Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2018.028

RESEARCH ARTICLE

Hematological and Serum Biochemical Effects of Aflatoxin B1 Intoxication in Broilers Experimentally Infected with Fowl Adenovirus-4 (FAdV-4)

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ARTICLE HISTORY (17-423)

Received: December 29, 2017
Revised: January 12, 2018
Accepted: January 14, 2018
Published online: January 16, 2018

Key words:
Adenovirus
Aflatoxins
Hematology/Blood
Kidney
Liver
Serum chemistry

ABSTRACT

Mycotoxins contamination of poultry feeds is a global issue faced by the poultry industry due to increase demand and provision of poor quality cereal grains. The current experimental study was designed to investigate concurrent administration of aflatoxins and fowl adenovirus in commercial broilers. Fowl adenovirus-4 is re-emerging in Pakistan including its horizontal and vertical transmission. A total of 120 one day old birds were divided into six equal groups (A-F). Groups A was kept as control, B and C were fed AFB1 100 and 200 ppb respectively, whereas group D, E and F were administered FAdV-4, AFB1 100 +FAdV-4, AFB1 200 + FAdV-4, respectively. Hemoglobin concentration, erythrocytic count and leukocytic count of all the groups significantly decreased as compared to control group. Hematocrit (%) of group C, E and F was significantly lower as compared to group A. Serum concentration of creatinine, urea, ALT, AST and GGT significantly increased in all groups as compared to control group in a dose dependent manner. It might be concluded that AFB1 intoxication in FAdV-4 infected birds potentiated the hepatic and renal damage as indicated by increased values of urinary and hepatic biomarkers.

To Cite This Article: Naseem MN, Saleemi MK, Abbas RZ, Khan A, Khatoon A, Gul ST, Imran M, Sindhu ZUD and Sultan A, 2018. Hematological and serum biochemical effects of aflatoxin B1 intoxication in broilers experimentally infected with fowl adenovirus-4 (FAdV-4). Pak Vet J, 38(2): 209-213.
http://dx.doi.org/10.29261/pakvetj/2018.028

INTRODUCTION

Mycotoxins are heat stable, low molecular weight secondary metabolites that are produced by several toxigenic fungi. Toxins producing fungi are present worldwide and their potential to grow and produce toxins on the different cereal crops makes them inevitable contaminants of human and animal food chain (Hassan *et al*., 2012; Saleemi *et al*., 2015). Tropical and subtropical areas having high temperature and humidity are most prone to detrimental effects of mycotoxins (Banerjee, 2010). These toxicogenic fungi proliferate mostly in damaged grains under anaerobic conditions considered ideal for mycotoxins production (Saleemi *et al*., 2017). The most important mycotoxins that have toxic and immunosuppressive effects mainly include aflatoxins, ocharatoxins, trichothecenes, DON and T-2 toxins (Berek *et al*., 2001). Among these mycotoxins, the aflatoxin and ochratoxin are the most important toxins that are mainly produced by storage fungi. *Aspergillus* species especially *A. flavus* and *A. parasiticus* are mainly found to produce aflatoxins in poultry feed and its ingredients throughout the world (Saleemi *et al*., 2012; Khan *et al*., 2017). There are four types of aflatoxins; AFB1, AFB2, AFG1 and AFG2 (Tedesco *et al*., 2004). Among these, AFB1 is considered the most toxic and its detrimental effects ranges from mild digestive problems to carcinogenesis. AFB1 has been identified as group 1 carcinogenic agent by the International agency for research on cancer (IRAC). In livestock and poultry, AFB1 is considered as potent hepatotoxic and nephrotoxic agent causing immuno-suppressive, mutagenic and teratogenic effects (Hassan *et al*., 2012; Khan *et al*., 2014). In poultry industry aflatoxicosis is considered an important issue by virtue of its occurrence and toxicities (Saleemi *et al*., 2015).

In Pakistan, aflatoxin contamination of poultry feed and feed ingredients have already been reported. Presence of high levels of AFB1 in poultry feed and feed ingredients can expose birds to severe immuno-suppression and stunted growth in the birds (Hassan *et al*., 2012). Aflatoxins are known to inhibit protein synthesis and can be injurious to cells and tissues involve in protein

synthesis like liver and gut epithelium. Hemoglobin concentration and hematocrit values also decreased due to intoxication of AFB1 and T-2 toxin (Abeena *et al.*, 2015). As these toxins are hepatotoxic so they lead to increased serum enzyme concentrations.

In the field conditions most of the time high levels of mycotoxins resulted in immunosuppression that exposed birds to infectious diseases. Hydropericardium syndrome (HPS) and Inclusion body hepatitis (IBH) are the most important infections caused by the fowl adenovirus in broiler birds. The causative agent of HPS and IBH belonged to serotype 4 of the fowl adenovirus. As the disease name indicates hepatitis and hydro-pericardium are the major pathological findings of FAdV-4. Besides these two pathological conditions, anemia, hemorrhagic disorder, pulmonary edema, nephritis and high mortality was also observed in FAdV-4 infected flock. This virus mostly effect fast growing birds at the 2-3 weeks of age. This virus was found to hinder the development of lymphoid organ in the birds. The birds infected with FAdV-4 showed nephritis, pancytopenia in bone marrow, characteristics intra-nuclear inclusion bodies in liver cells (Sandhu *et al*., 1995). Adenovirus infected birds showed severe anemia, decreased hematocrit value and erythrocytic count but increased in total Leukocytic count (Sandhu *et al.*, 1998).

It was observed that for development of clinical disease of FAdV-4, synchronous immunosuppression is required. So, immunosuppression due to mycotoxicosis, CIA and IBD were found to increase pathogenicity of adenovirus infection (Hafez, 2011). In Pakistan first outbreak of FAdV-4 was identified in 1987 at Angara Goth, Sindh Province (Anjum, 1990). Vertical transmission of FAdV is also reported in Pakistan (Rehman *et al*., 2011). Keeping in view this important unattended problem of the poultry industry, this study was planned to investigate the pathological effects of aflatoxin B1 intoxication in broiler birds infected with fowl adenovirus (FAdV-4). Most probably no information is available in accessible literature on the hematological and biochemical changes induced by concurrent administration of AFB1 and FAdV-4 infection.

Nowadays, high mortalities are reported showing lesions of both IBH/HPS and aflatoxins, such disease conditions remain undiagnosed or misdiagnosed. Keeping in view current situation of reemergence of FAdVs, this experimental study was designed to explore the hematological and biochemical changes occurred in AFB1 intoxication and FAdV-4 infection alone and in combination.

MATERIALS AND METHODS

Experimental design: A total of 120 day-old broiler chicks (Hubbard Classic) were purchased from local hatchery. These birds were kept under standard managemantal conditions. After acclimatization period of 2 days, these birds were divided into 6 groups (A-F) randomly, having 20 birds in each. Each group was further divided into 4 replicates having 5 birds in each. Group A was kept as control and offered normal commercial feed, while group B and C were offered feed intoxicated with AFB1 @ 100 and 200 ppb respectively. Birds in group D were fed with normal feed and infected with FAdV-4 virus. Group E and F were fed with AFB1 intoxicated feed @ 100 and 200 ppb respectively, along with FAdV-4 infection. Birds were vaccinated according to vaccination schedule recommended by national disease control committee of poultry diseases of Pakistan Poultry Association.

Preparation of AFB1 intoxicated feed: Lyophilized spores of *A. flavus*, obtained from University De Valencia Spain (link: Fries, A. NRRL 6540 and CECT 2687) were used for aflatoxin production. Aflatoxin was produced by inoculating this *A. flavus* culture on rice. The quantification total aflatoxin and AFB1 was done by using commercially available ELISA kit (MaxSignal® Aflatoxin B1 Elisa test Kit 1055-04, Bio Scientific Corp. TX 78744 USA). The level of AFB1 was estimated as 640 ppb.

Experimental infection of avian adenovirus: Previously isolated FAdV-4 was used for experimental infection of birds at day 16 of the experiment. Groups D, E & F were given infection with infective dose of FAdV-4. These birds were given subcutaneous injection of viral inoculum @ 0.3 ml/per bird. Each inoculum having a titer of $10^{5.6}$ $TCID^{50}$ per 0.3 ml of inoculum.

Parameter studied

Hematology: At 21st and 35th day of experiment, blood samples were collected with and without EDTA from each group. Serum was harvested from the coagulated blood. EDTA added blood was subjected to immediate analysis for hematology. Total erythrocytic and leukocytic count was made by using haemocytometer. Hematocrit % was determined by using micro-hematocrit method. Hb-concentration was measured by using method as previously described (Hussain *et al.*, 2017).

Serum biochemistry: Serum samples collected at 21st and 35th day of experiment were subjected to biochemistry analysis by using commercially available kits. These parameters included serum enzyme concentration by measuring alanine aminotransferase (ALT: Merck-France, Cat# 5.17531.0001), aspartate aminotransferase (AST: Merck-France, Cat# 5.17521.0001) and gamma glutamyl transferase (GGT: Merck-France, Cat# 5.17527.0001). Serum protein concentration was also measured including total protein (Merck-France, Cat# 5.17630.0001), serum albumin (Merck-France, Cat# 5. 17620.0001) and serum globulin. Serum urea (Merck-France, Cat# 5.17611.0001) and creatinine (Merck-France, Cat# 5.17551.0001) level were also measured.

Statistical analysis: The experimental data were collected and analyzed using ANOVA and the means of different groups were compared through Duncan's multiple range tests by using M-STATC statistical software (Michigan State University, East Lansing, MI). The level of significance was $P<0.05$.

RESULTS

Hematological analysis: At 21st day of experiment, erythrocytic count, leukocytic count and Hb-concentration of all the groups were significantly lower from control

group, whereas erythrocytic count, leukocytic count and Hb-concentration of group F was also significantly lower as compared to other groups (Table 1). Hematocrit (%) of group C, E and F was significantly lower as compared to control group. The mean corpuscular volume (MCV) of group E and F was significantly higher as compared to control group. Mean corpuscular hemoglobin (MCH) of group F was significantly lower as compare to control group. But MCH of other groups was non-significantly different from control group. Mean corpuscular hemoglobin concentration (MCHC) of group E and F was significantly lower as compare to group A. MCHC of group B, C and D was non significantly different from control group.

At 35th day of experiment, erythrocytic count of group F was significantly lower as compare to control group, whereas erythrocytic count of all the groups was non-significantly different from control group (Table 2). Leukocytic count and hematocrit % of all the groups except B was significantly lower as compared to control group. Hemoglobin concentration of groups that were given AFB1 alone and in combination with FAdV-4 was significantly lower as compare to control group. MCV of group C, E and F was significantly lower as compare to control group. Mean corpuscular hemoglobin (MCH) of all the groups except D, was non-significantly different as compare to control group. Mean corpuscular hemoglobin concentration (MCHC) of all groups were non-significantly different from the control group.

Serum biochemistry: At 21st day of experiment, serum alanine aminotransferase (ALT) level of all groups was significantly higher as compared to control group (Table 3). Serum aspartate aminotransferase (AST) level of C, D, E and F was significantly higher as compared to control group. Serum gamma glutamyl transferase (GGT) level of group F was significantly higher as compared to control group, whereas serum gamma glutamyl transferase (GGT) level of group C, D and E was non-significantly different from control group. At 35th day of experiment, ALT level of group B, C, E and F was significantly higher as compared to control group. AST level of all groups was significantly higher as compared to control group. GGT level of group C, E and F was significantly higher as compared to control group A, whereas serum GGT level of group B and D was non significantly different from control group. The concentration of hepatic markers including ALT, AST and GGT was significantly higher in all the groups as compared to control group. In combined groups these values were higher indicating hepatic damage.

At 21st day of experiment, total serum protein and albumin concentration (Table 4) of group B, C and E was significantly lower as compared to control group, whereas group D and F were non-significantly different from control group. Serum globulin level of all the groups was non-significantly different from control group. At 35th day of experiment, total protein concentration and serum albumin concentration of all the groups was non-significantly different from control group. Serum globulin level of group F was significantly lower as compared to control group, whereas globulin level of other groups was non-significantly different from control group.

Table 1: Hematological values of birds fed with various levels of AFB1 and experimentally infected with FAdV-4 at 21st day of experiment (Mean±SD)

Groups	Erythrocytes 10^6/µL	Leukocytes 10^3/µL	Hb (g/dL)	Hematocrit %	MCV (fL)	MCH (pg)	MCHC (g/dL)
A	3.66±0.28a	25.80±1.59a	14.50±0.71a	31.00±4.24a	84.38±5.23b	39.60±1.05a	47.06±4.16a
B	3.00±0.11b	19.75±1.63b	11.50 ±0.71b	26.50±2.12ab	88.26±3.74b	38.31±0.91ab	43.43±0.81ab
C	2.47±0.21c	17.60±0.71bc	9.50±0.71c	19.50±3.54c	78.46±7.78b	38.39±0.32ab	49.20±5.29a
D	3.10±0.11b	19.18±0.78b	12.00±0.00b	25.50±0.71abc	82.27±0.72b	38.73±1.41ab	47.07±1.31a
E	3.10±0.11cd	14.39±0.58cd	8.50±0.71c	23.00±1.41bc	108.27±8.08a	39.95±2.11a	36.93±0.80bc
F	1.93±0.06d	11.71±2.67d	7.00±0.00d	20.00±1.41c	103.29±3.91a	36.19±1.19b	35.08±2.48c

Values in each column followed different small letters are statistically different P≤0.05. (Group A=Normal Feed, Group B=AFB1 100µg/kg of feed, Group C=AFB1 200 µg /kg of feed, Group D=Normal Feed + FAdV-4, Group E=AFB1 100 µg/Kg of feed + FAdV-4, Group F=AFB1 200 µg/Kg of feed + FAdV-4).

Table 2: Hematological values of birds fed with various levels of AFB1 and experimentally infected with FAdV-4 at 35th day of experiment (Mean±SD)

Groups	Erythrocytes 10^6/µL	Leukocytes 10^3/µL	Hb (g/dL)	Hematocrit %	MCV (fL)	MCH (pg)	MCHC (g/dL)
A	4.06±0.17a	27.52±1.58a	15.76±0.60a	33.00±1.41a	81.27±0.09a	38.83±0.14b	47.77±0.23b
B	3.58±0.09a	25.29±0.88a	12.77±0.37bc	26.50±2.12ab	73.86±4.02ab	35.62±0.12b	48.29±2.47ab
C	3.10±0.18ab	20.01±1.44b	10.70±0.48cd	22.00±1.41bc	70.83±0.52b	34.47±0.41b	48.66±0.94ab
D	3.07±0.98ab	20.71±2.67b	14.81±1.52ab	24.50±7.78bc	79.81±0.03a	49.99±10.94a	62.64±13.68a
E	3.41±0.52ab	19.21±1.12b	12.57±1.48c	24.00±1.41bc	70.89±6.73b	36.96±1.32b	52.28±3.11ab
F	2.29±0.27b	18.21±1.57b	8.86±0.57d	16.50±2.12c	72.01±0.81b	38.81±2.08b	53.92±3.50ab

Values in each column followed different small letters are statistically different P≤0.05. (Group A=Normal Feed, Group B=AFB1 100µg/kg of feed, Group C=AFB1 200 µg /kg of feed, Group D=Normal Feed + FAdV-4, Group E=AFB1 100 µg/Kg of feed + FAdV-4, Group F=AFB1 200 µg/Kg of feed + FAdV-4).

Table 3: Serum enzymes concentration of birds fed with various level of AFB1 and experimentally infected with FAdV-4 (Mean±SD)

Day of experiment	At 21st day of Experiment			At 35th day of Experiment		
Group	ALT (IU/L)	AST (IU/L)	GGT (IU/L)	ALT (IU/L)	AST (IU/L)	GGT (IU/L)
A	10.33±1.15e	122.66±7.51d	0.49±0.08b	6.333±0.58d	135.000±4.36d	0.418±0.17c
B	19.00±3.00d	137.66±3.06d	0.63±0.45b	23.667±5.03c	182.333±11.15c	0.981±0.08bc
C	34.66±2.52b	173.66±26.31c	0.83±0.15ab	33.667±10.07b	217.000±14.73abc	1.767±0.27a
D	17.33±3.06d	170.66±5.03c	0.89±0.28ab	12.000±1.00d	213.667±42.57bc	0.948±0.35bc
E	28.00±6.00c	205.00±25.00b	0.79±0.10ab	32.333±3.51b	239.333±30.55ab	1.337±0.66ab
F	42.00±4.36a	243.33±17.56a	1.18±0.43a	51.667±2.52a	258.667±17.16a	1.274±0.32ab

Values in each column followed different small letters are statistically different P≤0.05. (Group A=Normal Feed, Group B=AFB1 100µg/kg of feed, Group C=AFB1 200 µg /kg of feed, Group D=Normal Feed + FAdV-4, Group E=AFB1 100 µg/Kg of feed + FAdV-4, Group F=AFB1 200 µg/Kg of feed + FAdV-4).

Table 4: Serum proteins concentration of birds fed with various level of AFB1 and experimentally infected with FAdV-4 (Mean±SD)

Day of experiment	At 21st day of Experiment			At 35th day of Experiment		
Group	Total Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Total Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)
A	2.71±0.51ab	1.96±0.32a	0.75±0.76a	2.823±0.57a	2.087±0.39a	0.736±0.19a
B	1.74±0.44bcd	1.08±0.12b	0.66±0.35a	2.496±0.18a	1.938±0.08a	0.558±0.26ab
C	1.12±0.39d	0.68±0.24c	0.43±0.15a	2.634±0.06a	1.754±0.20a	0.880±0.16a
D	3.17±1.16a	1.74±0.08a	1.43±1.09a	2.342±0.27a	1.771±0.11a	0.570±0.29ab
E	1.62±0.16cd	1.12±0.17b	0.49±0.06a	2.301±0.33a	1.651±0.30a	0.650±0.22ab
F	2.50±0.13abc	1.76±0.21a	0.74±0.10a	2.282±0.54a	1.986±0.57a	0.296±0.13b

Values in each column followed different small letters are statistically different P≤0.05.

Table 5: Serum urea and creatinine concentrations of birds fed with various level of AFB1 and experimentally infected with FAdV-4 (Mean±SD)

Day of experiment	At 21st day of Experiment		At 35th day of Experiment	
Group	Urea (mg/dl)	Creatinine (mg/dl)	Urea (mg/dl)	Creatinine (mg/dl)
A	7.000±1.00c	2.203±0.16c	4.667±3.06c	1.563±0.11c
B	15.000±1.73b	2.582±0.32bc	20.333±1.15b	2.710±0.07ab
C	17.667±3.51b	3.148±0.07ab	21.667±3.51b	2.470±0.77b
D	14.000±1.00b	2.043±0.23c	16.667±2.08b	2.319±0.22b
E	16.333±2.08b	2.606±0.16bc	35.000±8.00a	2.609±0.39b
F	26.667±1.53a	3.679±0.99a	38.333±6.51a	3.312±0.24a

Values in each column followed different small letters are statistically different P≤0.05.

The serum urea and creatinine concentrations (Table 5) at 21st day of experiment showed that urea level of all groups was significantly higher as compared to control group. Serum creatinine value of group F was significantly higher as compared to group A, whereas other groups was non significantly different from control group. At 35th day of experiment, serum urea concentration of all the groups was significantly higher in comparison with control group. Serum creatinine value of all the groups was also significantly higher as compared to control group.

DISCUSSION

Mycotoxins are known to cause severe immunosuppression that may expose the birds to different infectious diseases. Among these, Hydro-pericardium syndrome (HPS) is one of the important diseases caused by FAdV-4 that can occur in mycotoxin intoxicated flock. Mycotoxins especially aflatoxins are toxic to liver, kidney and also known to cause severe anemia. Anemia in birds is indicated by pale liver and lower values of hemato-logical parameters including hematocrit and hemoglobin.

In current study, the hematological parameters including erythrocytic and leukocytic count, hematocrit% and hemoglobin concentration were significantly decreased in birds fed higher doses of AFB1 as compared to control group A. A significant decrease in red blood cell counts, Hb concentration and hematocrit % with aflatoxicosis could be due to toxic effects of aflatoxins on hemopoietic organs (Sakhare *et al*., 2007). Hussain *et al*. (2016) and Khan *et al*. (2017) also observed lower hematological values in birds fed with moldy feed containing aflatoxin. Difference among erythrocyte indexes including MCV, MCHC and MCH in all groups was non-significantly different from group A. In opposite to our findings, Kubena *et al*. (1993), Valchev *et al*. (2014) observed reduction of RBC indices that could occur due to inhibition of protein synthesis. The difference in our findings might be due to low dosage of AFB1 used in our experiment as compared to them. Same anemic changes were also observed by Sadhu *et al*. (1998) in ochratoxin intoxicated birds experimentally infected with FAdV-4.

In present study, serum enzyme concentration that included ALT, AST and GGT was examined at 21st and 35th day of experiment indicating liver damage. Serum enzyme activity was increased in groups fed with AFB1 @ 200 ppb alone and in combination with FAdV-4. The experimental findings of Zaho *et al*. (2010) and Khan *et al*. (2017) were in line with our results. Serum biomarker concentration index represents the activity if vital organs (Hassan *et al*., 2012). The increased level of ALT and GGT is related with hepatocytes damage which is main target organ of AFB1 and FAdV-4.

In present study, serum total proteins, albumen and globulin concentration was significantly lower in groups offered with higher doses of AFB1 alone and in combination with FAdV-4 as compared to control group. The lessen concentration of protein in serum might be due to the failure of mRNA transcription or amino acid transfer that leads to inhibition of the protein synthesis (Kubena *et al*., 1993). The findings of Gowda *et al*. (2008) and Zhao *et al*. (2010) were same with our findings. These lower values of protein indicated the hypoproteinemia. Groups that were fed with AFB1 @ 200 ppb of feed alone and in combination with FAdV-4 had high concentration of urea and creatinine as compared to other groups indicating severe kidney damage. The increase in serum urea and creatinine concentration might be due to severe injury to renal tubules (Abdu *et al*., 2011). Similar findings was also observed by Jindal *et al*. (1994) and Bhatti *et al*. (2016).

Conclusions: These hematobiochemical findings indicate that AFB1 caused severe kidney and liver damage in broiler birds infected with FAdV-4. The morbidity and severity of HPS/IBH increased as concentration of AFB1 increased in feed.

Authors contribution: MNS, MKS and AK contributed towards research planning, data analysis and manuscript writing, MNS, MI, STG, RZA, AK and MKS remained involved in execution of experiment and laboratory analysis and data interpretation. MNS, MKS, AS and ZDS participated in manuscript preparation and statistical analysis.

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