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ORIGINAL ARTICLE

Influence of Dietary Inclusion of Zinc in Ameliorating Adverse Effects of Aflatoxin on Immunity, Pathology of Organs and Jejunal Morphometry in Broiler Chickens

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Abstract

To test the efficacy of zinc in ameliorating aflatoxicosis, day-old broiler chicks (n=240) were divided into 6 treatment groups containing 5 replicates of 8 birds each (T_1-control; T_2-T_1+250ppb AFB_1; T_3-T_1+20ppm Zn; T_4-T_1+40ppm Zn; T_5-T_2+20ppm Zn; T_6-T_2+40ppm Zn diet) and the experiment was conducted from day 1 to 42 days of age. The results showed that the CMI and HA titre values in T_2 was lower ($P<0.05$) than T_1. The CMI and HA titre values of T_5 did not vary ($P<0.05$) from T_1 and T_2. The CMI value in T_6 was higher ($P<0.05$) than T_2 and statistically similar to T_1. Grossly, swelling, enlargement and paleness with focal dark area; and histopathologically, moderate to severe degenerative changes in hepatic cells with greater disorganization in tissue marking the hepatotoxicity and proliferation of bile ducts along with periportal infiltration of heterophils and MNCs (mononuclear cells) in liver of T_2 were observed. In intestine, degenerative changes, sloughing and focal area of severe necrosis along with infiltration of inflammatory cells were seen in T_2. Mild gross and histopathological lesions were also reported in liver and intestine of T_5 and T_6. The crypt depth in T_2 was higher ($P<0.05$) than T_1. The crypt depth in T_3 to T_6 was statistically similar to T_1. The villus length/crypt depth ratio in T_1 was higher ($P<0.05$) than T_2. The villus length/crypt depth ratio in T_3 to T_6 was statistically similar to T_1. It was concluded that aflatoxin contamination at 250 ppb level in diet resulted in reduced immunity; gross and histopathological lesions in liver and intestine; increased crypt depth and decreased villus length: crypt depth ratio. Inclusion of zinc (40 mg/kg diet) partially ameliorated the adverse effect of aflatoxicosis on immunity; pathology of liver and intestine; and jejunal morphometry in broiler chickens.

Keywords: Aflatoxin, Immunity, Pathology, Jejunal morphometry, Broiler chickens.

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1. Introduction

Aflatoxins cause serious economic losses in the poultry industry because they prevent birds from achieving optimum body weight gains. Aflatoxins affect the feed consumption and body weight gain and feed efficiency of the broiler. Modern broilers are known to gain more weight by utilizing less feed in shorter time. As AFB_1 is known as hepatotoxic, it might result in more profound negative effects in birds with more efficient nutrient conversion demanding faster hepatic metabolism. Aflatoxicosis in poultry causes lowered performance in terms of reduced body weight gain, feed intake and feed efficiency (Patil *et al*., 2013; Sharma *et al*., 2014a; Silambarasan *et al*., 2013; Singh *et al*., 2015a; Singh *et al*., 2016c; Singh and Mandal, 2013; Singh *et al*., 2013a; Singh *et al*., 2013b; Khatke *et al*., 2012b; Sharma *et al*., 2015; Shamsudeen *et al*., 2013; Singh *et al*., 2011; Sharma *et al*., 2016; Singh, 2019a; Singh, 2019b; Singh, 2019e), reduced nutrient utilisation (Silambarasan *et al*., 2013), increased mortality (Silambarasan *et al*., 2015; Khatke *et al*., 2012b; Sharma *et al*., 2014; Sharma *et al*., 2015; Shamsudeen *et al*., 2013; Singh *et al*., 2011; Singh, 2019c; Singh, 2019d; Singh, 2019f), anemia (Singh *et al*., 2015a; Singh *et al*., 2016c), hepatotoxicosis and haemorrhage (Singh *et al*., 2015a; Singh *et al*., 2013a;

Singh *et al*., 2013b; Singh *et al*., 2016c), gross lesions in organs (Singh *et al*., 2015a; Singh *et al*., 2013a; Singh *et al*., 2013b; Singh *et al*., 2016c), altered relative weight of organs (Patil *et al*., 2013; Singh *et al*., 2013b; Sharma *et al*., 2015; Shamsudeen *et al*., 2014; Singh, 2019a; Singh, 2019b; Singh, 2019e), altered carcass quality traits (Shamsudeen *et al*., 2014), altered biochemistry (Patil *et al*., 2013; Singh and Mandal, 2013; Singh *et al*., 2013a; Singh *et al*., 2013b; Singh *et al*., 2011; Singh, 2019a; Singh, 2019b; Singh, 2019e; Silambarasan *et al*., 2016; Sharma *et al*., 2014b) and histopathological lesions in organs (Khatke *et al*., 2012a; Singh, 2019c; Singh, 2019d; Singh, 2019f; Silambarasan *et al*., 2016; Sharma *et al*., 2014b). It impairs humoral and cellular immune responses in poultry and increases susceptibility to environmental and infectious agents (Khatke *et al*., 2012a; Patil *et al*., 2013; Sharma *et al*., 2016; Singh, 2019c; Singh, 2019d; Singh, 2019f; Silambarasan *et al*., 2016; Sharma *et al*., 2014b) leading to severe economic losses. Mycotoxins can substantially decrease antioxidant assimilation from the feed and increase their requirement to prevent damaging effects of free radicals produced as a result of mycotoxin exposure. Zinc supplementation is helpful in aflatoxicosis because it acts as antioxidant by different mechanism like it is a cofactor of the main antioxidative enzyme Cu-Zn- super-oxide dismutase that inhibits the NADPH-dependent lipid peroxidation (Prasad and Kucuk, 2002), induces production of metallothionein that acts as a free radical scavenger (Oteiza *et al*., 1996). The objective of the present study was to evaluate the effectiveness of zinc in ameliorating adverse effects of aflatoxicosis on immunity and pathology of liver and intestine in broiler chickens.

2. Materials and Methods

2.1 Production and Analysis of Aflatoxin

Aflatoxin was produced using the fungal strain *Aspergillus flavus* NRRL 6513. To get the fresh spores, the culture was regularly subcultured on potato dextrose agar (PDA) medium slants and stored at 5°C. Aflatoxin was produced on maize substrate as per the method of Singh and Shrivastava (2012) and on liquid medium as per the method of Singh and Shamsudeen (2008). The extraction and estimation of aflatoxin was done as per Pons *et al*. (1966). Aqueous acetone was used for extraction of the toxin. Aflatoxin contents were finally quantified using UV-spectrophotometry.

2.2 Experimental Design

Experimental design was completely randomised design (CRD). There were 6 dietary treatments. Each dietary treatment consisted of 5 replicates and each replicate had 8 chicks. The experiment was conducted in broiler chickens from day-old to 6 weeks of age. The basal diet was mixed with the required quantity of mouldy maize to get the desired concentration of 250 ppb AFB_1 (Table 1).

Table 1: Experimental groups and treatment

Group	Dietary Treatment
T_1	Basal diet
T_2	Basal diet+250ppb aflatoxin B_1
T_3	Basal diet+20ppm Zinc
T_4	Basal diet+40ppm Zinc
T_5	T_2+20ppm Zinc
T_6	T_2+40ppm Zinc

2.3 Biological Experiment and Analysis

Day-old broiler chicks were obtained from experimental hatchery, ICAR-Central Avian Research Institute, Izatnagar. The chicks were wing banded, weighed individually and distributed randomly into 6 treatment groups. All the birds were reared under standard management conditions from 0-6 weeks. All birds were fed with broiler starter ration from 1-21 days and broiler finisher ration from 22-42 days. Weekly individual body weight and feed consumption of each group were recorded. The composition of broiler starter and finisher ration are presented in Table 2. The protein content as per AOAC (1995) and calcium as per Talapatra *et al*. (1940) were estimated, while the concentration of lysine, methionine, available P and metabolizable energy value were calculated. The microtitre haemagglutination procedure as described by Siegel and Gross (1980) with slight modifications was followed to measure total HA antibody titres in chickens. The cell mediated immune response to PHA-P antigen was evaluated by the method described by Corrier and DeLoach (1990). After 42nd day of age ten birds of equal sex from each dietary treatment were selected as per body weight closer to mean for carcass studies. The blood samples from each treatment group were collected. The serum was separated and stored at -20^{0}C and analyzed for various biochemical parameters. At the end of experiment, liver and intestine samples were collected and fixed in 10% formal saline. The formal saline fixed samples were cut into pieces of 2-3 mm thickness and washed thoroughly in tap water overnight before dehydrating the tissues in ascending grades of alcohol (50%, 60%, 70%, 80%, 90% absolute alcohol I and II). The dehydrated tissues were cleared in benzene and embedded in paraffin blocks. Serial -

Table 2: Ingredients and chemical composition of basal feed

Ingredient	Starter (%)	Finisher (%)
Maize	55.505	61.715
Soybean	41	35
Limestone	1	1.1
Di-calcium phosphate	1.75	1.5
Common salt	0.3	0.3
DL-methionine	0.11	0.02
TM premix*	0.1	0.1
Vitamin premix**	0.15	0.15
B complex***	0.015	0.015
Choline chloride	0.05	0.05
Chemical composition of basal feed		
Crude protein (%)	21.50	19.50
ME (kcal/kg)	2859.82	2919.78
Calcium (%)	1.04	0.99
Available phosphorus (%)	0.45	0.40
Lysine (%)	1.29	1.14
Methionine (%)	0.52	0.43

**TM premix supplied mg/kg diet: Mg, 300; Mn, 55; I, 0.4; Fe, 56; Zn, 30; Cu, 4.*
***Vitamin premix supplied per kg diet: Vit A, 8250 IU; Vit. D3, 1200 ICU; Vit. K, 1 mg.*
****B complex supplied per kg diet: Vit. B1, 2 mg; Vit. B2 4 mg; Vit. B12, 10 mcg; niacin, 60 mg; pantothenic acid, 10 mg; choline, 500 mg, Vit. E, 40 IU.*

sections of 5-micron thickness were cut and stained with hematoxyline and eosin (Culling, 1968) and examined for various histopathological changes.

2.4 Statistical Analysis

The collected data was subjected to statistical analysis using Statistical Package for Social Sciences (SPSS Version 16.0). The recorded data were subjected to one-way analysis of variance with comparison among means was made by Duncan's multiple range test with significance level of P<0.05.

3. Results and Discussion

3.1 Immune Response

Aflatoxicosis suppresses both humoral and cell mediated immunity. Immunosuppression caused by aflatoxicosis has been demonstrated in poultry as well as laboratory animals (Sharma, 1993). The data pertaining to CMI response to PHA-P measured as foot web index and humoral immune response measured as haemagglutination titre (HA) against SRBCs of broiler chickens fed different dietary treatments was statistically analyzed and presented in Table 3 while its graphical representation is given in Fig 1 and 2, respectively.

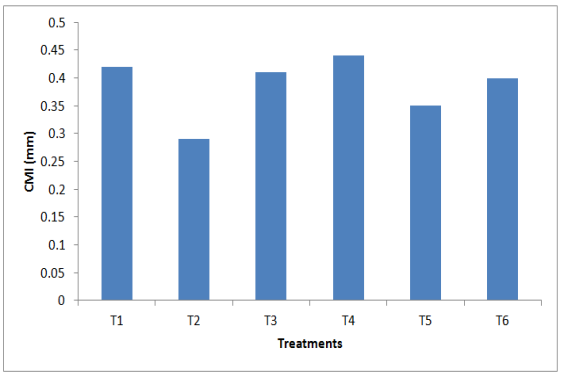

Fig 1: Cell mediated immune response to PHA-P.

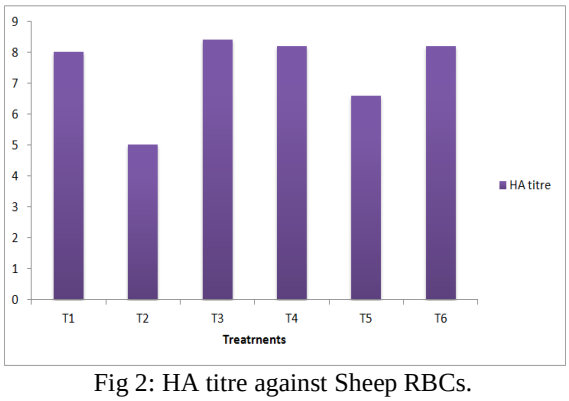

Fig 2: HA titre against Sheep RBCs.

Table 3: Cellular and humoral immunity of broilers fed different treatments

Treatment	Identification	CMI (mm)	HA Titre
T_1	Control (C)	0.42 ± 0.40^{b}	8.00 ± 0.54^{b}
T_2	C+AF 250ppm	0.29 ± 0.04^{a}	5.00 ± 0.63^{a}
T_3	C+20ppm zinc	0.41 ± 0.06^{b}	8.40 ± 0.50^{b}
T_4	C+40ppm zinc	0.44 ± 0.04^{b}	8.20 ± 0.58^{b}
T_5	T_2+20ppm zinc	0.35 ± 0.05^{ab}	6.60 ± 0.67^{ab}
T_6	T_2+40ppm zinc	0.40 ± 0.02^{b}	8.20 ± 0.86^{b}

Values bearing different superscripts in a column differ significantly (P<0.05).

3.1.1 Effect on Cell Mediated Immunity (CMI)

The CMI value in control group (T_1) was 0.42 mm which decreased (P<0.05) to 0.29 mm in the aflatoxin fed group (T_2). This revealed that inclusion of dietary aflatoxin at 250 ppb level in feed decreased (P<0.05) the CMI response to PHA-P compared to that of control. The CMI value in T_3 and T_4 was statistically similar to that of control. The CMI values of group T_5 did not vary (P<0.05) from T_1 and T_2. The CMI value in group T_6 was higher (P<0.05) than that of T_2 and statistically similar to that of control, indicating that supplementation of zinc at 40 mg/kg diet ameliorated the ill effects of aflatoxin on CMI response. The present study showed that aflatoxin contamination at 250 ppb level in diet caused significant (P<0.05) reduction in CMI response to PHA-P. This result was in agreement with other reports wherein reduction (P<0.05) in CMI response due to aflatoxicosis was reported in broiler chickens (Khatke *et al*., 2012a; Patil *et al*., 2013; Sharma *et al*., 2016; Singh, 2019c; Singh, 2019d; Singh, 2019f; Silambarasan *et al*., 2016; Sharma *et al*., 2014b; Kadian *et al*., 1988; Deo *et al*., 1998; Bakshi, 1991). Suppression of CMI response may be due to impaired lymphoblastogenesis (Chang *et al*., 1976) and impairment of lymphokine production (McLouglin *et al*., 1984). In the present study, supplementation of zinc (40 mg/kg diet) ameliorated the ill effect of aflatoxicosis on CMI response in broiler chickens.

3.1.2 Humoral Immune Response

The HA titre value in aflatoxin fed group (T_2) was lower (P<0.05) than that of control (T_1). The HA titre value of other groups did not vary (P<0.05) from that of control. In the present study, dietary aflatoxin at 250 ppb level reduced (P<0.05) the HA titre against sheep RBCs. This result was in agreement with other reports wherein reduction (P<0.05) in humoral immune response due to aflatoxicosis was reported (Khatke *et al*., 2012a; Patil *et al*., 2013; Sharma *et al*., 2016; Singh, 2019c; Singh, 2019d; Singh, 2019f; Silambarasan *et al*., 2016; Sharma *et al*., 2014b; Giambrone *et al.*, 1985; Virdi *et al*., 1989; Bakshi, 1991). Also, Verma (1994) reported decrease (P<0.05) in HA titre against SRBCs with inclusion of 0.5 and 1 ppm level of aflatoxin in feed in broiler chickens. In present study, the HA titre value of group T_5 did not vary (P<0.05) from T_1 and T_2. The HA titre value in group T_6 was higher (P<0.05) than that of T_2 and statistically similar to that of control, indicating that inclusion of zinc (40 mg/kg diet) to the aflatoxin contaminated diet ameliorated the ill effect of aflatoxicosis on humoral immune response.

3.2 Gross and Histopathology of Liver and Intestine

Liver is the primary organ for the metabolism of aflatoxin therefore the alterations were observed in the liver parenchyma as liver is main target organ in aflatoxicosis. The results of gross and histopathology of liver and intestine are illustrated in Fig 4.13 to 4.26.Grossly, the liver samples of T_1 (Fig 4.13), T_3 and T_4 were normal. The liver samples collected from T_2 (Fig 4.14) grossly showed swelling, enlargement and paleness with focal dark area on liver. Similar gross lesions were reported in several studies (Singh *et al*., 2015a; Singh *et al*., 2013a; Singh *et al*., 2013b; Singh *et al*., 2016c). Mild gross lesions were also reported in livers of groups T_5 and T_6. The architectural and cellular organization of liver parenchyma was normal in group T_1 (Fig 4.15), therefore this group was adopted as a reference standard for comparison of tissues from other groups. In group T_2 (Fig 4.16) tissues showed moderate to severe degenerative changes in hepatic cells with greater disorganization in tissue marking the hepatotoxicity. This section showed the proliferation of bile ducts along with periportal infiltration of heterophils and MNCs (mononuclear cells). The cellular organization was again normal in T_3 (Fig 4.17) and T_4 (Fig 4.18) groups. Micrograph of liver in group T_5 (Fig 4.19) showed mild changes like sinusoidal dilatation with mild changes in hepatocytes. Group T_6 (Fig 4.20) revealed only mild congestion with normal appearing hepatocytes. In the present study, diet containing 250 ppb of aflatoxin resulted in alterations in the liver tissue like degeneration, focal areas of necrosis, bile duct hyperplasia, and MNCs infiltration around portal vein in broiler chickens. Similar histopathological lesions were reported in several studies conducted by several researchers (Khatke *et al*., 2012a; Singh, 2019c; Singh, 2019d; Singh, 2019f; Silambarasan *et al*., 2016; Sharma *et al*., 2014b; Tessari *et al.*, 2006; Karaman *et al.*, 2010). The present study further revealed that inclusion of zinc at 40 mg/kg diet level to the 250 ppb aflatoxin contaminated diet partially ameliorated the ill effects of aflatoxin on gross and histopathology of liver. In the case of intestine, cellular organization of intestine was normal in control group (T_1) (Fig 4.21). Degenerative changes,

Fig. 4.13: Liver of control group

Fig. 4.14: Liver of AF fed group: Paleness, haemorrhage and rounded margin

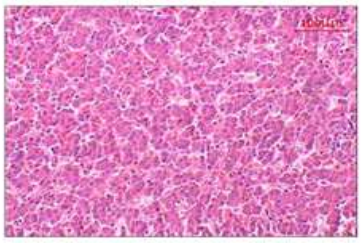

Fig. 4.15: Basal diet
Normal liver parenchyma

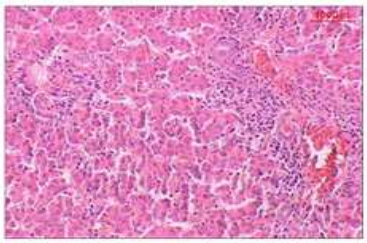

Fig. 4.16: Basal diet and AF
Proliferation of bile duct along with periportal infiltration

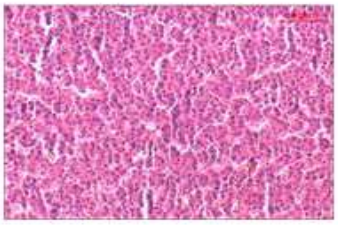

Fig. 4.17: Basal diet +20 ppm zinc
Normal liver parenchyma

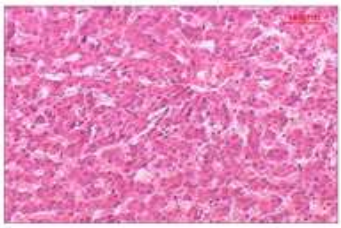

Fig. 4.18: Basal diet +40 ppm zinc
Normal liver parenchyma

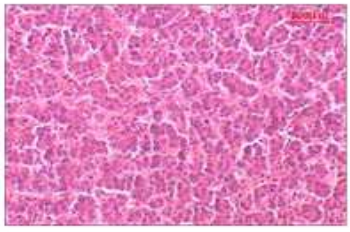

Fig. 4.19: Basal diet +AF+20 ppm zinc
Mild sinusoidal dilatation with mild changes in hepatocytes

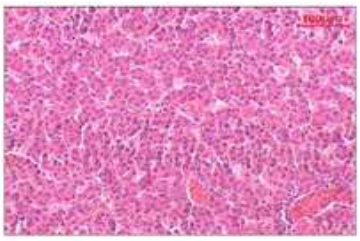

Fig. 4.20: Basal diet+AF+40 ppm zinc
Mild congestion with normal appearing hepatocytes

sloughing and focal area of severe necrosis along with infiltration of inflammatory cells were seen in aflatoxin fed group (T_2) (Fig 4.22). T_3 (Fig 4.23) and T_4 (Fig 4.24) groups showed normal histopathology as control. In T_5 (Fig 4.25) group degeneration and severe necrosis in villus were seen and in T_6 (Fig 4.26), focal area of necrosis with normal histology as control was seen. It indicated that 40 mg/kg diet zinc level was partially effective in amelioration of aflatoxicosis on intestinal histopathology in broiler chickens. The present study revealed severe histopathological lesions in T_2, due to feeding of 250 ppb aflatoxin contaminated feed. Similar histological changes were also observed by Kumar and Balachandran (2009) wherein they reported catarrhal enteritis with lymphocytic or mononuclear cell infiltrations in the intestine of broilers fed on 1 ppm aflatoxin contaminated feed. The present study further revealed that inclusion of 40 mg/kg diet zinc to

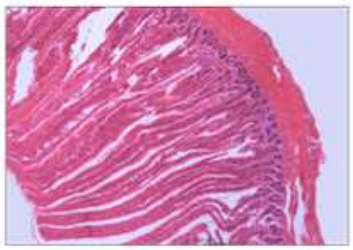

Fig. 4.21: Basal diet
Normal intestinal tissue

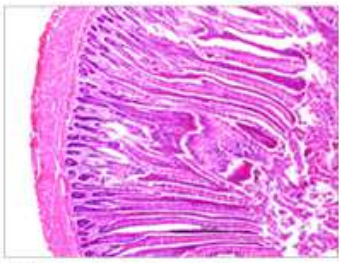

Fig. 4.22: Basal diet and AF
Degeneration and sloughing of villus

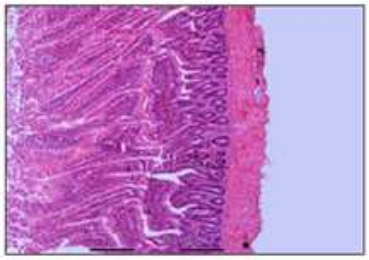

Fig. 4.23: Basal diet +20 ppm zinc
Normal intestinal tissue

Fig. 4.24: Basal diet +40 ppm zinc
Normal intestinal tissue

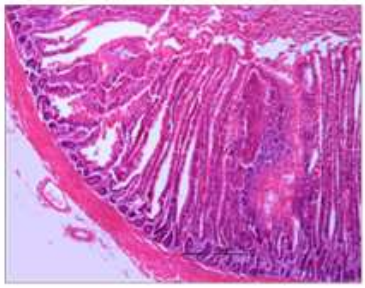

Fig. 4.25: Basal diet+AF+20 ppm zinc
Degeneration and severe necrosis in villus

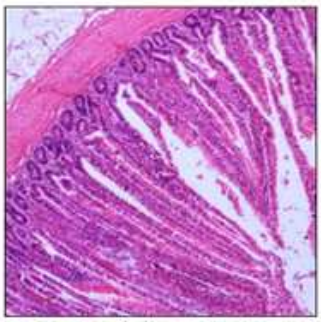

Fig. 4.26: Basal diet +AF+40 ppm zinc
Focal area of necrosis with normal villus

the 250 ppb aflatoxin contaminated feed partially ameliorated the adverse effects of aflatoxicosis on histopathology of intestine in broiler chickens.

3.3 Jejunal Morphometry

The parameters pertaining to morphometry of distal jejunum of GIT as influenced by various dietary treatments are given in Table 4. There was no significant change in villus length among various treatment groups. The crypt depth in aflatoxin fed group (T_2) was higher ($P<0.05$) (129.66 µm) than that of control (109.66 µm). The crypt depth in T_3 to T_6 was statistically similar to that of control. In the present study, aflatoxicosis resulted in increased ($P<0.05$) crypt depth compared to that of control. The study further revealed that inclusion of zinc to the aflatoxin contaminated feed restored the crypt depth value equal to that of control. The villus length/crypt depth ratio in control (T_1) was 7.42 which reduced ($P<0.05$) to 6.34 in aflatoxin fed group (T_2), indicating that aflatoxicosis resulted in reduced ($P<0.05$) villus length/crypt depth ratio in broiler chickens. This finding was in agreement with that of Jahanian *et al.* (2016). Similar result was also reported by Applegate *et al.* (2009), wherein intestinal crypt depth, but not villus length increased linearly with increasing aflatoxin concentration in the diet (0, 0.6, 1.2, and 2.5 mg/kg) which had an bearing on the villus length: crypt depth ratio. The villus length/crypt depth ratio in T_3 to T_6 groups was statistically similar to that of control. The present study

Table 4: Morphometric study of distal jejunum of broilers fed different treatments

Treatment	Villus length (µm)	Crypt Depth (µm)	Villus length/ Crypt Depth
T_1	813.33±2.40	109.66±2.90[a]	7.42±0.19[a]
T_2	822.33±5.83	129.66±1.20[b]	6.34±0.09[b]
T_3	815.33±2.18	113.76±1.86[a]	7.16±0.10[a]
T_4	815.30±2.81	112.86±5.38[a]	7.26±0.36[a]
T_5	817.03±4.07	117.53±1.52[a]	6.95±0.12[a]
T_6	817.16±4.18	115.86±2.45[a]	7.06±0.16[a]

Values bearing different superscripts in a column differ significantly (P<0.05).

revealed that inclusion of zinc to the aflatoxin contaminated diet improved (P<0.05) the villus length/crypt depth ratio equal to that of control.

4. Conclusion

It was concluded that aflatoxin contamination at 250 ppb level in diet resulted in reduced immunity; gross and histopathological lesions in liver and intestine; increased crypt depth and decreased villus length: crypt depth ratio. Inclusion of zinc (40 mg/kg diet) partially ameliorated the adverse effect of aflatoxicosis on immunity; pathology of liver and intestine; and jejunal morphometry in broiler chickens.

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