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Toxicological and Pathological Effects of Mycotoxins in Poultry in Saudi Arabia

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ABSTRACT

Background: Mycotoxins are secondary metabolites of low molecular weight produced by a wide range of fungi, principally molds. There are over 200 species of molds that produced mycotoxins of fungi. This study was carried out in Al-Qassim region-Saudi Arabia to investigate the occurrence of mycotoxins in poultry feed to identify their different groups and study the toxicological and the pathological effects of these toxins.

Methods: Samples of the poultry feeds were collected from different poultry farms and were analyzed by quantitative using ELISA. A portion of the tissues were collected for histopathology.

Results: Mycotoxins were reported to be ubiquitously present as 67.86, 50, 50, 35.71 and 32.14% of the analyzed samples, which reported positive for aflatoxins, ochratoxins, fumonisin, T-2 toxin, and zearalenone, respectively. Representative tissue sections showed necrosis of hepatocytes, glomerular, tubule-interstitial nephritis and necrotic myocarditis. The results indicate that most of these samples were below the maximum regulatory limit.

Conclusion: The co-occurrence of several mycotoxins was demonstrated and the combined action of mycotoxins can generate an interactive effect such as additive, synergism, or antagonism.

Key-words: Aflatoxins, Fumonisin, Mycotoxins, Ochratoxins, Poultry, T-2 toxin

INTRODUCTION

The term “mycotoxin” is derived from “mykes” meaning fungi and “Toxicon” meaning poison. Mycotoxins are secondary metabolites of low molecular weight produced by a wide range of fungi, principally molds.

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There were over 200 species of molds that produce mycotoxins. Aflatoxins, zearalenone, ochratoxin A, fumonisins, trichothecenes such as deoxynivalenol, and T-2 toxin are some of the mycotoxins that can significantly impact the health and productivity of poultry species [1].

Aflatoxins, a class of mycotoxins produced by fungal species of the genus *Aspergillus flavus* and *A. parasiticus)* are often found in feed ingredients used for poultry rations. Most prevalent forms of AF include B1, B2, G1, and G2, with aflatoxin B1 (AFB1) being the most common and biologically active component [2]. Aflatoxins cause a variety of effects in poultry, including decreased weight

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gain, poor feed efficiency, reduced egg production and egg weight, increased liver fat, changes in organ weights, reduction in serum protein levels, carcass bruising, poor pigmentation, liver damage, decreased activities of several enzymes involved in the digestion of starch, protein, lipids, and nucleic acids, and immunosuppression [3-5]. Evidence suggests that immunosuppression caused by AF results in many disease outbreaks, vaccination failures, and poor antibody titers [5]. At necropsy, livers are usually pale and enlarged, as a result of aflatoxicosis. Histologically, liver lesions included congestion of the hepatic sinusoids, focal hemorrhages, centrilobular fatty cytoplasmic vacuolation and/or necrosis, biliary hyperplasia, and nodular lymphoid infiltration [4]. Osborne *et al.* [6]. Found, AF at levels that did not affect growth, produced a malabsorption syndrome characterized by steatorrhea, hypocarotenoidemia, and decreased concentrations of bile salts and pancreatic lipase, trypsin, amylase, and RNase. At a cellular level, chicks fed 1.0 mg/kg AFB1 had decreased hepatic gene expression of superoxide dismutase, glutathione S-transferase, and epoxide hydrolase and increased gene expression of Interleukin 6 and cytochrome p450 1A1 and 2H1 [7]. In chicks fed 2.0 mg/kg AFB1, various hepatic genes associated with energy production and fatty acid metabolism (carnitine palmitoyltransferase), growth and development (insulin-like growth factor 1), antioxidant protection (glutathione S-transferase), detoxification (epoxide hydrolase), coagulation (coagulation factors IX and X), and immune protection (interleukins) were down-regulated, whereas genes associated with cell proliferation (ornithine decarboxylase) were upregulated [8].

Ochratoxin type mycotoxin which is most commonly found in the field is ochratoxin A (OTA), which has a primary target organ on the kidneys as it is known to be nephrotoxic [9]. Residues of OTA may be found in liver, kidneys, muscle, and eggs [10] and possess carcinogenic effects, which may be harmful when consumed by humans [9]. Ochratoxins are a group of structurally related metabolites that are produced by fungi belonging to the genera Aspergillus, Penicillium, and Ochratoxin A (OTA) is the most prevalent mycotoxin of this group. Signs of OTA toxicity in poultry include weakness, anemia, decreased feed consumption, reduced growth rate and egg production, poor feathering, and excessive mortality at high dietary concentrations [11-13].

Pathophysiological changes include decreased urine concentration and glomerular filtration rate, impairment of proximal tubular function, and degeneration and ultrastructural alterations in renal integrity [14]. Increases in the relative weights of liver, spleen, pancreas, proventriculus, gizzard, and testes have also been reported in poultry fed OTA [12,13]. Ochratoxin A consists of an isocoumarin moiety linked through the 7-carboxy group to the amino acid L-β-phenylalanine. At a cellular level, OTA interferes with DNA, RNA, and protein synthesis by inhibiting the enzyme phenylalanine t-RNA synthetase [15]. Ochratoxin A also affects renal carbohydrate metabolism through a reduction of the renal mRNA coding for phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme in gluconeogenesis [4]. The effects of OTA on DNA, RNA, and protein synthesis are thought to be due to the phenylalanine moiety of the toxin competing with phenylalanine in the enzyme-catalyzed reaction [15]. Ochratoxin A also causes hypocarotenidemia in broilers [14] that is more severe than that caused by AF [6,16].

Fumonisin B1 (FB1) has been reported to be the predominant form produced by *Fusarium verticillioides* [17]. In comparison to horses and swine, 2 susceptible species, chicks, and turkeys are relatively resistant to the toxic effects of FB1. The primary changes in chicks, ducks, and turkeys have decreased body weight gain and liver pathology [18-21]. Hepatic changes in chicks were multifocal hepatic necrosis and biliary hyperplasia [20,21]. Hepatocellular hyperplasia and increased extramedullary hematopoiesis were also noted in a study done by Weibking *et al.* [21]. The primary liver pathology observed in ducklings and turkeys fed FB1 was diffuse hepatocellular hyperplasia, with biliary hyperplasia evident in turkeys fed 150 - 300 mg FB1/kg [21] and in ducklings fed 400 mg FB1/kg [22]. In studies designed to evaluate the chronic effects of FB1, chick performance up to 7 weeks was not affected by up to 50 mg FB1/kg diet, whereas turkeys fed 50 mg FB1/kg diet had lower feed intakes than birds fed 0 or 25 mg FB1/kg diet [23]. The mechanism by which the FUM cause toxicity in animals appears to be due to the disruption of sphingolipid metabolism [24]. Current evidence indicates that the FUM are specific inhibitors of ceramide synthase (sphinganine/sphingosine N-acyltransferase) a key enzyme required for the synthesis of ceramide and more complex sphingolipids. Inhibition

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of this enzyme system leads to an increase in tissue concentrations of the sphingolipids sphingosine (SO) and sphinganine (SA), and a change in the SA: SO ratio. An increase in the SA: SO ratio, has been observed in tissues of broilers, turkeys, and ducklings fed FB1 [18,20,23].

The interaction between mycotoxins often leads to synergistic effects, when the negative effects of one mycotoxin are amplified by the presence of another. In the case of poultry, synergistic effects were frequently described in instances where aflatoxins were involved, with the same for ochratoxin A, T-2 toxin, and fumonisin B1. Aflatoxin B1, which is known to be a hepatotoxin and ochratoxin A, a nephrotoxin, acted synergistically when fed simultaneously to broiler chicks [13]. Synergistic effects were also seen in broilers fed aflatoxin B1 and T-2 toxin [25], or T-2 toxin and deoxynivalenol, whereas T2 toxin and ochratoxin A caused additive effects in broilers [24].

MATERIALS AND METHODS

Sample collection- The study was conducted in poultry farms in Al-Qassim region, Buraida, Saudia Arabia and the feedstuff samples were collected in 2015 from poultry Farm 1 and poultry Farm 2 and were analyzed for the occurrence of mycotoxins. A portion of organs was collected for histopathology.

Mycotoxins analysis- To evaluated mycotoxins occurrence, feed samples were subjected to quantitative analysis using ELISA based analytical test kits for aflatoxin, Ochratoxin A, T2-toxin, fumonisin, and zearalenone (RIDASCREEN FAST, R-Biopharm AG, Darmstadt, Germany).

Poultry feeds samples preparation and Extraction- Poultry feeds samples were collected for analysis. These samples were finely grounded. Five grams of poultry feed samples were blended with 25 ml of 70% v/v methanol/ water solution for 3 minutes. Extracts were filtered through a Whatman No. 1 filter paper then aflatoxins, Ochratoxin A, T2-toxin, and zearalenone filtrates were diluted with distilled water at a ratio of 1:1 and fumonisin filtrates at a ratio of 1:4. Fifty of the diluted filtrate per well was used for testing.

ELISA test- To evaluated mycotoxins occurrence, feed samples were subjected to quantitative analysis using ELISA-based analytical test kits for aflatoxin, Ochratoxin A, T2-toxin, fumonisin, and zearalenone (RIDASCREEN FAST, R-Biopharm AG, Darmstadt, Germany).

Procedure of mycotoxins analysis- A sufficient number of wells were inserted into the microwell holder for all standards and samples for running, standard and sample positions were recorded. 50 µl of standard or prepared sample was pipetted into separate wells by using a new pipette tip for each standard or sample. 50 µl of enzyme conjugate was added to each well. 50 µl of anti-mycotoxin antibody solution (black cap) was added to each well. Wells was moved back and forth for well mixing and incubated for 10 min at room temperature (20-25^{0}C/ 68-77^{0}F). A Liquid was dumped out of the wells into a sink, microwell holder was tapped upside down onto a clean filter towel (three times in arrow) to remove all remaining liquid from the wells. Wells were filled (250 µl per well) with distilled deionized water, remaining liquid was removed by empty in the wells again. The washing step was repeated two more times.100µl of substrate/ chromogen (brown cap) was added to each well. The plate was mixed gently by shaking manually and wells were incubated for 5 minutes (+/- .05) at room temperature (20–25^{o}C/ 68 - 77^{o}F) in the dark. 100 µl of the stop solution (yellow cap) was added to these wells, mixing was done by shaking the plate manually and the absorbance at 450 nm was measured. Results were read within 10 minutes after the addition of the stop solution.

Histopathology- For routine paraffin wax histopathology, a portion of 1 cm^3 of tissue from different visceral organ was processed and stained with Hematoxylin and Eosin as described by Hewitson and Dabry [26].

RESULTS

Study of mycotoxins in poultry feeds samples- Feed samples were analyzed to scrutinize the concentration of mycotoxins in feedstuff. Results in Table 1 shows the concentration means of Aflatoxin, Ochratoxin, Fumonisins, T-toxin and Zearalenone in poultry feed farm 1 during 6 months. Aflatoxin and Ochratoxin were not detected in the first three months, while the mean concentration of Fumonisins, T-toxin and Zearalenone were 76.46 ppb, 2.18 ppb and 89.53 pbb, respectively.

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The concentrations of Aflatoxins, Ochratoxin A, Fumonisins, T-toxin and Zearalenone in poultry feed were 0.11 ppb, 52.42 ppb, 2.12 ppb, 4.44 ppb, and zero, respectively.

Table 1: Comparison of means and SD of mycotoxins at different times on Farm 1

Mycotoxins	Farm (1) First three month	Farm (1) Second three month
Aflatoxin	–	0.11±0.19
Ochratoxin	–	52.42±49.72
Fuminpsin	76.41±22.50	2.12±20658.09
T-toxin	2.18±250.15	4.44±500.53
Zearatoxin	89.54±113.74	–

Comparison between mycotoxins in Tow farms- Table 2 shows, the concentrations means of Aflatoxin, Ochratoxin, Fumonisin, T-toxin and Zearalenone in Farm 1 and Farm 2 poultry feed Farm 2 during the study period Aflatoxins, T-toxin and Zearalenone were not detected in Farm 2, while the concentration means of ochratoxin was high in the two farms (26.21, 7.3500).

Table 2: Farm 1 and Farm 2 means and SD of mycotoxins concentrations

Mycotoxins	Farm 1	Farm 2
Aflatoxin	0.0550±0.13	0
Ochratoxin	26.21±42.58	7.3500±0.64
Fuminpsin	1.06±17438.20	1.98±279.35
T-toxin	3.31±374.82	–
Zearatoxin	44.77±87.06	–

Survey of mycotoxins in poultry feeds stuff- A total of 28 feed samples were collected from different poultry farms in Al-Qassim area during 2015. The results of mycotoxins occurrence in the poultry feedstuff were presented in Table 4 & Table 5. From these 28 samples, Aflatoxin was not detected in 9 samples. The higher concentration recorded for aflatoxin was 9 ppb. Zearalenone and T-toxins were not detected in 14 samples and ranged from 1.3–240.2 ppb and 51.95–327.1, respectively. Total 18 samples were free from T-toxins and the higher recorded concentration was 715.2 ppb. Zearalenone was not detected in 19 samples. Percentage and levels of detected mycotoxins in PMFS tested were presented in Table 4. Through the study period, out of 28 samples, 19 samples were positives for Aflatoxins (67.87%) and 14 samples were positives for Ochratoxin and Fumonisins (50%). The lower positive samples were T-2 Toxin (35.71%), and Zearalenone (32.14%).

Table 3: Concentrations of mycotoxins in Al-Qassim region

S. No.	Aflatoxin	Ochratoxin	Fumonisins	T-toxin	Zearalenone
1	1.3	0	0	0	0
2	4.8	0	312.9	0	0
3	5.5	3.6	211.9	0	0
4	3,7	2.1	0	413.92	0

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5	0	0	0	0	0
6	7.11	3.8	315.72	177.44	211.6
7	0	2.7	0	0	0
8	2.2	0	0	0	55.1
9	4.1	0	53.2	0	107.22
10	0	0	0	0	0
11	0	1.5	217.7	0	0
12	3,5	0	0	0	0
13	0	3.5	0	0	0
14	2.8	0	67.33	101.33	327.1
15	6.3	0	311.67	411.6	0
16	0	1.5	0	715.2	0
17	2.4	1.85	51.95	0	63,1
18	2.75	1.3	0	0	0
19	0	2.1	322.96	155.33	0
20	4.8	0	0	0	0
21	2.9	240.2	0	101.7	212
22	4.8	0	211.7	0	0
23	5.45	0	0	0	63.6
24	1.7	3.8	77.34	352	0
25	N D	2.7	65.11	0	301.96
26	0	0	0	155.7	0
27	9	0	135.1	0	0
28	2.3	1.85	327.1	99.8	77,2
Means	2.79	9.73	95.77	95.86	49.18

Table 4: Number of tested positive sample, percentage, and levels of detected mycotoxins in PMFS tested

Parameters	Aflatoxins	Ochratoxin	Fumonisins	T-2 Toxin	Zearatoxin
No. of samples tested	28	28	28	28	28
No. of positive samples	19	14	14	10	9
Percentage positive (%)	67.86%	50%	50%	35.71%	32.14%
Average level	3.08	9.73	95.77	95.86	49.18
Highest level	9	240.2	327.1	715.2	327.1

PMFS = Poultry Manufactured Feeds Sample

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Toxopathological effects of mycotoxins in poultry- Feedstuff mycotoxin concentration, clinical signs of chicken, postmortem, histopathological changes of heart, liver, kidneys, spleen, and serum from Poultry farm 1 were analyzed.

Feedstuff mycotoxins concentration- The mean concentration of Aflatoxin, Ochratoxin, Fumonisins, T-toxin, and Zearalenone in farm 1 during months was shown in Table 1.

Clinical signs- The common clinical signs were a reduction in feed intake. There was a sharp decrease in egg production, egg size, and egg weight; weak eggshell decreased response to antibiotic treatment, general weakness, paralysis, and in-coordination of movement, and increased mortality rate.

Post-mortem lesions- Post-mortem was done in some chickens. The liver appeared enlarged, pale yellow in color. There were hemorrhages on subcutaneous tissues and muscles. Kidneys were extremely swollen and pale. Other lesions commonly found were hemorrhages on the intestinal mucosa Fig. 1 & Fig. 2.

Fig. 1: Enlarged and pale yellow Liver

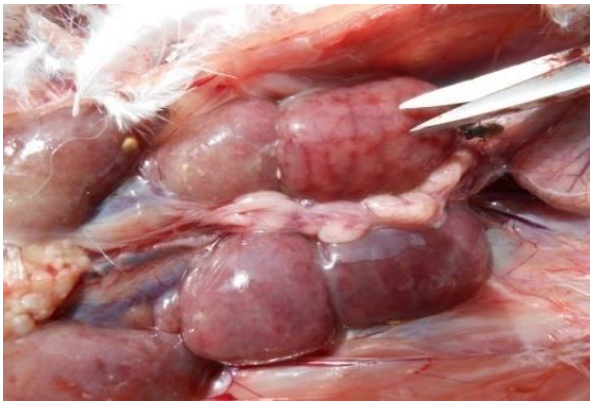

Fig. 2: Enlarged, extremely swollen and pale Kidney

Histopathological analysis- Representative tissue sections of the liver, spleen, kidney, Proventriculitis, and heart shown the followings-

Liver- Liver sections revealed massive hepatocellular necrosis, perivascular pleomorphic lymphocytic proliferation and deposition of fat droplets (Fatty degeneration) resulted in ballooning and necrosis of hepatocytes. Liver sections showed also multifocal perivascular cuffing of pleomorphic lymphocytes (some coalesce to each other), severe hepatic necrosis and deposition of intranuclear acidophilic viral inclusion bodies (Fig. 3 & Fig. 4).

Spleen- Spleen sections exhibited multifocal diffused perivascular pleomorphic lymphocytic proliferation and deposition of intranuclear acidophilic inclusion bodies (Fig. 5).

Kidneys- Kidney revealed embolic glomerular and tubulointerstitial nephritis with diffused infiltration of pleomorphic lymphoproliferation (Fig. 6).

Proventriculitis- Proventriculus exhibited diffused proliferation of lymphocytes combined by glandular hyperplasia, congestion and epithelial necrosis (Fig. 7).

Heart- Heart revealed multifocal necrotic myocarditis with severe atrophy of myofibrils (Fig. 8).

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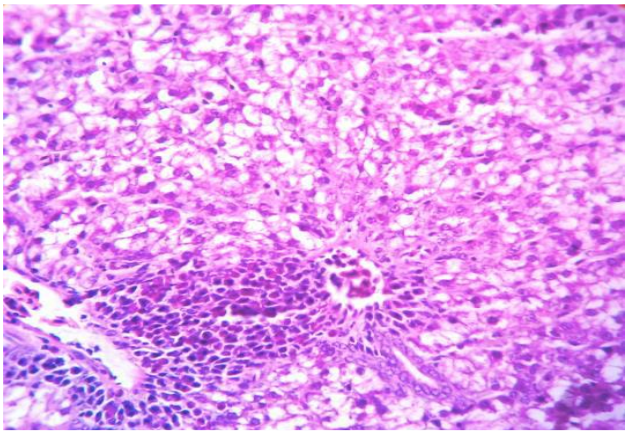

Fig. 3: Liver section showing Hepatocellular necrosis, Fatty degeneration and perivascular hepatitis (H&E stain)

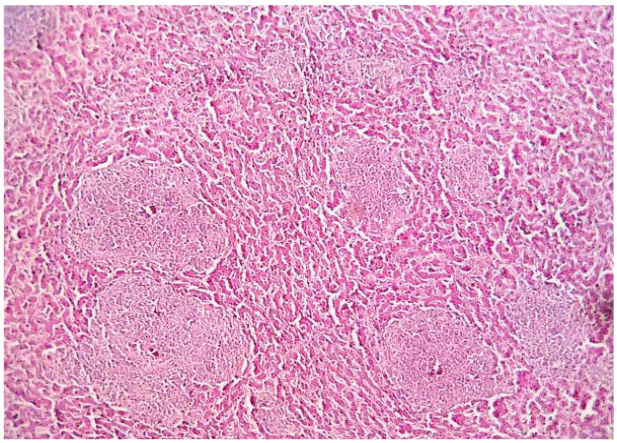

Fig. 4: Liver: Diffused Perivascular lymphoproliferation, Intranuclear inclusion body (H&E stain, 10,20,40X)

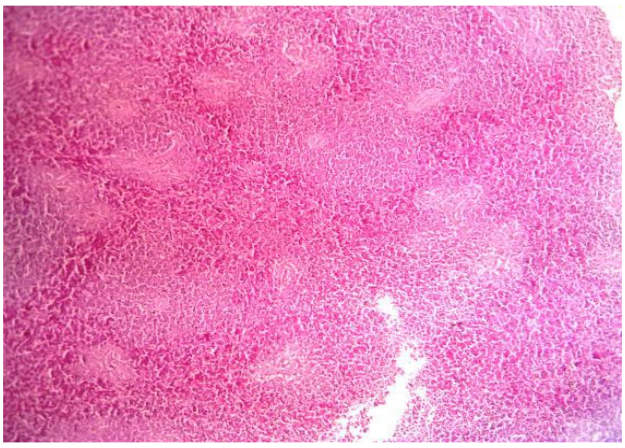

Fig. 5: Spleen: Perivascular Lymphoproliferations spleenic necrosis and vacuolation (Lymphocytic depletion) (H&E stain, 10,20,40X)

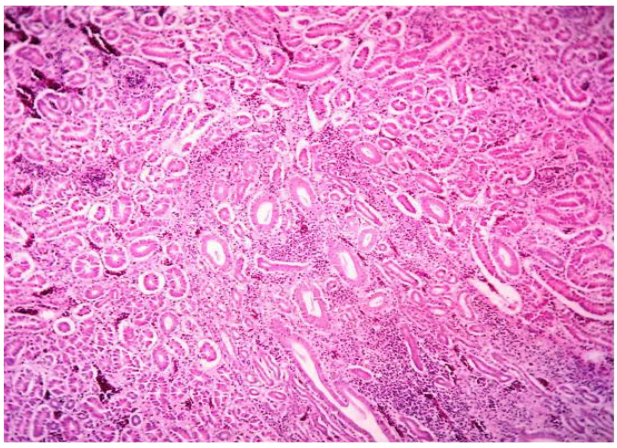

Fig. 6: Kidney: Diffused Glomerulo-embolic and tubulonephritis necrosis and hemorrhage (H&E, 10,20,40X)

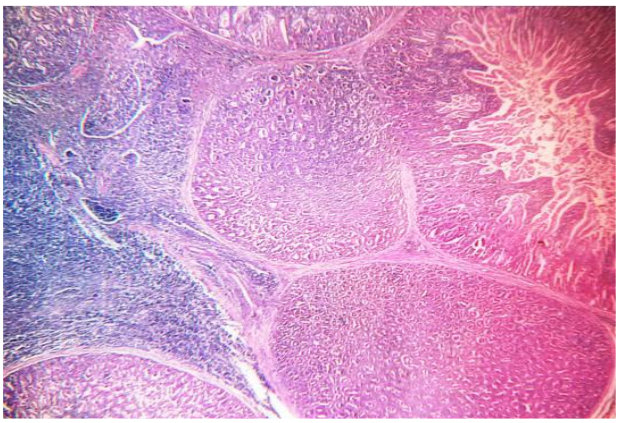

Fig. 7: Proventriculitis: necrosis, hemorrhages, lymphocytic infiltrations, neoplastic and hyperplasia (H&E stain, 10,20,40X)

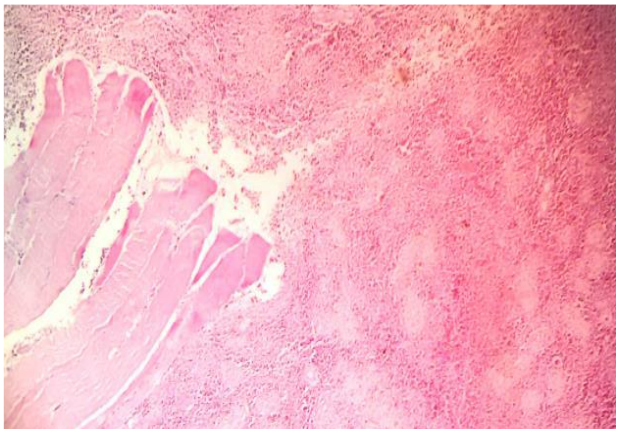

Fig. 8: Heart: Myocarditis, infiltration of inflammatory cells (H&E stain, 10,20,40X)

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DISCUSSION

Mycotoxin contamination of animal fodder and processed or compound feed is common in developing countries. Millions of tons of foodstuffs were lost each year as a direct mycotoxin infestation of world's food grains [27]. In the present study, Samples of poultry feeds were tested for the occurrence of mycotoxins using an ELISA technique. The results revealed that mycotoxins prevalence in poultry feeds were 55%, 50%, 61.11%, 44.12%, and 35.48% for Aflatoxin, ochratoxin, fumonisin, T2 toxin and zearalenone, respectively. Contamination with aflatoxins was common. In Sudan, Huda and Elham [28] reported that more than 50% of poultry feed were found contaminated with aflatoxin. Similarly, Mursal and Saad [29] showed contamination of all samples of broilers rations with aflatoxin on surveying poultry feed in Sudan. In this study, the concentration of aflatoxins in all samples showed low levels of contamination. The highest value 9 ppb was far below the accepted level of 20 ppb in poultry feeds. This result is similar to that reported in Donna *et al*. [30]; Rossi *et al*. [31] in Brazil where 97% of the samples analyzed were below the European Union Commission (EUC), but differ from the results obtained in Argentina, where 48% of the analyzed samples had a range from 17 to 197 ppb [32-29] reported. In India, Banerjee and Shetty [33] reported 5.5 to 90 ppb concentrations of aflatoxin in feedstuff samples from different poultry farms. This variation might be due to environmental and storage conditions, which favor fungal growth and subsequent aflatoxin production. However, half of the samples were found positive for ochratoxin. Five samples were above the European Permissible Limit E U.P.L (5 ppb). Fumonisins were detected in 22 of the analyzed samples with 61% positive samples, 3 samples were above 2000 ppb. These results differed from Mariana *et al.* [34] where Fumonisins were detected in all samples (100%) and agreed with Shareef [35]. T-2 toxin and zearalenone were found in 44.12%, 35.48%, respectively. This finding was in agreement with that of Mariana *et al*. [34]. Contamination of poultry feed with such mycotoxins pose significant hazards to poultry and humans. The residual toxin in poultry tissues or their products, eggs predispose human to immunity suppression or even cancer on dealing with products with a high concentration above the permissible standard or level. In the present study, there were histopathological changes in different organs. The liver was enlarged, friable, haemorrhagic and pale. Liver sections revealed massive hepatocellular necrosis, perivascular pleomorphic lymphocytic proliferation. These findings coincided with those reported by Mursal and Saad [29]. Similar results were obtained by Abdel Gabbar and Saad [36], when experimentally induced chronic aflatoxicosis in laying chicks was done. These pathological liver changes might be attributed to aflatoxin action on the liver as it is the target organ involved and is mostly affected organ when poultry fed contaminated feed [37]. Furthermore, the kidneys were found pale, swollen, enlarged and revealed embolic glomerular and tubulointerstitial nephritis. This result might be due to Ochratoxin A as it was a nephrotoxin. The combined action of these mycotoxins can generate an interactive effect such as additive, synergism, or antagonism. Synergistic interaction causes the most toxic effects in the case of aflatoxins and OTA or aflatoxins and toxin T-2. In our study, co-occurrence of at least two mycotoxins was determined in many of the analyzed samples. The combined action of mycotoxins can generate interactive pathological and toxicological effects. Our results agreed with Mariana *et al.* [34]; Shareef [35]; Abdel Gabbar and Saad [36]; Charlton [37]; Rajendra *et al.* [38] and were in partial agreement with Adel *et al.* [39]. The negative effects of mycotoxins on chicken performance have been demonstrated. In a study carried by Smith and Moss [40] reduction in body weight and increased liver and kidney weights were observed when feeding a high level (3.5 mg/kg of feed) of an AF mixture (i.e. 79% AFB1, 16% AFG1, 4% AFB2, and 1% AFG2) to broilers. Also, increased blood urea-N and decreased serum levels of total protein, albumin, triglycerides, and phosphorus were observed. This finding is similar to our result. Furthermore, feeding OTA (0.3-1 mg/kg of feed) to broilers reduced glycogenolysis and resulted in dose-dependent glycogen accumulation in the liver. These negative metabolic responses were attributed to inhibition of cyclic adenosine 3´,5´- monophosphate-dependent protein kinase and were reflected in decreased efficiency of feed utilization and teratogenic malformations [18]. The activities of other enzymes (e.g. alkaline phosphatase, acid phosphatase, lactate dehydrogenase, and succinate dehydrogenase) in several organs (e.g. heart, liver, spleen, and pancreas) of 1-week-old chicks also were

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altered by ingesting feed contaminated with *Fusarium roseum*. Such a change in enzyme activity resulted in metabolic and cellular respiratory disorders, reduced body weight gain, and tissue necrosis [22]. Moreover, Fusarium mycotoxins have been shown to adversely affect poultry. In the present study, reduced feed intake and body weight gain in addition to necrosis, hemorrhages, lymphocytic infiltrations, neoplastic and hyperplasia of Proventriculitis of studied chickens were observed on H&E stained tissue.

CONCLUSIONS

Contamination of poultry feed with mycotoxins is very dangerous and increasing problems for both man and animals. Many authors reported aflatoxin as being the most potent naturally occurring carcinogen known. In this work, although the amounts of the mycotoxins detected on poultry feed were lower than the regulation limits established. The co-occurrence of several mycotoxins was demonstrated. The combined action of mycotoxins can generate an interactive effect such as additive, synergism, or antagonism. Synergistic interaction causes the most toxic effects. Although high level can cause mortality; low level can be detrimental if continually fed. As a general rule, chicks should not receive more than 20ppb aflatoxin in the diet. However, feeding levels lower than 20 ppb may reduce their resistance to diseases and ability to withstand stress by inhibiting their immune system.

The study showed that there is a high need to perform a further study regarding the Mycotoxins in poultry feeds. More studies are vital to determine the toxic effects of the combined action of mycotoxins and their synergistic additional interaction.

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