODS

Production and quantification of aflatoxins: Aflatoxins were produced on rice (Hussain *et al*., 2008). Briefly, 25ml distilled water plus 25µl trace metal ($ZnSO_4 \cdot 7H_2O$ = 1.0g, $CuSO_4 \cdot 5H_2O$ = 0.50g, in 100 ml distilled water) solution was added to the rice. Spores of *Aspergillus flavus* (link: Fries. A. NRRL 6540 and CECT. 2687) were inoculated into rice. Quantifications of aflatoxins were carried out by HPLC method 990.33 (Anonymous, 2000).

Preparation of experimental feeds: Feed (corn, soy meal) with 22% total protein, providing 3000 Kcal/kg metabolizable energy was prepared and was analyzed for Aflatoxin, Ochratoxin and Zearalenone by TLC (Howell and Taylor, 1981). AFB1 quantity to be mixed with feed for every group was calculated. Fermented rice comprising the required amount of AFB1 were weighed and extracted by soaking into a threefold quantity of chloroform (100:300) for overnight and filtered through cotton cloth. All solvent was evaporated through rotary evaporator. Then concentrated residues were re-suspended into 100ml Polyethylene glycol and evenly mixed with 0.5kg, then in 2kg and finally in the required quantity of feed.

Experimental Birds: Broiler chicks (n=480) of 14 days of age were divided equally into 6 groups (A-F). Group A was kept as a nontoxic control group. Aflatoxin B1 suspended in polyethylene glycol was mixed in the feed of these groups at a level of 0, 50, 100, 200, 400, and 800µg/ kg feed dose levels. On days 1, 5, 10, 15, 20, 25, 28, 31, 36 and 42 of the experiment, six birds were killed humanely from each group. The remaining chickens were killed humanely at 56 days of age, i.e., end of the experiment.

Clinical signs and behavior alterations: Clinical signs and behavior alterations were recorded two times daily for desirability to feed and water, alertness, feces consistency, feather appearance and nervous signs. Each clinical signs were subjectively assigned a score from 0-3 according to its absence (0) and intensity (1-3) and number of birds showing the sign in each group. Two observations for each condition was recorded every day and averaged. Daily score was added at the end of the experiment to get a total score of each group in each experiment (Hussain *et al*., 2010). Mortalities of experimental birds in each group, their organ absolute and relative weights were recorded at each killing.

Gross and microscopic pathology: Six birds were individually weighed, sacrificed for gross examinations of visceral organs. Liver and kidneys were collected from each bird and fixed in formalin (10% neutral buffered) for further tissue studies. The gross lesions were subjected to score (0-2) depending upon the absence (0) and intensity (1-2). Values assigned for a particular lesion for all birds of a group necropsied were added to obtain an individual lesion score and summed up to obtain a lesion score for a particular time. Finally, a cumulative lesion score for a group was obtained by adding of all lesion scores of that group.

Statistical analysis: One-way ANOVA test was applied to analyze the data by using MSTAT C statistical software. The group means were compared by using DMR test and significance level was $P \leq 0.05$.

RESULTS AND DISCUSSION

In this experiment different feed levels of Aflatoxins were calculated on the basis of AFB1 concentration in the fermented rice, ranging from 0, 50, 100, 200, 400 and 800µg/kg feed. Feed aflatoxins at concentrations varying from 50 to 2000µg/kg for variable time periods have been used to induce aflatoxicosis of subacute, acute and chronic (Rosa *et al*., 2001; Ortatatli *et al*., 2005). In this study different dose levels (50, 100, 200, 400 and 800 µg AFB1/ kg feed) were offered to chicks of 14 days of age for 28.

Clinical signs and behavioral alterations: A subjective comparison of different clinical signs and behavioral alterations based upon the visual assessment of has been shown in Table 1. All the parameters in this table indicated surge in severity of the clinical signs with an increase in the dietary levels of aflatoxins.

Birds of group A (control), B (50µg/kg) and C (100µg/kg) behaved normally in terms of attraction toward feed, water intake, fecal consistency, feather condition and nervous signs throughout the length of the experiment. The lesser attraction toward the feed and increased interest in drinking water was observed in groups D (200µg/kg), E (400µg/kg) and F (800µg/kg) as compared with other groups. The consistency of feces of the birds of groups A (0µg/kg), B (50µg/kg) and C (100µg/kg) was firmed while those of Groups D (200µg/kg) had loose droppings. Birds of Groups E 400µg/kg) and F (800µg/kg) had watery droppings. Feather of birds of group A were shiny while the feathers of birds of group B (50µg/kg), C (100µg/kg) and D (200µg/kg) did not vary from those of Group A. Groups E and F showed less shiny, ruffled and broken feathers as compared with control group A. Similar clinical signs have already been reported (Kubena *et al*., 1998; Hussain *et al*., 2008; Khan and Zahoor, 2014). All these researchers described unthriftiness, increased water intake, anorexia and mortality as general changes during aflatoxicosis. During toxicity, increased water intake could be an effort to dodge desiccation thus regaining water loss from the body due to diarrhea. Nervous signs comprising of torticollis (Fig. 1) were observed in some birds of group F (800µg/kg) only started on day 20th of the experiment, while other birds of other groups did not show signs of nervous derangement. Nervous derangement resulting as torticollis was observed only in some of the birds given 800µg AFB1/kg feed for 28 days in the present study. Similar nervous signs have also been reported by Chohota *et al*. (2000) during a concurrent outbreak of chlamydiosis and aflatoxicosis in chickens having 500ppb aflatoxin in feed. Signs of nervous derangement have been observed in ducks suffering from aflatoxicosis (Hoerr, 2003). No clinical signs were observed in the present study in group A (0µg AFB1/kg) and B (50µg AFB1/kg). Similar results were reported by Ortatatli and Oguz. (2001). In this experiment a subjective scoring of clinical signs revealed an increase in severity of signs with the escalation of AF consumption through feed in the same age birds, suggested a dose related increase and age related decrease in severity of clinical signs of aflatoxicosis.

Mortality of the birds in different groups given different dietary AFB1 for 28 days from 14 days of age has been presented in Table 2. In groups A, B and C mortality was nil throughout the course of the experiment. In the present study, non-presence of clinical signs and mortality highlighted the needs of new assessment of permissive levels for poultry birds. A decrease in body weight of birds during experimental aflatoxicosis has been observed which are in line with (Tessari *et al*., 2006; Jakhar and Sadana, 2004; Dos Anjos *et al*., 2016) they all reported a significant decrease in body weight of broiler chicks below 21 days of age fed up to 5 mg/kg aflatoxin.

Table 1: Clinical signs and behavior score of broilers given different levels of dietary aflatoxin B1 from 14 days to 42 days of age

Clinical signs and behavior	Score range	Groups (Aflatoxin B1 µg/kg feed)					
		A (0)	B (50)	C (100)	D (200)	E (400)	F (800)
Alertness normal – depressed	0-3	-	-	-	30	30	62
Attraction to feed normal – less interest	0-3	-	-	-	110	120	132
Attraction to water normal – more/less interest	0-3	-	-	56	86	112	168
Feces consistency normal form – watery	0-3	-	-	-	138	142	150
Feather normal shiny – ruffled & Broken	0-3	-	-	-	-	56	112
Nervous derangement: no – present	0-3	-	-	-	-	-	9
Cumulative score		0	0	56	364	460	633

Table 2: Mortality of broiler chicks given different dietary levels of aflatoxin B1 from 14 days to 42 days of age

Experiment (weeks/Days)		Age of birds (Days)	Groups (Aflatoxin B1 µg/kg feed)					
Weeks	Days		A 0	B 50	C 100	D 200	E 400	F 800
1	-	14-21	-	-	-	-	-	-
2	-	22-24	-	-	-	-	-	-
2	10	25	-	-	-	-	-	1
	11	26	-	-	-	-	-	1
	12	27	-	-	-	-	-	2
	13	28	-	-	-	-	-	1
3	-	29	-	-	-	2	2	-
		30-35	-	-	-	-	-	-
4	-	36-42	-	-	-	-	-	-
5	-	43-49	-	-	-	-	-	-
	Mortality (No.)	-	-	-	-	-	4	5
	Mortality (%)	-	-	-	-	3.33	6.33	8.33

Relative organ weights: The relative organ weights of different organs on each killing were shown in Table. 3. These results showed significantly higher values with an increase in feeding AFB1 levels. The relative weight of each organ was increased up to 31st day of the experiment. On day 42 weights of all the organs were non-significantly different from control.

Same dietary levels of aflatoxins, however, resulted in a non-significant difference in body weight of birds above 21 days of age suggesting an age related development of resistance in chicks towards aflatoxins. Quezada *et al*. (2000) reported a non-significant change in body weight of 4 weeks old broiler chicken by feeding AFB1 up to 2.0µg/g of feed. Our result showed a similar trend of age related development of resistance.

Gross lesions: Birds in Group A and B did not show any abnormality and gross lesions throughout the length of the experiment. Liver was normal in color and consistency. Kidneys, heart, spleen, thymus, intestine and subcutaneous tissues were normal in appearance throughout the length of the experiment.

The constant gross lesion observed in the current study was hepatomegaly and anemic liver of birds. This change was followed by moderately enlarged kidneys and hemorrhages on different organs/tissues of the body of the intoxicated birds. Birds in Group C (100µg/kg) showed moderate enlargement of the liver and kidneys on 25th and 28th days of the experiment. In group D and E no such changes were observed in any organ or tissue on days 1, 5, 10 and 15. On days 20 and 25 there was a moderate enlargement of liver and kidneys. On day 28 livers and kidneys were larger than those of group A and pale in color some of the birds also showed some petechial hemorrhages on these organs (Fig. 2A). On day 31, liver was moderately enlarged, but no hemorrhages were present on it. On days 36 and 42 all organs appeared normal, when compared with those of the control birds. Birds in group F (8200µg/kg) showed gross enlargement of liver and kidneys on day 15 of the experiment. On day 20 and 25 enlarged livers were light in color and friable in nature (Fig. 2B). On day 28 different organs also showed the presence of petechial and ecchymotic hemorrhages. On day 31 no hemorrhagic lesions were present in any organ, but swelling of liver and kidneys was still persistent. On day 36 and 42 all the organs were normal in size, appearance and consistency compared with those of group A. Similar lesions in the liver have been reported by Khan et *al*. (2014) in the layer breeders.

Enlarged livers and kidneys in the present study were also evident by the significant increase in relative weights of these organs in different groups. A subjective comparison of different gross lesions based upon the visual assessment of birds of different treatment groups has been shown in Table 4. Pallor of the liver attained the highest score in group F (800µg/kg), which was significantly different from groups D (200µg/kg) and C (100µg/kg). Group C (100µg/kg) showed significantly lower values than B (50µg/kg) and F (800µg/kg).

Fig. 1: Torticollis in bird on 20th day of experiment fed AFB1 800µg/kg (group F).

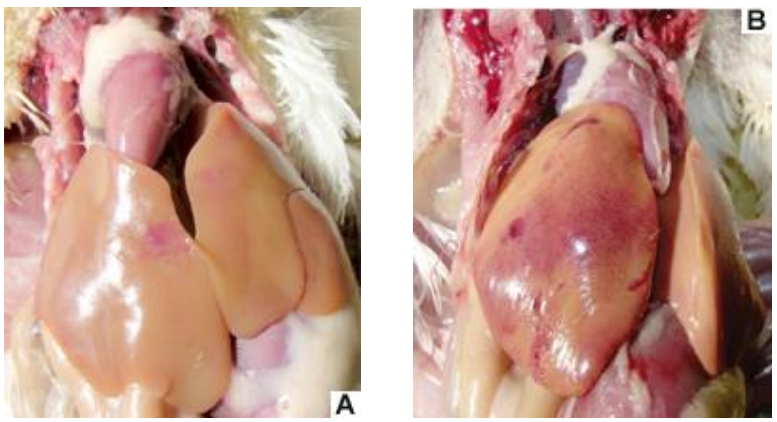

Fig. 2: A) Pale yellow colored liver on 28th day of experiment in group E fed AFB1 400µg/kg, B) pale hemorrhagic liver on 20th day of experiment of bird fed AFB1 800µg/kg.

Table 3: Relative weights (% of body weight) of different organs of birds given different levels of dietary aflatoxin B1 from 14 days to 42 days of age

Days of experiments	Groups (Aflatoxin B1 µg/kg feed)					
	A (0)	B (50)	C (100)	D (200)	E(400)	F (800)
Livers						
1	3.20±0.13	3.22±0.09	3.46±0.16	3.58±0.01	3.44±0.28	3.46±0.38
10	3.02±0.01c	3.27±0.07b	3.09±0.09c	3.10±0.07c	3.17±0.03b	4.31±0.12a
20	4.01±0.04c	3.92±0.12c	3.53±0.09d	3.61±0.05d	4.22±0.00b	4.77±0.06a
28	2.91±0.10e	3.23±0.12d	3.80±0.04c	3.84±0.03c	4.41±0.02b	4.78±0.05a
30	3.01±0.02d	2.97±0.17d	2.94±0.07d	3.30±0.05b	3.12±0.04c	3.40±0.13a
40	3.37±0.02a	2.97±0.17c	2.64±0.07c	3.30±0.05a	3.12±0.04b	3.40±0.13a
Kidneys						
1	0.57±0.03	0.58±0.10	0.66±0.13	0.58±0.08	0.47±0.08	0.49±0.14
10	0.54±0.09	0.48±0.11	0.48±0.09	0.59±0.11	0.42±0.01	0.56±0.10
20	0.57±0.01b	0.43±0.01c	0.51±0.04c	0.67±0.08a	0.61±0.09a	0.71±0.06a
28	0.37±0.11c	0.45±0.09c	0.56±0.07b	0.59±0.09b	0.75±0.12a	0.84±0.09a
31	0.43±0.02c	0.41±0.01c	0.53±0.11b	0.56±0.01b	0.51±0.04b	0.76±0.07a
42	0.48±0.14	0.59±0.05	0.64±0.12	0.58±0.07	0.62±0.12	0.51±0.04
Spleens						
1	0.02±0.01	0.02±0.01	0.04±0.01	0.02±0.02	0.04±0.01	0.03±0.01
10	0.02±0.01b	0.06±0.02a	0.02±0.01b	0.06±0.02a	0.02±0.01b	0.02±0.01b
20	0.01±0.02b	0.09±0.03a	0.04±0.02b	0.11±0.03a	0.02±0.01b	0.03±0.01b
28	0.06±0.01b	0.11±0.03a	0.04±0.01b	0.14±0.05a	0.03±0.01b	0.09±0.04a
31	0.05±0.01b	0.14±0.04a	0.07±0.01b	0.14±0.03a	0.05±0.01b	0.18±0.04a
42	0.06±0.01	0.07±0.02	0.10±0.03	0.18±0.03	0.09±0.03	0.14±0.04
Bursa of Fabricius						
1	0.09±0.02	0.09±0.010	0.08±0.03	0.10±0.03	0.09±0.02	0.07±0.02
10	0.07±0.02	0.05±0.01	0.07±0.03	0.06±0.03	0.10±0.02	0.09±0.03
20	0.12±0.01a	0.05±0.01b	0.05±0.01b	0.06±0.02b	0.06±0.01b	0.11±0.02a
28	0.06±0.01	0.05±0.01	0.08±0.03	0.07±0.02	0.06±0.02	0.05±0.02
31	0.09±0.03	0.05±0.01	0.08±0.04	0.07±0.04	0.08±0.02	0.12±0.05
42	0.05±0.03	0.04±0.02	0.06±0.02	0.05±0.03	0.05±0.02	0.05±0.02

Values (mean+SD) with different letters in a row differ significantly (P≤0.05).

Table 4: Gross lesions score in different organs of broiler chicks given different levels of dietary aflatoxin B1 from 14 days to 42 days of age

Lesions		Groups (aflatoxin B1 µg /kg feed)					
		A (0)	B (50)	C (100)	D (200)	E (400)	F (800)
Pallor Liver		-	-	2c	6bc	14ab	16a
Friable liver		-	-	-	-	-	15
Hemorrhages	Subcutis	-	-	-	-	-	12
	Muscular	-	-	-	-	10b	21a
	Hepatic	-	-	26b	34ab	32ab	43a
	Kidneys	-	-	10c	28b	31ab	38a
	Enteric	-	-	-	-	16	22
	Heart	-	-	-	-	-	3
Enlargement of organs	Liver	-	-	37c	42bc	52b	72a
	Kidneys	-	-	18c	28bc	36ab	40a
	Heart	-	-	-	-	-	20
Cumulative Score for each group		-	-	93d	138c	19b	287a

Values with different letters in each row differ significantly (P≤0.05). The maximum possible for each organ was 96 and cumulative score 1056.

The score for hemorrhages on muscles was significantly higher in group F as compared with E. A score of hemorrhages on the liver were significantly higher in group F as compared with group A. The score for hemorrhages on kidneys were highest in group F and significantly different from groups D and C. Group C was also significantly lower from group B. Liver enlargement attained a maximum score in group F (800µg/kg), followed by E, D and C. Group F was significantly different from E which in term was significantly higher than group C. Enlargement of kidneys had highest score in Group F and was significantly different from groups D and C. The cumulative score for gross lesions were significantly different between the groups C, D, E and F, being highest in F followed by E, D and C. These results are in line with (Ortatatli *et al*., 2005; Shivachandra *et al*., 2003). Enlarged livers due to aflatoxicosis have also been reported in other birds like Japanese quails (Parlat *et al*., 1999).

Conclusions: Aflatoxin B1 at level of 50µg/kg feed did not show any behavioral alterations, gross and pathological effect in broilers when fed continuously for 28 days. In other groups pathological changes were seen in a dose related manner. Severe changes were seen in higher dose levels.

Author's contribution: MKS and MZK conceived the idea and tailored the project. ZH executed the project, laboratory analysis and manuscript preparation. AK and SR contributed in data analysis, interpretation of research findings and manuscript preparation. All authors approved the final version of manuscript.

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