

Studies on Detoxification of Aflatoxins Contaminated Rabbits' Rations Treated with Clay and Ammonia

Helal S.H. Abu-EL-Zahab^{1,2} Ahmed Abdel Aziz Baiomy^{1,2} and Dalia Yossri Saad^{1,2}

¹Medical Biotechnology Department, Faculty of Applied Medical Sciences, Taif University, KSA

²Zoology Department, Faculty of Science, Cairo University, Egypt

Aabdelaziz.baiomy@gmail.com

Abstract: The aim of the present study is to investigate the effect of ammonia and clay on detoxification of aflatoxins contaminated Rabbits' rations. Four rations (control, aflatoxin contaminated ration, contaminated ration treated with ammonia (1%) and contaminated ration treated with clay (2%)) fed to 40 New Zealand male rabbits (10 animals each). The average daily weight gain of rabbits fed contaminated ration was reduced, whereas rabbits fed ration in addition to clay or ammonia showed weight gain. The increase in relative weight of the studied rabbit's organs (liver and kidneys) was reported in this study. However, the improvement in relative weight of internal organs of rabbits fed aflatoxin contaminated ration treated with clay, could be due to the protection effect of bentonite against aflatoxin and the alkaline effect of ammonia treatment on depressing the growing of fungi which reflect on less production of aflatoxin. Histopathological changes in liver of rabbits fed aflatoxin contaminated ration treated with clay at 45 and 90 days period and kidneys at 45 days period showed mild to moderate in severity. While the kidneys at 90 days period showed normal histological structure. Histopathological changes which occurred in liver and kidneys at 45 and 90 days period of rabbits fed contaminated ration treated with ammonia are considered mild changes and reversible. So, ammoniation or clay addition proved to be recommended as a cheapest way to inhibit the fungus growth and can detoxify its effect in rabbits feeds.

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1 Introduction

Aflatoxins refer to a group of extremely poisonous mycotoxins produced by two common fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Mycotoxins are chemical compounds produced by fungi while growing on organic substances such as corn and peanuts (Wood, 1989). When animals or humans consume these compounds, they may produce severe undesirable health effects. Aflatoxins affect animal performance via reducing feed intake and growth and can cause serious economic problems for animal production industry (Brekke *et al.*, 1977; Rodgers *et al.*, 2002; Pfohl-Leszkowicz *et al.*, 2007; Asi *et al.*, 2012 and Somorin *et al.*, 2012). Aflatoxins are of great concern as carcinogenic, mutagenic and immunosuppressive substances (Eaton and Gallagher, 1994 and Theumer *et al.*, 2003). Signs of acute aflatoxicosis are seen as severe liver and kidney damage, hemorrhage, suppression of immune system and death (Moorthy *et al.*, 1985; Huff *et al.*, 1986 and Pier, 1992). Aflatoxin is toxic to embryonic human liver in tissue culture (Zuckerman *et al.*, 1967). Indeed rat fibroblasts in tissue culture are so sensitive to aflatoxin was able to develop a useful bioassay method on this basis (Daniel, 1965). In chronic aflatoxicosis, most of the effects are still referable to hepatic injury, but on a milder scale, reduction of feed intake and growth rate of young

animals and reduction of reproductive performance of adult animals (Diekman and Green, 1992; Lindemann *et al.*, 1993; Schell *et al.*, 1993 and Nowar *et al.*, 2000). The rabbit appears to be even more sensitive to the acute effects of aflatoxin than mammalian species (Abdelhamid *et al.*, 1985). In addition aflatoxin binds with DNA and RNA and prevents the protein synthesis in the body (Pier, 1992). There are different means for aflatoxin inactivation, physical and chemical methods (János *et al.*, 1995). Removing aflatoxins from contaminated foods and feedstuffs remaining a major problem and there is a great demand for effective decontamination technology (Janos *et al.*, 2010). Ammoniation of any infected materials with fungi and used in animal feed can come over the problem of aflatoxins produced by such fungi (Norred, 1990; Fayed, 1999 and Mahendra *et al.*, 2012). Clays are generally inert and non toxic to animals (Oliver, 1997) and have a capacity to bind aflatoxin (Phillips *et al.*, 1990 and Mahendra *et al.*, 2012). The dietary addition of zeolites (Kececi *et al.*, 1998; Bailey, 2006 and Dixon, 2008), bentonite (Oguz, 1997 and Magnoli *et al.*, 2008) and Egyptian tafla (Nowar and Abd EL-Mageed, 1996) have been used for reduction of aflatoxins toxicity *in vivo*.

2. Materials and Methods

Animals:

Forty growing New Zealand white male rabbits

aged one month and half were divided into four groups. All groups were equal in number (10 rabbits each). All rabbits were approximately similar in their initial body weight at the beginning of the experiment. They were fed the experimental rations to meet nutrient requirements of rabbits during the growing period according to Nutrient Research Council (NRC, 1977).

The experimental groups:

Group 1 (G1): Aflatoxin free ration (Control group). Group 2 (G2): Aflatoxin contaminated ration. Group 3 (G3): Aflatoxin contaminated ration treated with 2% clay (Nowar *et al.*, 2001). Group 4 (G4): Aflatoxin ammoniated ration, treated with anhydrous ammonia at 1% concentration (Sundstol *et al.*, 1978). For each dietary treatment three rabbits were randomly chosen at the middle and the end of the experimental period, all rabbits were fasted before slaughtering. The slaughter test was performed at 45 and 90 days of the experimental period. The three rations group was compared to a control ration free from aflatoxin contamination.

Microorganism:

Aspergillus flavus, NRRL 2999 (Chohal and D. Howks Worth laboratory U.S.A.). Aflatoxin was produced according to the method by Shotwell *et al.* (1966) and Sharon *et al.* (1973).

Average of live body weight (LBW):

Live body weight of rabbits was recorded individually at beginning and at weekly intervals to the nearest gram until the end of the experimental period. Weighing was done in early morning before receiving feed or water.

Average of live body weight gain (BWG):

The BWG was calculated at the end of the experimental period, by subtracting the LBW from the weight at the end of the experiment, then divided by the experimental period.

Histopathological examination:

Samples for histopathological examination were taken from liver and kidney at 45 and 90 days period. Liver and Kidney were fixed in 10% neutral buffer formalin solution then washed in tap water and dehydrated by different grades of alcohol and cleared by xylene then embedded in paraffin. The paraffin embedding blocks were cut at 4-5 μ m thick. The sections were routinely stained with haematoxylin and eosin (Bancroft and Cook, 1994).

Statistical analysis:

All statistical analysis were done according to SAS (1998).

3. Results

Growth performance:

Data in tables (1, 2 and 3) presented the growth performance of rabbits fed the experimental rations. Average daily gain of rabbits fed

contaminated ration (G2) was reduced by about 56.1% when compared with the control group (G1) after 45 days. While the reduction was 64.3% at 45-90 days period, whereas the overall period (90 days) was 64.2%. Rabbits fed ration in addition to clay (G2) gained 29.8% more than the control after 45 days; 2.4% at 45-90 days and 15.3% after 90 days. Rabbits in group G4 showed a percentage of 17.9 more gain after 45 days and 8.2% at the overall period (0-90 days) compared to the control (G1), while it had less gain reached 1.1% at 45-90 days period than G1.

Table 1: Animal growth performance for rabbits fed the experimental rations for 45 days (Mean \pm SE). a,b,c and d: Means in the same row with different

Items	G1	G2	G3	G4
IBW (g)	939.80 \pm 3.13	940.6 3 \pm 6.17	940.00 \pm 2.14	940.63 \pm 3.11
FBW (g)	1860.8 5 ^c \pm 12.40	1345.00 ^c \pm 11.11 ^d	2135.63 ^a \pm 23.10	2026.25 ^a \pm 18.19
Daily gain (g)	21.93 ^b \pm 1.22	9.63 ^c \pm 0.16	28.47 ^a \pm 2.14	25.85 ^a \pm 3.13
% difference from G1	-	-56.1	+29.8	+17.9

superscripts are significantly different (P<0.05).

Table 2: Animal growth performance for rabbits fed the experimental rations from 45 to 90 days (Mean \pm SE).

Items	G1	G2	G3	G4
IBW (g)	1851.82 ^c \pm 24.16 ^d	1267.78 ^e \pm 37.14	2180.83 ^a \pm 20.11	2063.85 ^b \pm 19.18
FBW (g)	3330.85 ^c \pm 44.15	1796.25 ^e \pm 48.17	3695.20 ^a \pm 41.14	3527.46 ^b \pm 39.15
Daily gain (g)	35.22 ^a \pm 1.20	12.58 ^c \pm 2.15	36.06 ^a \pm 0.85	34.85 ^a \pm 0.91
% difference from G1	-	-64.3	+2.4	-1.1

a,b,c,d and e: Means in the same row with different superscripts are significantly different (P<0.05).

Table 3: Animal growth performance for rabbits fed the experimental rations from 0 to 90 days (Mean \pm SE).

Items	G1	G2	G3	G4
IBW (g)	939.80 ± 2.14	940.63 ± 2.34	940.00 ± 1.95	940.63 ± 3.02
FBW (g)	3330.85 ± 6.15	1796.25 ± 11.11	3695.20 ± 9.17	3527.46 ± 13.20
Daily gain (g)	28.46 ^b ± 0.16	10.19 ^c ± 1.13	32.80 ^a ± 2.21	30.80 ^{ab} ± 1.90
% difference from G1	-	-64.20	+15.30	+8.20

a,b,c,d and e: Means in the same row with different superscripts are significantly different ($P < 0.05$).

Relative organs weight to live body weight of rabbits:

Relative organs weight to live body weight of rabbits fed the experimental rations are presented in table (4) after 45 days and table (5) after 90 days. Rabbits fed aflatoxin contaminated ration (G2) showed heavier ($P < 0.05$) relative weight of liver (3.70%), kidneys (0.92%) after 45 days and (3.52 and 1.08) for both liver and kidney respectively after 90 days than those fed aflatoxin free ration (G1). Other groups of rabbits had no significant relative weight of liver and kidneys to live body weight after both 45 and 90 days periods.

Table 4: The effect of the experimental rations on relative organs weight (Liver and kidney) to live body weight of rabbits after 45 days of the experiment (Mean \pm SE).

Items	G1	G2	G3	G4
Live body weight (g) "Pre slaughter"	1900.00 ^b ± 4.87	1436.67 ^c ± 7.72	1933.33 ^a ± 8.89	1863.33 ^c ± 11.20
Liver weight (g)	49.00 ^b ± 1.11	53.13 ^a ± 2.02	51.67 ^{ab} ± 2.12	49.33 ^b ± 1.00
Relative liver weight (%)	2.58 ^b ± 0.61	3.70 ^a ± 0.06	2.67 ^b ± 0.72	2.65 ^b ± 0.12
Kidneys weight (g)	14.33 ^a ± 0.01	13.31 ^a ± 0.55	14.77 ^a ± 0.11	14.07 ^a ± 0.21
Relative kidneys weight (%)	0.75 ^b ± 0.49	0.92 ^a ± 0.04	0.77 ^b ± 0.91	0.75 ^b ± 0.84

a,b,c,d and e: Means in the same row with different superscripts are significantly different ($P < 0.05$).

Histopathological lesions:

No histopathological lesions were detected in liver and kidneys of rabbits fed the normal ration (control group) from beginning up to the end of the experimental period.

Table 5: The effect of the experimental rations on relative organs weight (Liver and kidney) to live body weight of rabbits after 90 days of the experiment (Mean \pm SE).

Items	G1	G2	G3	G4
Live body weight (g) "Pre slaughter"	3063.67 _{bc} ± 66.14	1913.33 _d ± 38.42	3478.34 _a ± 21.41	3165.67 _b ± 48.24
Liver weight (g)	74.58 ^a ± 6.33	67.31 ^c ± 5.35	85.09 ^a ± 2.37	75.81 ± 1.07 ^b
Relative liver weight (%)	2.43 ± 0.22 ^c	3.52 ± 0.10 ^a	2.45 ± 0.15 ^c	2.39 ^c ± 0.10
Kidneys weight (g)	22.55 ^{bc} ± 0.11	20.70 ^c ± 3.14	26.03 ^a ± 0.81	23.83 ^{ab} ± 2.12
Relative kidneys weight (%)	0.74 ^b ± 0.33	1.08 ^a ± 0.13	0.75 ^b ± 0.03	0.76 ^b ± 0.11

a,b,c,d and e: Means in the same row with different superscripts are significantly different ($P < 0.05$).

The liver of (G2) after 45 days of treatments showed congestion of central vein, sinusoids and portal blood vessel (Fig.1A). Hepatocytes showed increase in its size with vacuolation of their cytoplasm (Hydropic degeneration), there was midzonal necrosis characterized by deeply eosinophilic cytoplasm, pyknosis of nucleus (Fig.1B). Cholangiofibrosis was detected in the portal areas characterized by proliferation of fibrous connective tissue around portal triad accompanied with mononuclear cells aggregation (Fig.1C). After 90 days of the experiment, the same previous pathological lesions were detected in liver but in sever degree. There were multiple areas of hemorrhage dispersed hepatocytes from each others (Fig.1D). The fibrous connective tissue proliferation extended in-between the hepatic lobules to form periportal cirrhosis (Fig.1E). There were hyperplasia of bile ducts accompanied with infiltration of mononuclear cells mainly macrophages and lymphocytes (Fig.1F). The kidneys of (G2) group after 45 days of the experiment showed different degenerative changes in epithelial lining proximal and distal convoluted renal tubules as hydropic degeneration, renal cast and coagulative necrosis. Renal epithelium appeared enlarged in size with vacuolation of the cytoplasm and even necrosis, (Figs., 2A, 2B and 2C). After 90 days of the experiment, kidneys showed hydropic degeneration in the epithelial lining of most of renal tubules, there was interstitial nephritis characterized by proliferation of fibrous connective tissue and mononuclear cells aggregation mainly macrophages and lymphocytes (Fig.2D).

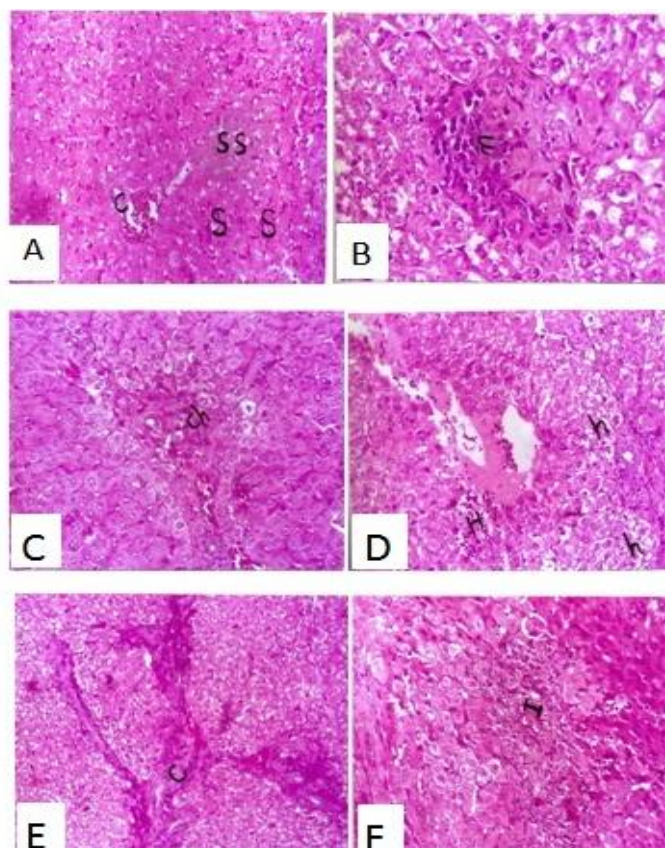
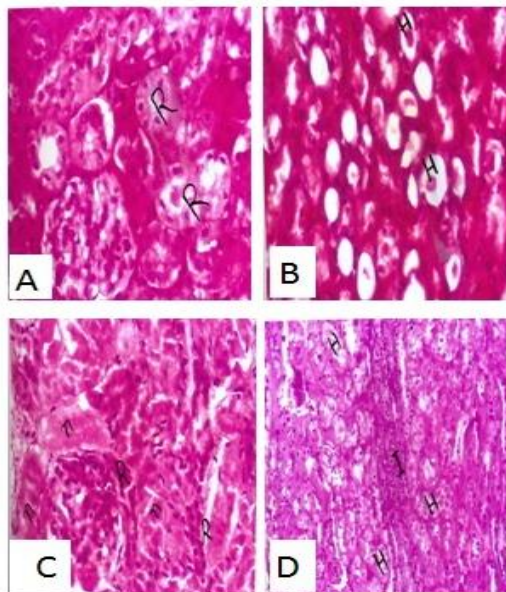


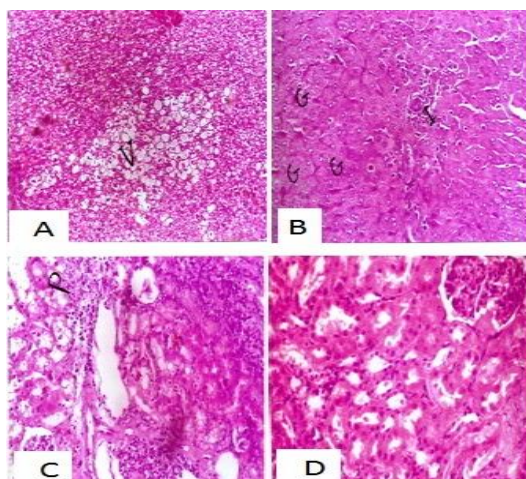
Figure 1.

- (1A): Photomicrograph of Liver of rabbits fed aflatoxins contaminated ration and sacrificed at 45 days showing congestion of central vein (C) and sinusoids (S). H&E x100
- (1B): Photomicrograph of Liver of rabbits fed aflatoxins contaminated ration and sacrificed at 45 days showing midzone necrosis and inflammatory cells aggregation (n). H&E x400
- (1C): Photomicrograph of Liver of rabbits fed aflatoxins contaminated ration and sacrificed at 45 days showing cholangiofibrosis (ch). H&E x200
- (1D): Photomicrograph of Liver of rabbits fed aflatoxins contaminated ration and sacrificed at 90 days showing hemorrhagic (H) areas and hydropic degeneration of hepatocytes (h). H&E x200
- (1E): Photomicrograph of Liver of rabbits fed aflatoxins contaminated ration and sacrificed at 90 days showing periportal cirrhosis (C). H&E x100
- (1F): Photomicrograph of Liver of rabbits fed aflatoxins contaminated ration and sacrificed at 90 days showing proliferation of connective tissue between hepatocytes with inflammatory cells (I). H&E x400

The liver of rabbits fed on ration treated with clay (G3) after 45 days of the experiment showed moderate congestion of central vein, sinusoid and portal blood vessel. The hepatocytes were suffered from vacular degeneration of the cytoplasm (moderate hydropic degeneration). There were focal areas of vacular nodules in midzonal area characterized by ballooning of hepatocytes with losing of most of their nuclei (Fig.3A). After 90 days of the experiment, there was slight enlargement of hepatocytes accompanied with granulation of the cytoplasm (cloud swelling). There was mild changes in mononuclear cells aggregation mainly macrophages and lymphocytes were detected in portal triad (Fig.3B). The kidneys after 45 days of the experiment showed moderate congestion of glomerular tuft and renal blood vessels. Some cases showed perivascular cuffing represented by aggregation of lymphocytes around the renal blood vessels (Fig.3C). The kidneys after 90 days of the experiment showed normal histological structure (Fig.3D).

**Figure 2.**

- (2A): Photomicrograph of Kidneys of rabbits fed aflatoxins contaminated ration and sacrificed at 45 days showing enlargement of renal epithelium and vacuolation of its cytoplasm (R). H&E x400
- (2B): Photomicrograph of Kidneys of rabbits fed aflatoxins contaminated ration and sacrificed at 45 days showing hyaline cast in the lumen (H) of most of renal tubules. H&E x400
- (2C): Photomicrograph of Kidneys of rabbits fed aflatoxins contaminated ration and sacrificed at 45 days showing coagulative necrosis of most renal tubules (n). Notice the regenerating tubules (R). H&E x400
- (2D): Photomicrograph of Kidneys of rabbits fed aflatoxins contaminated ration and sacrificed at 90 days showing fibrous connective tissue proliferation accompanied with mononuclear cells aggregation (I). Notice hydropic degeneration of epithelial lining of most renal tubules (H). H&E x200

**Figure 3.**

- (3A): Photomicrograph of Liver of rabbits fed aflatoxins contaminated ration treated with clay and sacrificed at 45 days showing vacuolar nodules (V). H&E x200
- (3B): Photomicrograph of Liver of rabbits fed aflatoxins contaminated ration treated with clay and sacrificed at 90 days showing few inflammatory cells aggregation in portal area (I). Notice the granulation of most of cytoplasm of hepatocytes (G). H&E x200
- (3C): Photomicrograph of Kidneys of rabbits fed aflatoxins contaminated ration treated with clay and sacrificed at 45 days showing perivascular cuffing (P). H&E x200
- (3D): Photomicrograph of Kidneys of rabbits fed aflatoxins contaminated ration treated with clay and sacrificed at 90 days showing normal histological structure. H&E x200

The liver of rabbits fed on ration treated with ammonia (G4) after 45 days of the experiment showed slight enlargement of hepatocytes and mild hydropic degeneration of cytoplasm (Fig.4A). Whereas after 90 days of the experiment the liver blood vessels showed signs of vasculitis represented by desquamation of endothelial lining, mild destruction wall and inflammatory cells aggregation (Fig.4B). The kidneys after 45 days of the experiment showed mild hypercellularity of the glomerular tuft. The epithelial lining of proximal convoluted tubules showed slight degenerative changes as granulation and vacuolation of the cytoplasm with narrowing of their lumen (Fig.4C). After 90 days of the experiment, most of the renal tubules showed slight enlargement of the size of tubular epithelium (Fig.4D).

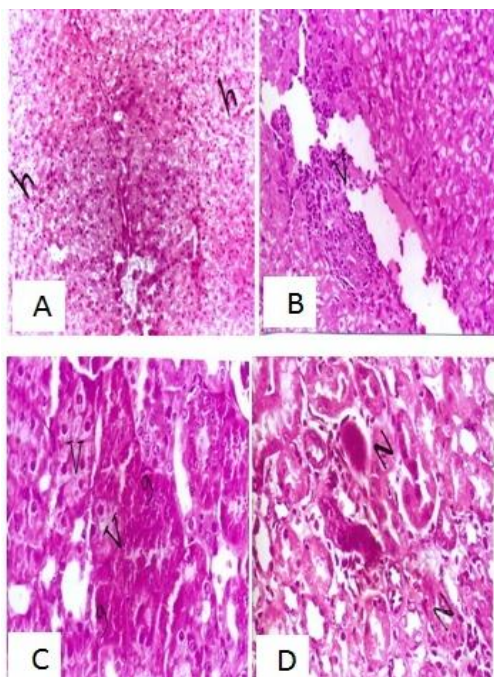


Figure 4.

- (4A): Photomicrograph of Liver of rabbits fed aflatoxins contaminated ration treated with ammonia and sacrificed at 45 days showing mild hydropic degeneration of hepatocytes (h). H&E x100
- (4B): Photomicrograph of Liver of rabbits fed aflatoxins contaminated ration treated with ammonia and sacrificed at 90 days showing vasculitis and rupture of portal blood vessels (V). H&E x200
- (4C): Photomicrograph of Kidneys of rabbits fed aflatoxins contaminated ration treated with ammonia and sacrificed at 45 days showing granulation (g) and vacuolation of cytoplasm of epithelial lining of the renal tubules (V). H&E x400
- (4D): Photomicrograph of Kidneys of rabbits fed aflatoxins contaminated ration treated with ammonia and sacrificed at 90 days showing enlargement of the nucleus of some epithelial lining of the renal tubules (N). H&E x400

4. Discussion

In the present study, the effect of clay and ammonia on aflatoxin contaminated ration had been studied on rabbits. The average daily body gain of rabbits fed aflatoxin contaminated ration was linearly decreased with the advance of the feeding period. The decrease in body weight was associated with decline in the average of daily feed intake. Feed intake may have been depressed as a result of decreased palatability of aflatoxin contaminated ration. In addition, aflatoxins impair nitrogen and energy utilization on the ingested diet through its

adverse affects on the liver, as it is considered as the center of body metabolism (Reddy *et al.*, 1991).

In the present study, seven rabbits fed aflatoxin contaminated ration were died through the experimental period. This could indicate a sign of chronic aflatoxicosis in rabbits fed contaminated ration with aflatoxin. The obtained results are agreed with Nowar *et al.* (2001) they noticed a reduction in average of live body weight and gain of rabbit fed diet naturally contaminated with 860 ppb aflatoxins. When aflatoxin ration treated with clay (2%) (bentonite), the performance of rabbits were

improved, this could be explained by the basic mechanism of bentonite and the adsorbents in preventing the aflatoxin toxicity, it appears to involve sequestration of aflatoxin in gastrointestinal tract and chemisorptions to the adsorbent, which reduces the bioavailability of aflatoxins (Davidson *et al.*, 1987 ; Lindemann *et al.*, 1993 and Mahendra *et al.*, 2012). The effect of ammonia treatment of aflatoxin contaminated rations in improving animal performance could be due to the effect of ammonia releases as alkaline compound on preventing the production of aflatoxicosis by fungi and verified the conversion of B1 aflatoxin (more toxic) to D1 aflatoxin (non toxic) (Southern and Clawson, 1980 ; Norred, 1982; Fayed, 1999 and Mahendra *et al.*, 2012).

The increase in relative weight of organs (liver and kidneys) of rabbits fed aflatoxin contaminated ration was reported in this study, this result was agreed with Nowar *et al.* (1996 and 2001) they explained that the relative weight of internal organs could be a result of increased lipids in the liver which associated with harmful effect of aflatoxin. However, the improvement in relative weight of internal organs of rabbits fed aflatoxin contaminated ration treated with clay, could be due to the protection effect of bentonite against aflatoxin (Nowar *et al.*, 2000 and 2001; Bailey *et al.*, 2006 and Dixon *et al.*, 2008) and the alkaline effect of ammonia treatment on depressing the growing of fungi which reflect on less production of aflatoxin (Jones *et al.*, 1996; Fayed, 1999 and Mahendra *et al.*, 2012).

Aflatoxin B1 is one of the most common mycotoxin and it is a potent hepatoxins and hepatocarcinogen. The liver histopathological results observed in this study could be due to the metabolism of aflatoxins occurs in liver by cytochrom P450 enzyme. In the liver cell, aflatoxins converted to classes of metabolites which bound to cellular macromolecules such as essential enzymes blockages of RNA polymerase and ribosomal translocase and formation of DNA adduct. Aflatoxins can bind to various proteins which may affect structural and enzymatic protein functions. Also, aflatoxins and their metabolite are mainly secreted by bile, so this explains the pathological lesions observed in bile duct (Hsieh, 1985; Hsieh and Atkinson, 1990 and Leesson *et al.*, 1995). Although the kidney was not the target organ for the effect of aflatoxins; there were histopathological changes recorded in this study. Aflatoxin and its metabolite can be excreted via the kidneys producing damage to kidney's tissue (Leesson *et al.*, 1995 and Agag, 2004). Histopathological changes were showed in liver of rabbits fed aflatoxin contaminated ration treated with clay after 45 and 90 days of the experiment, whereas

kidneys after 45 days showed a mild to moderate histopathological severity. While the kidneys after 90 days of the experiment showed normal histological structure. Histopathological changes which occurred in liver and kidneys at 45 and 90 days period of rabbits fed contaminated ration treated with ammonia are considered as mild changes and reversible. The histopathological findings clearly indicated that the addition of clay or ammonia to aflatoxins contaminated ration greatly diminished the deleterious effect of aflatoxicosis on liver and kidneys (Jones *et al.*, 1996; Fayed, 1999; Nowar *et al.*, 2001 and Mahendra *et al.*, 2012).

So, it can be concluded from this study, that aflatoxin contaminated ration treated with either clay 2% (G3) or ammonia 1% (G4) had proven to be the best detoxification methods. However, ammonia treatment can be recommended as a cheapest way to inhibit the fungus growth and can effectively detoxify aflatoxin in animal feeds. Clay (bentonite) at the rate of 2% could be also another cheap way for the detoxification of aflatoxin effect by adsorption of it from gastrointestinal tract of animals on the surface of the bentonite layers, consequently prevent the bioavailability of aflatoxin.

Corresponding Author:

Dr. Ahmed Abdel Aziz^{1,2}

¹Medical Biotechnology Department, Faculty of Applied Medical Sciences, Taif University, KSA

²Zoology Department, Faculty of Science, Cairo University, Egypt

Aabdelaziz.baiomy@gmail.com

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