

Aflatoxin B1 Residues in Eggs and Flesh of Laying Hens Fed Aflatoxin B1 Contaminated Diet

Saqer Mohammad Herzallah

Department of Nutrition and Food Science, Faculty of Agriculture, Mu'tah University, Karak, Jordan

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ABSTRACT

Aflatoxin B1 (AFB1) and total Aflatoxins (AFT) contaminated feed effect on aflatoxins residue level in eggs, muscles (breast, leg), organs (liver, kidney, gizzard) and excreted aflatoxins in chicken litter of layer hens were monitored. Laying hens were on four levels of aflatoxins for 6 weeks and monitored weekly for the change in both AFB1 and AFT levels. Pronouncedly, the AFB1 and AFT were detected in eggs, muscles (legs, breast), organs (liver, kidney and gizzard) and litter in noticeable amounts. Total Aflatoxin (AFT) level was lowest in chicken breast (0.63 ppb) and highest in liver (2.12 ppb) and gizzard (1.22 ppb) of chicken fed diet with 965.12 ppb. Whereas, AFB1 residue was 0.66 ppb in eggs, 1.59 ppb in liver tissues of hens given feed contaminated with 894.12 ppb. Residue level of AFB1 was high in liver and kidney of all treatments. The chicken breast tissues were lowest in AFB1 and AFT of values 0.72 and 0.63, respectively. Eggs production was significantly ($p < 0.05$) affected with AFB1 contaminated feed and egg production was decreased by more than 30%.

Keywords: Aflatoxins, AFT, AFB1, Dose/Residue, Layers

1. INTRODUCTION

Mycotoxins are group of toxic compounds detected in 1960s (Asao *et al.*, 1965) known as bisfuranocoumarines compounds found in grains contaminated with *Aspergillus flavus* and *Aspergillus parasiticus*, with aflatoxin B1 (AFB1) as the most potent toxins of teratogenic, mutagenic and carcinogenic effect (Mishra and Das, 2003; IARC, 2002; Williams *et al.*, 2009; Manning *et al.*, 2005; Bintvihok *et al.*, 2003; Khan *et al.*, 2010; Arana *et al.*, 2011). Animals are considered the most group exposed to high concentration of aflatoxins through feedstuffs that develop several health problems which lead to large economical losses. These losses are pronouncing in meat and eggs in terms of quality and quantity as a result of contamination with aflatoxins residues (Bintvihok *et al.*, 2002; Farombi, 2006; Hall and Wild, 2003). Feeds of cereal grain origin demonstrate the most susceptible commodities along with nuts for contamination with AFG1, AFG2 and AFB2 in addition to AFB1 as the main toxins contaminants. Grain

contamination with aflatoxins recognized as a threat to human and animals through consumption of contaminated foods and feeds and considered by FDA as an avoidable contaminant (Kim *et al.*, 2000). Food and Agricultural Organization (FAO) estimated the contamination with the mycotoxins by 25% of the produced world's crops (Fink-Gremmels, 1999). Therefore, the food chain of animal origin are considered the most vulnerable and the most affected by the presence of aflatoxins which varies with age and species of the animals (Williams *et al.*, 2009; Manning *et al.*, 2005). The level of AFB1 adopted in most of the world countries is 5 ppb regulatory as a level of human contamination risk (Yosef *et al.*, 2003). Marine animals also could be exposed to AFB1 contamination through feed chain and thus exerts threat to human when the concentration were higher than the permitted levels (Farabi *et al.*, 2006). Epidemiological research proves the effect of AFB1 as a causative agent for liver cancer and human health hazards threats associated with food chain contamination with AFB and their metabolites

such as M1 in milk are of food safety authorities to avoid entrance of the contaminated commodities into suitability for consumption and to avoid AFB1 contaminated food that pose threat and hazards to human through food consumption. The objective of this study was to determine the residual effect of AFB1 and Total aflatoxins in poultry flesh and eggs.

2. MATERIALS AND METHODS

Sixty of 23-week-old layers chicken (Hubbard) were used and fed *ad libitum* with a standard layer diet for 2-week-period, during which daily egg production were recorded. The birds were divided into 4 groups each of 15 birds fed with standard layer and broiler diet (Table 1) obtained from a commercial feed company was used as a basal feed and with artificially challenged with 0, 190.02, 395.31 and 894.12 ppb AFT B1. The eggs from all groups were collected, recorded daily and the AFB1 residues were determined weekly as well. The residual of the AFB1 in flesh of the layer chickens used in the experiment were determined at the end of 5 week of production.

2.1. Chemical and Reagent

Anhydrous sodium sulphate $\geq 99\%$, diatomaceous earth, NaCl (99%), aflatoxins Kit standard of AFB1, AFB2, AFG1 and AFG2 of 98% purity, Methanol of 99.5% and Hexane were purchased from Sigma-Aldrich (St Louis, MO, USA). Acetone (BDH chemicals, Ltd, Liverpool, UK) SPE-CN was purchased from Varian (Palo Alto, California, USA) and aflatoxin Immunoaffinity Columns (IAC) purchased from r-biopharm (R-Biopharm, Darmstadt, Germany).

2.2. Test Protocol

Aflatoxins of a finally ground feed samples (2-g) was extracted by mixing with 10-mL of 70:30 (methanol: distilled water) for 30 min at room temperature (20-25°C) using a shaker at 2000 rpm (IKA, Mount Holly, NJ, USA). The mixture was filtered through Whatman no. 1 filter paper (Whatman, Hamburg, Germany) and a 100- μ L of the eluate diluted 1: 6 with sample dilution buffer (Phosphate buffer solution, pH 7.2). The aflatoxin was determined using r-biopharm kit. Poultry muscles, eggs and organs (liver, kidney, gizzard) were extracted by a method used by Herzallah (2009), in brief, 50-g of poultry muscles, eggs and organ representative homogenized samples were blended at high speed blender (balck and Dickers, UK) with 100 mL of water and acetone (1:1) mixture for 10 min, then

diatomaceous earth was added to the mixture, stirred gently for 5 min, filtered through fast filtering paper and 10-mL of the filtrate was mixed with 5% NaCl and Hexane (1:1). The mixture was shaken at 1200 rpm for 20 min using a mechanical shaker (IKA, Hamburg, Germany), the hexane layer was removed out of the solution with care and discarded. The AFB1 was extracted with chloroform (3 \times 50 mL); the chloroform layer collected, dried over anhydrous sodium sulphate and evaporated using rotary evaporator. The residues were redissolved in 1 mL chloroform and cleaned over SPE-CN. The eluted aflatoxins were evaporated to dryness under a stream of N₂, redissolved in methanol and analyzed by HPLC.

2.3. HPLC Determination

HPLC (Waters Co., MA, USA) equipped with 1525 binary HPLC pump, column oven 5CH model and fluorescence detector (Model FL 2475) at wavelength 365 and 425 nm for excitation and emission, respectively, was used in AFB1 analysis.

Table 1. Feed composition of experimental layer diet used in the study^{1,2,3}

Ingredients	%
Corn	60.50
Soybean meal	21.50
Vegetable oil	3.00
Limestone	8.66
DCP	0.49
Salt	0.20
DL-Methionine	0.11
L-Lysine	0.94
Cocciostat	0.00
Vitamin premix	0.25
Mineral premix	0.10
Choline chloride	0.10
Antioxidant	0.10
Antifungal	0.10
Analysis: Metabolizable energy (kcal/kg of dry matter)	2907.48
CP (%)	18.00
NPP (%)	0.43
Ca (%)	3.80
Na (%)	0.18

¹Each kg of vitamin premix contains 2.4 \times 10⁶ IU vitamin A; 3.2 \times 10⁵ vitamin D3; 5.6 \times 10³ mg vitamin E, 640 mg vitamin K3; 500 mg vitamin B1; 1120 mg vitamin B2, 3200 mg niacin; 1600 mg Ca-D-pantothenate; 800 vitamin C, 2.4 mg vitamin B12, 160 mg folic acid; 7.2 mg D-biotin; 8000 mg vitamin C; 20000 mg choline chloride; ²Each kg of mineral premix contains 8 \times 10⁴ mg manganese; 6 \times 10⁴ mg zinc; 200 mg cobalt; 100 mg iodine; 150 mg selenium; ³DCP, dicalcium phosphate; CP, crude protein; NPP, non phytate phosphorous

Thermo LC-Si column (250×4.6 mm id) kept in column oven at 40°C and the flow rate of isocratic mobile phase composed of toluene, ethyl acetate, formic acid and methanol (90:5:2.5:2.5, v/v/v/v) was set at 2.0 mL min⁻¹.

2.4. Recovery of Aflatoxins

The standard curve for AFB1 was linear with correlation coefficient of 0.999 and the Mean coefficient of Variation was 1.32% for AFB1 with a Minimum Detection Limit (MDL) of 0.05 ppb.

2.5. Statistical Analysis

Results were analyzed for statistical significance (p<0.05) by analysis of variance using GLM and Duncan's Multiple Range of SAS version 9.0 software (SAS, 2007) to reduce the data. The significance was evaluated through mean values that showed significant differences in the Least Significant Difference (LSD) procedure if p<0.05.

3. RESULTS

Eggs produced by layer hens fed AFB1 contaminated feed with 894.12 ppb found to contain higher level of AFB1 (0.66 ppb) than eggs and/or flesh from hens fed a diet of 190.02 ppb AFB1 concentration that cause a residual effect of 0.33 ppb as shown in **Table 2**. The muscle and organs of layer hens after 7 weeks of production and fed diet contaminated with AFB1 and Total AF were found to contain higher levels of AFT and AFB1 with an increase in the levels feed aflatoxin concentration, of feed contaminated with 894.12 or 965.61 ppb and 190.02 or 192.61 ppb for AFB1 and AFT, respectively (**Table 2 and 3**).

Liver of layer hens was higher in residue level of AFT (2.12 and <0.51) with significant (p<0.05) difference. The results found in this study for kidney, gizzard, leg (drumstick and thigh), liver, feed and litter are presented in **Table 2 and 3**. The egg production by layer hens was decrease as the level of AFB1 or AFT in contaminated feed increased by ≥30% as shown in the data presented in **Table 4**.

Table 2. Aflatoxin B1 content of Eggs from hens fed aflatoxin contaminated feeds^{1,2,3}

Sample Description	Treatment		
	T1	T2	T3
	B1 (ppb)		
Feed	894.12 ^a ±5.12	395.31 ^a ±3.05	190.02 ^a ±3.16
Eggs	0.66 ^d ±0.11	0.43 ^c ±0.21	0.33 ^d ±0.12
Breast	0.72 ^d ±0.19	0.52 ^c ±0.17	0.33 ^d ±0.08
Liver	1.59 ^c ±0.14	0.86 ^c ±0.13	0.46 ^{cd} ±0.07
Kidney	0.88 ^d ±0.13	0.59 ^c ±0.12	0.44 ^{cd} ±0.11
Legs + Thigh	0.78 ^d ±0.12	0.45 ^c ±0.11	0.30 ^d ±0.12
Gizzard	1.22 ^c ±0.16	0.84 ^c ±0.12	0.76 ^c ±0.12
Litter	26.06 ^b ±0.17	16.30 ^b ±2.01	6.50 ^b ±1.04

¹values are means of three readings ± SD. ²control samples less than <0.05 the MDL; ³values of different superscript letter within column are significantly differ at p<0.05

Table 3. Residual effect of mycotoxin-contaminated feed on eggs and poultry flesh total aflatoxins (AFT)

Sample	Treatment ^{1,2}		
	T1	T2	T3
	AFT (ppb)		
Feed	965.12 ^a ±3.11	467.27 ^a ±4.01	192.61 ^a ±3.14
Eggs	1.54 ^{bc} ±0.25	0.71 ^b ±0.29	0.42 ^b ±0.21
Breast	0.63 ^d ±0.09	<0.05 ⁴	<0.05
Liver	2.12 ^b ±0.21	1.02 ^b ±0.12	0.51 ^b ±0.16
Kidney	1.41 ^c ±0.19	0.61 ^b ±0.10	0.47 ^b ±0.09
Legs (drumstick and thigh)	1.01 ^{cd} ±0.11	0.41 ^b ±0.02	<0.05
Gizzard	1.20 ^{cd} ±0.11	<0.05	<0.05

¹Values are mean of three readings ± SD; ²Values of different superscript letter within column are significantly differ at p<0.05; ³Total aflatoxins represent the sum values of B1, B2, G1 and G2; ⁴Control samples contain <0.05 ppb

Table 4. Eggs production of layer hens challenged with aflatoxin B1 in their ration^{1,2}

Treatment	Eggs production (%)	
	Group 1	Group 2
Control	90.0 ^a ±2.10	95.8 ^a ±2.10
Treatment 1	50.0 ^c ±1.80	65.0 ^c ±1.60
Treatment 2	70.0 ^b ±2.00	70.5 ^b ±1.80
Treatment 3	70.0 ^b ±2.30	70.7 ^b ±1.60

¹Values are mean of three readings ± SD; ²Values of different superscript letter within column are significantly differ at p<0.05

4. DISCUSSION

The production of eggs largely affected by the concentration of AFB1 in the diet as its concentration increase the residue level increased. The residue level of 1.54 and 0.66 ppb in eggs produced by layer hens fed AFT and AFB1, respectively, while, 0.71 and 0.42 ppb AFT in eggs of hens fed diet of 467.27 and 192.61 ppb, respectively (**Table 2 and 3**). The increase in residue level of AFT (compared to control group) was less than the increase in residue level made by 965.12 ppb AFT contaminated feed with no significant ($p>0.05$) difference. The results of the residual effect of AFT on the residue levels in eggs was in agreement with Hussain *et al.* (2010) who found direct relationship between AFB1 in the diet and the residue level in liver, muscle and eggs. Also, the present results were in compliance with other of detectable residue in meat and eggs from chicken fed contaminated diet reported by previous reports (Hussain *et al.*, 2010). The residual effect of feeds on the residue level in eggs for both AFB1 and AFT were obvious (**Table 2 and 3**) and the AFB1 was increased by 127%, whereas, AFT increased by 294% for treatment 1 and 13 and 7% for treatment 3(T3). The level of the residue was dose dependent and varies between treatments with significant ($p<0.05$) difference between treatment1 (T1) and treatments 2 and 3 (T1 and T3).

Egg production of laying hens fed a diet contaminated with different levels of AFB1 (894.12, 395.31, 190.02, 0) were found to be significantly ($p<0.05$) affected (**Table 4**). The reduction in egg production was $\geq 35\%$ for treatment 1 and $\geq 30\%$ for treatments 2 and 3. These results were in agreement with data reported by Hamilton and Garlich (1971) that hens receiving 1.25 to 20 ppm AFB1 in their diet decrease the egg production by 3 wk. However, AFB1 contaminated feed were found to be dose dependent in decreasing the egg production (**Table 4**). Also in agreement with reports by Denli *et al.* (2009) who found that AFB1 of 1 mg kg⁻¹ affect strongly the performance of Chicken and Iqbal *et al.* (1983) who also found that diets with AFB1 levels of 600 ppb affect egg characteristics and production by laying hens. Furthermore, the results of low egg production of layer hens could be attributed to the changes that AFB1 and AFT affect liver metabolism function that considered as a targeted organs by AFB1 (Osweiler *et al.*, 2010; Zaghini *et al.*, 2005; Bailey *et al.*, 2006; Pasha *et al.*, 2007; Miazzo *et al.*, 2005). AFB1 also, cause drop in

feed intake, decreased digestibility and liver lesions that hitherto lead to drop in egg production (Van Rensburg *et al.*, 2006; Kermanshahi *et al.*, 2007; Al-Shawabkeh *et al.*, 2009; Shen *et al.*, 2009; Kang and Lang, 2009).

Muscles of layer hens were tested for AFB1 and AFT residue level after 7 weeks production and found to follow same increasing approach of aflatoxin dose in feed of the given diet. The liver showed a significant ($p<0.05$) rise in AFT against the residue level found in breast, kidney, gizzard and leg (drumstick and thigh) of the same treatment (**Table 3**). Leg (drumstick and thigh) had lower residue level of AFT this could be explained by the removal of part of the skin that contains the subcutaneous fat and hereafter aflatoxin level reduction. On the other hand, muscles or organs of challenged chicken such as liver, gizzard, legs (drumstick and thigh) and kidney, showed maximum value of AFT. A 2.12, 1.02 and 0.51 ppb found in liver tissues of treatments 1, 2 and 3, respectively.

AFB1 residue in chicken muscles (**Table 2**) was similar in response of Dose/Residue (D/R) inclination level in eggs and poultry muscle. For example, the residue levels in layer hens liver was 1.59 ppb with insignificant ($p>0.05$) difference among treatments 1, 2 and 3. It was noticed that aflatoxin AFB1 amount in the diet determine the level of AFB1 residue level in poultry meat. The results were in agreement with the results found by Hussain *et al.* (2010) who found that the increase in the level of AFB1 in the diet increase the residue level in muscles of layer chickens and also in accordance with results reported by Zaghini *et al.* (2005) who found the increase in liver AFB1 and AFM1 with dose increase of AFB1 in feeds provided to broiler and laying hens. Metabolite disturbances and the withdrawal of aflatoxin in hens litter increases with egg production time after wk 7. The results were in agreement with the results found by Zaghini *et al.* (2005) who found that the use of mannanoligosaccharides increase adsorption of AFB1 to the polysaccharides and decrease the level in poultry muscles. AFB1 and AFT residue levels in eggs, muscles and organs of the layer hen and egg production were found to be dose dependent affected with the increase in the residue levels in eggs, animal flesh and egg production. The results found in the present study were in agreement with data reported by Denli *et al.* (2009) who found that AFB1 of 1mg/kg affect strongly the performance of chicken and Iqbal *et al.* (1983) who found also that diets with AFB1 levels of 600 ppb reduce egg characteristics and production by laying hens.

5. CONCLUSION

Reduction in egg production and egg quality were resulted from using AFB1 contaminated chicken diet in feeding laying hens. AFB1 residue in laying hens' tissues and organs of liver, kidney, breast, legs, gizzard increased with increasing feed AFB1 concentration. AFB1 and AFT could contaminate the food produced from laying hens or eggs raised on AF contaminated feed. Additionally, litter generated from chicken fed artificially contaminated AFB1 had high AF residues and the level increased with increasing AFB1 contamination diet.

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