Ameliorative Effects of Cholestyramine and Oxihumate on Aflatoxicosis in Broiler Chickens

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INTRODUCTION

Mycotoxins are significant global issue with strong economically negative impact on livestock, poultry and humans. The big issue associated with mycotoxin-contaminated animal feed is not only acute disease outbreaks (Murugesan et al., 2015; Haque et al., 2020), but also severe metabolic disorders contributing to poor animal productivity (Bryden, 2012). Aflatoxins are chemically difuranocoumarin derivatives with a bifuran group attached to the coumarin nucleus with a pentanone ring (in case of AFBs) or a lactone ring (in case of AFGs). Among the 20 identified AFs, the most important members are AFB1, AFB2, AFG1 and AFG2 (Kumar et al., 2017; Abdel-Sattar et al., 2019).

Liver is the main site for AFB1 accumulation and metabolization (Yunus et al., 2011). A survey was conducted on a total of 300 samples (100 feed stuff, 100 liver 100 and breast muscle samples) from different farms at age of marketing. The results revealed higher levels of AFB1 residues in liver samples than those detected in muscle tissues (Khaled et al., 2019). In livers, AFB1 induced hepatic lipidosis or necrosis accompanied by bile duct hyperplasia and fibrosis beside portal inflammatory cell infiltrates (Mendieta et al., 2018).

A mycotoxin binder are nutritionally inert materials that are added to animal feed in a limited amount to firmly bind mycotoxins restraining them from entering the blood stream. The supplementation of mycotoxin binders to contaminated diet has been deemed a promising result in counteracting the mycotoxicosis (Chen et al., 2014; Pappas et al., 2016; Saleemi et al., 2020). Cholestyramine is a synthetic chemical drug contains high doses from insoluble quaternary ammonium anion exchange resins which strongly combines with anionic compounds, bile acid and some cationic compounds (Underhill et al., 1995). It exerted efficient adsorption capacity for ochratoxin A, fumonisins and zearalenone on many in vitro and...
laboratory models but not in poultry (Avantaggiato et al., 2005). Oxihumate is a specific bituminous carbon humic acids by reversing the mechanism by which the coal was produced (Bergh et al., 1997). It is physical antimycotoxins had a high adsorption capacity for several mycotoxins especially AFB1. Oxihumate adsorbed many concentrations of aflatoxin at different pH levels. Its supplementation at a concentration of 3.5 g/kg ration was potent in reducing the growth inhibitory effects of aflatoxicosis.

However, published data regarding the uses of cholestyramine as a mycotoxin binder for aflatoxicosis in poultry feed have so far not been available. Furthermore, it is not known if it could alleviate the mycotoxicosis lesion in different organs. Therefore, the present work was aimed to evaluate the protective role of cholestyramine against aflatoxicosis in broiler chickens in a comparative study with oxihumate. The study evaluated some parameters such as clinical signs, mortalities, pathological and immunohistochemical analysis as well as toxin residues in different organs.

MATERIALS AND METHODS

**Birds:** Sixty-four healthy, one-day-old, baby chicks of Cubb breed, were purchased from the Alkahirah Poultry Company. The birds were housed separately in animal house research facility at the Faculty of Veterinary Medicine, Zagazig University and were kept under standard hygienic conditions. All the bird procedures were performed in strict accordance with the recommendations in the guidelines of the Institutional Animal Care and Use Committee (ZU-IACUC) of Zagazig University.

Preparation of Mycotoxin (Aflatoxin): Aspergillus flavus MD 341 strain was kindly obtained from the Central Laboratory of Residues of Agricultural Product, Agriculture Pesticides Residues Centre, Dokki, Egypt. The Aspergillus flavus MD 341 strain was incubated at 28°C for 8 days on liquid media containing 2% yeast extract and 20% sucrose. AFB1 concentration in the culture was determined using High performance liquid chromatography (HPLC) according to the method described by (AOAC, 2000). The media was sprayed and mixed in the chicken home-made diet at a concentration of 2 ppm which was rechecked using HPLC (Kumar et al., 1993).

Antimycotoxin drugs: Cholestyramine, it was manufactured by Bristol- Myers Squibb (Questran ®) 4 gm/sachet. Oxihumate, it was purchased from El-Nasr pharmaceutical Chemical Company, Egypt.

Experimental design: A total of 64, one-day-old, Cubb broiler chicks were divided evenly into four groups. In the group (Gp) A treatment, birds were fed on home-made maize-soyabean meal diet contaminated with AFB1 at 2ppm. In Gp B treatment, broilers were fed on the Gp A contaminated diet plus cholestyramine at a dosage of 170 µg/ mg mycotoxin (340 µg / kg diet). Birds on Gp C were fed on the Gp A contaminated diet with 3.5 g/kg ration of oxihumate, whereas Gp D was fed on a home-made maize-soybean meal diet with neither mycotoxins nor drug treatment. Hence, the diet ingredients had no detectable AFB1 mycotoxin level. The clinical signs and mortalities were recorded daily in all the experimental groups. Postmortem lesions of dead birds were recorded. At the end of the experiment (36) days, all the live birds were sacrificed and examined for any gross lesions. Tissue specimens from livers and kidneys were homogenized and frozen at -20°C in the dark till the time of the analysis of the AFB1 residues. Furthermore, tissue specimens from livers, kidneys, spleens, bursa of fabricius and intestine were fixed in 10% buffered neutral formalin. Paraffin sections 5 micron thick was prepared and stained with hematoxylin and eosin stain and examined microscopically (Survarna et al., 2013). Lesion scores of different examined organs in the experimental groups were estimated based on the examination of ten fields for each investigated section.

Immunohistochemical (IHC) analysis: To estimate the DNA integrity, further sections on positively charged coated slides were used for IHC technique using Anti-P53 antibody (Abcam, Cambridge, UK; ab131442) and IHC kits were purchased from Dako (Glostrup, Denmark) according to the manufactures’ instructions.

Residual investigation: Residues of AFB1 were determined in livers, kidneys and breast muscles at the end of the experiment in all the experimental groups, 36 days post initial feeding. High performance liquid chromatography (HPLC), Agilent Series 1200 using fluorescence (FLD) detector with excitation at 360 nm and emission at 440 nm was used. The procedures were performed according to the method described by (Beg et al., 2006).

Statistical analysis: The obtained data were analyzed using the statistical package for social science (SPSS, version 18 software, 2011) for obtaining means and standard deviation. The data were statistically analyzed using one-way ANOVA. Duncan’s test was used for making a multiple comparisons among the groups for testing the inter-grouping homogeneity (Tamhane and Dunlop, 2000).

RESULTS

Chickens of AFB1 intoxicated group (Gp A) showed general signs of an illness, mainly in the form of ruffled feather, droopy head and wings with depression, lameness and yellowish diarrhea. The cumulative mortality rate was 25% (4 birds/group) during the whole experiment. Chickens orally supplemented with cholestyramine at a dosage of 170 µg/ mg mycotoxin (Gp B) appeared clinically healthy with mild diarrhea and mortalities reached 12.5% (2 birds/group). Five chicks of Gp C, orally supplemented with mycotoxin contaminated diet plus 3.5 g/kg ration of oxihumate, had poor performance and depression beside the mortality rate in this group was 18.75% (3 birds/group). While neither clinical signs nor mortalities among chickens of Gp D could be detected.
Birds fed on AFB1 contaminated diet have friable enlarged livers with yellow coloration and greyish necrotic areas on their cut surface. The kidneys were enlarged or pale in color and four birds had urates deposition. The intestinal mucosal surface showed severe congestion with presence of undigested food particles. Atrophied bursa of fabricius was observed in twelve cases (75%) while splenomegaly was also noticed in six cases (37.5%).

The histological lesions of the examined organs are summarized in Table 1. Briefly, samples from AFB1 treated group (Gp A) had moderate to severe lesions. Random areas of coagulative necrosis of the hepatocytes with or without a few inflammatory cells were abundant (Fig. 1a). Portal fibrosis, cholestasis, leukocytic infiltration and numerous bile ductules were prevalent (Fig. 1b). Some cases showed focal extravasated erythrocytes replacing the hepatic parenchyma (Fig. 1c). Some apoptotic hepatic cells were intermingled with degenerated cells. Besides the hepatic lesions, renal lesions were also included such as individualization and dissociation of other renal epithelium with massive leukocytic cells infiltration (Fig. 1d). The lymphoid organs (bursa of fabricius and spleen) were characterized by mild to moderate depletion of lymphocytes from the medulla of lymphoid follicles and white pulps (Fig. 1e). Moreover, enteric lesions in group A represented by destruction of villous epithelium with severe periglandular leukocytic cells infiltration in submucosa (Fig. 1f).
In the present work, chickens orally supplemented with cholestyramine at a dosage of 170 µg/mg mycotoxin (Pappas et al., 2011) appeared clinically healthy and were in better clinical conditions compared with AFB1 intoxicated group (Gp D) showed AFB1 intoxication clinical signs as well as the highest cumulative mortality rate, 25%. Consistently, an earlier study reported that feeding of broiler chickens with diet containing 0.1 mg/kg AFB1 led to 10% cumulative mortality throughout the experimental period (Pappas et al., 2016). The variation in the mortality rate between the two experiments could be attributed to the difference in the dosage of the mycotoxin included in the chicken diet. Hence, there is established a dose-effect relationship between aflatoxin B1 level and broiler performance (Yunus et al., 2011).

In order to assess the ameliorative effects of the used antimycotoxin binders on AFB1 contaminated ration, the AFB1 residues were measured in liver, kidney and breast muscles using high performance liquid chromatography (Table 2). The highest residues level was detected in the liver tissues of Gp A then kidneys while both residues levels of liver and kidneys were significantly higher than AFB1 residues levels in muscles. Highly significant lowering of residual concentrations of AFB1 in all in kidney and liver were detected in Gp B and C due to inclusion of either cholestyramine or oxihumate in birds ration. No detectable value of AFB1 residues could be detected in chickens of Gp D.

**Table 1: Lesion scores of different examined organs in the experimental groups**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Lesions score in different examined organs among experimental groups</th>
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<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Liver</td>
<td>++</td>
</tr>
<tr>
<td>Kidney</td>
<td>++</td>
</tr>
<tr>
<td>Spleen</td>
<td>++</td>
</tr>
<tr>
<td>Bursa</td>
<td>++</td>
</tr>
<tr>
<td>Fabricus</td>
<td></td>
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<tr>
<td>Intestine</td>
<td>+</td>
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**Table 2: AFB1 residues in different examined organs of the experimental groups**

<table>
<thead>
<tr>
<th>Organ groups</th>
<th>Liver*</th>
<th>Kidney*</th>
<th>Muscle*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>14.74±0.79a</td>
<td>23.19±2.86b</td>
<td>6.93±1.04c</td>
</tr>
<tr>
<td>B</td>
<td>18.70±1.72c</td>
<td>27.80±0.23c</td>
<td>8.21±1.54d</td>
</tr>
<tr>
<td>C</td>
<td>21.9±3.17c</td>
<td>39.28±3.17d</td>
<td>10.28±1.70e</td>
</tr>
<tr>
<td>D</td>
<td>----</td>
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*Results showed as (mean ±SD): Superscripts within the same column are significantly different at P<0.05, based on Duncan’s multiple comparison.

Addition of cholestyramine to AFB1 contaminated diet led to milder histological lesions in comparison to Gp A and C in all the examined organs. The majority of hepatic parenchyma appeared normal with slightly dilated hepatic blood vessels and sinusoids beside proliferation of Von kupffer’s cells (Fig. 2a). Minimal perivascular fibrosis could be seen in few cases. Moreover, kidneys suffered from mild leukocytic cells infiltrations with mild reversible degenerative changes of some renal tubules, meanwhile the majority of renal tissues were within the normal histomorphological picture (Fig. 2b). Spleen almost exhibited normal lymphoid tissue architecture (Fig. 2c). Mild vacuolation of the epithelial covering the lymphoid follicles of bursa of fabricius with apparently normal follicular zones were common. Mild to moderate intestinal lesions in Gp B characterized by restore of the absorptive surface with hyperplastic villous epithelium and fusion of some intestinal villi beside massive mucosal lymphohcytic cells infiltration (Fig. 2d). Regenerative attempts in some renal tubules were prevalent in the majority of cases (Fig. 3). Spleen of some cases showed mild lymphoid depletion, while others showed apparently normal lymphoid tissues. Bursa of fabricius revealed regenerative tissues particularly in the medulla with or without vesicle formation in the covering epithelium (Fig. 3c). A few cases exhibited normal or partial destruction of intestinal mucosa meanwhile, the majority of birds had apparently normal intestinal coats (Fig. 3d).

**DISCUSSION**

The present study assessed the efficacy of two antimycotoxin binders to reduce or eliminate the toxic effects of AFB1 on mortality, gross & histological lesions, DNA damage and AFB1 residues in different organs of broiler chickens. AFB1 is one of the most abundant mycotoxin that contaminate bird ration. Intoxication of broiler chickens with AFB1 leads to liver impairment, weight loss, immune suppression, and increasing mortality (Yunus et al., 2011). In accordance with the previous, chickens of AFB1 intoxicated group (Gp A) showed AFB1 intoxication clinical signs as well as the highest cumulative mortality rate, 25%. Consistently, an earlier study reported that feeding of broiler chickens with diet containing 0.1 mg/kg AFB1 led to 10% cumulative mortality throughout the experimental period (Pappas et al., 2016). The variation in the mortality rate between the two experiments could be attributed to the difference in the dosage of the mycotoxin included in the chicken diet. Hence, there is established a dose-effect relationship between aflatoxin B1 level and broiler performance (Yunus et al., 2011).

In the present work, chickens orally supplemented with cholestyramine at a dosage of 170 µg/ mg mycotoxin (Gp B) appeared clinically healthy and were in better clinical conditions compared with oxihumate treated birds or AFB1 only intoxicated birds with lower mortality rate.
Reduction of both clinical signs and mortality, particularly in group B could be attributed to the ameliorative effect of the used antimycotoxin binders, cholestyramine or oxihumate. The variation in mortality declared the beneficial effect of sequestering agent (cholestyramine), which was reported here for the first time as antimycotoxin for poultry. Sequestering agents are non-absorbable materials with large surface area and high adsorptive capacity. They are capable of binding toxins in the gastrointestinal tract, thereby reducing enterohepatic recirculation and ultimately the body burden of toxins (Hope, 2013). Additionally, these agents are nonspecific and can bind additional toxins, helping to reduce overall body burden of toxins.

In a previous work, oxihumate (humic acid) could alleviate some of the toxic effects of aflatoxin in growing broilers, especially when used in combination with other mycotoxin management practices (Jansen van Rensburg et al., 2006).

 Necropsy of bird of Gp A revealed remarkable liver injuries. In addition, variable gross lesions were recorded in kidneys, intestinal mucosal surface and immune organs (bursa of fabricius and spleens). Similar pathological changes were observed earlier when broiler chickens were fed on AFB1 at 0.5 ppm for 6 weeks (Kumar and Balachandran, 2014). Similar to the gross lesions, variable degenerative and necrotic alterations in the hepatic and renal ultrastructures were detected, which were concurred with those of (Kumar and Balachandran, 2014). The toxic effects of AFB1 in the kidney were clearly studied in details in HEK 293 cells and mice model (Li et al., 2018). Results of the aforementioned study showed that AFB1 (0.5 mg/kg) caused nephrotoxicity by activating oxidative stress via reducing the expression of proline dehydrogenase and L-proline levels which led to downstream apoptosis. Additionally, aflatoxicosis had a toxic effect on all the lymphoid organs and severe lymphoid depletion, which was obvious in our study (Yunus et al., 2011; Chen et al., 2014).

Addition of cholestyramine to AFB1 contaminated diet led to milder gross and histological lesions in comparison to Gp A and C in all examined organs. Reduced lesions in Gp B due to the ability of cholestyramine to combine with the mycotoxins in the gut and then, the toxin binder complex passes outside and removed through feces. This minimizes and prevents the exposure to mycotoxins (EFSA, 2009). Cholestyramine drug act like ‘chemical sponge’ and adsorb AFB1 in the gastrointestinal tract, thus preventing toxin uptake in the blood and subsequent distribution to target organs. It was proved to be an effective binder for AFB1 in vitro and in laboratory animal models only (Avantaggiato et al., 2005). Adding of oxihumate to the initial composition of feedstuffs should alleviate aflatoxicosis efficiently by forming of an oxihumate-aflatoxin complex that inhibits aflatoxin absorption from the gastrointestinal tract (Jansen van Rensburg et al., 2006).

Apoptosis is a process of programmed cell death that occurs in multicellular organisms. Mycotoxins induce apoptosis in chicken liver tissue, which can be easily revealed using apoptosis-related antibodies p21 and p53 that mediated p53/p21 apoptotic signaling pathway (Hussar et al., 2018). As confirmed earlier, AFB1 mode of action lead to DNA adduct formation (AFB1-formamidopyrimidine). This adduct causes mutation at codon 249 of the p53 tumour suppressor gene leading to malignant transformation (Liew and Mohd-Redzwan, 2018). In accordance with the previous, ingestion of maize-soybean meal diet contaminated with AFB1 at 2ppm for nearly five weeks (Gp A) led to an intense immune staining reaction for p53 marker in the immunohistochemical analysis. On the other hand, feeding of broilers on contaminated diet plus either cholestyramine or oxihumate at a dosage of 340µg/kg or 3.5g/kg ration resulted in only mild to moderate immune expressions for p53 marker, respectively. The previous confirms the value of the p53 Ab to estimate DNA integrity and apoptosis incidence results from AFB1 exposure. In addition, it verifies the induction of either liver necrosis or apoptosis via death receptor pathway in chickens, which fed on AFB1 contaminated diet only (Mughal et al., 2017). Reduction of p53 expression in both groups (B&C) was arising from the ameliorative effect of mycotoxin-induced liver apoptosis via the two used antimycotoxin binders.

The absorption of mycotoxins in birds takes place in the proximal part of the jejunum into vascular circulation. The absorbed mycotoxins transfers via the portal vein and reaches the liver and other organs. In the present work the highest residues level was detected in the livers, kidneys, and then muscles. Constantly, metabolites of AFB1 were clearly appeared in livers of broilers fed on 2.5 or 5 mg of aflatoxin/ kg ration for a period of 32 days more than kidney and muscle tissues (Fernández et al., 1994). However, Hussain and his colleagues (2010) detected AFB1 residue in muscle the highest AFB1 residue levels are usually detected in liver and muscles and residues clearance is slow from those tissues (Hussain et al., 2010). On the other hand and consistent with the other findings, highly significant lowering of residual concentrations of AFB1 in all kidney and liver were detected in Gp B and C due to inclusion of either cholestyramine or oxihumate in birds ration.

Conclusions: In conclusion, cholestyramine by dose (340µg/kg ration) had a pronounced ameliorative action to aflatoxicosis in broiler chickens by alleviating the majority of its adverse effects on vital organs particularly when compared with oxihumate (3.5g/kg ration).

Authors contribution: AMAA, MFF and MMM conceived and designed the study as well as executed the bird experiment and analyzed the ration & tissue samples. OH analyzed and revised the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

REFERENCES