Study of Toxicity and Pathogenicity of Aflatoxin B1 and G1 in Mice

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Abstract

The results of this investigation had demonstrated the production of aflatoxins from *Aspergillus flavus* and study the toxicity and pathological effect in mice. Aflatoxin B1 and G1 were produced by two methods. (Corn method and YES method). The concentration of aflatoxin B1 was 2 ppm while aflatoxin G1 was 5.25 ppm by using TLC and HPLC. Three groups of animals (mice) were taken. First and second groups treated with aflatoxin B1 and G1 respectively by (25µg/0.2 ml olive oil/animal/day) for 21&30 days. This study showed that aflatoxin G1had higher effect from B1 on the liver than kidney, while aflatoxin B1had higher effect from G1 on the kidney than liver .This result was confirmed by biochemical tests for liver enzymes and kidney salts. So, the result of liver enzymes in serum of mice treated with aflatoxin G1revealed a significant increase ($p<0.05$) in GOT, GPT and ALP at 30 days of experiment comparing with other groups. Whereas the result of kidney enzymes in serum of mice treated with aflatoxin B1 revealed a significant increase ($p<0.05$) in urea, creatinin and uric acid at 30 days of experiment comparing with other groups. Therefore, we could conclude that aflatoxines have acute toxicity effect in target organs of mice that treated with short period (30 days) and may develop to induce tumors when treated for longer period (6 months).

دراسة سمية وامراضية سموم الافلا ب1,ج1 في الفئران

شيماء نبهان ياسين وزينب رزاق زغير
كلية الطب البيطري/ جامعة بغداد

الخلاصة

هدفت هذه الدراسة إلى إنتاج سموم الافلا من فطر Aspergillus flavus ودراسة تأثيراتها السمية وامراضيتها على الفئران. تم إنتاج سموم الافلا B1 و G1 بطريقتين مختلفتين (طريقة وسط الذرة الطبيعي وطريقة الوسط السائل) حيث بلغ تركيز سم الافلاB1 (2) جزء من المليون وسم الافلا G1 (5.25) جزء من المليون باستخدام الصفائح الكروماتوكرافي الرقيقة TLC وجهاز الكروماتوكرافي السائل ذو الأداء العاليHPLC. أخذت 3 مجاميع من الحيوانات (الفئران) حيث اعطيت المجموعة الأولى سم الافلا B1 والمجموعة الثانية سم الافلا G1 وبجرعة 25 مايكروغرام/0.2 مل زيت الزيتون/للحيوان يوميا ولمدة 21 و30 يوما. أظهرت الدراسة أن سم الفلا G1 يمتلك أعلى تأثير على الكبد من سم B1 بينما الأخير أثر بشكل ملحوظ على الكلية أكثر من الكبد وقد تعززت هذه النتائج من خلال الفحوصات الكيموحيوية لأنزيمات الكبد وأملاح الكلية فظهر ان سم الافلاG1 كان الأكثر تأثيرا مهما إحصائيا (0.05%) على ALP,GPT,GOT في مصل الفئران المعاملة بالسم بعد 30 يوما من إجراء التجربة مقارنة بباقي المجاميع. أما سم B1 فكان الأكثر تأثيرا على أملاح الكلية Creatinin,Uric acid,Urea مهما إحصائيا (0.05%) في مصل الفئران المعاملة بالسم بعد 30 يوما من اجراء التجربة مقارنة بباقي المجاميع. ومن هذا نستنتج ان سموم الافلا لها تأثيرات سمية حادة إذا أعطيت لفترة قصيرة (30 يوما) والتي قد تتطور الى احاث اورام سرطانية عند تناولها لفترات طويلة.

Introduction

Aflatoxins are potent carcinogens produced by certain *Aspergillus* fungi. The aflatoxins were first discovered in the 1960s, and since then have been found to be distributed worldwide in a variety of commodities, foods, and feeds (1). Mycotoxins are very resistant fungal metabolites that can remain in foods after processing and, sometimes, even after cooking (2). Of the mycotoxin class,aflatoxins are considered harmful, being both acutely and chronically toxic. Aflatoxin B1 and B2 are some of the most known potent hepatocarcinogens. Along with aflatoxins G1 and G2, even extremely low levels of these aflatoxins in the diet are important public-health concerns (3,4). The hazards presented by mycotoxins may be conveniently divided into two classes. The first is related to the economic loss to producers of agricultural products, which will result from poor growth of livestock and subsequent failure to reach marked weight. The second hazard is that to man.This may result from direct ingestion of mycotoxins from the food such as groundnuts and cereals contaminated with fungi and also from secondary contamination from eating meat from animals with residues of mycotoxins or their metabolites in the tissues (3). Commodities and products frequently contaminated with mycotoxins include corn,wheat, barley, rice, oats, nuts, milk, cheese, peanut sand cottonseed. Mycotoxins produce a wide range of adverse and toxic effects in animals in addition to being food borne hazards to humans. There are many highly specific and sensitive methods for determining aflatoxin concentration in commodities or in culture, such as high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), thin-layer chromatography (TLC), and fluorescence polarization assay (5,6,7). Usually these methods are expensive and time-consuming. Chromatographic methods require extraction procedures to remove interfering substances, by using a mixture of water and a polar organic solvent. Commercially available ELISA kits provide a relatively easy assay for quantification of total aflatoxin concentration but do not identify individual aflatoxins present in the sample (8,9). So, this study was carried out to notice the effect of aflatoxins on the some internal organs of mice by using some of these methods to detect the concentration of these toxins.

Materials and Methods

- ***Aspergillus flavus*** isolates were obtained after cultivation of contaminated maize samples which was placed in 2% of hypochloride solution (NaOCI) for 1 min then washed for 2 times with sterilized distilled water for 1 min,then dried by filter paper and then cultured on Sabouraud Dextrose Agar (SDA) for 5 days at 25˚ C. (10).
- Testing the ability of isolated A. flavus for production aflatoxin: Fungal species belong to *A. flavus* had been tested to verify their ability for Aflatoxin production by cultivation these isolates on Coconut Extract Agar (CEA) according to (11,12).
- Production of aflatoxin: The present study involved 2 methods of culturing to produce aflatoxin. The first one by culturing on natural substrate maize and the second by culturing on liquid media.

A. Production of aflatoxin on natural substrate maize (Method A): According to the method described by (13) with some modification.

1. A quantity of 100 gm of maize was placed in 500 ml conical flask, 60 ml distelled water was added then sealed by cotton and silver foil, then autoclaved at 121˚C under 15 pound/inch 2 for 60 min. After that the flask was leaved at room temperature for 24 hrs. and the sterilization process was repeated with the frequent mixing to prevent clumps.

2. The flask was inoculated with (1ml) of spore suspension ($1X10^7$ spore/ml) and incubated at 28±2˚C for 21 days with shaking everyday(for 5 first days)by hand to lose any clumps of mold growth.
3. After incubation, the flask was dried in oven at 60˚C for 24 hrs. to kill the fungus and then grinding the sample with electric grinder.

- **Extraction and Detection of Aflatoxin:**
- **Extraction of Aflatoxin: According to (14), the extraction involved the following steps:**

1. Fifty gm of contaminated grind samples was placed in 500 ml conical flask and mixed with 100 ml of extracted solution which consist of 450 ml of acetonitrile, 50 ml potassium chloride 4% and 10 ml HCL 5N and blended by flask shaker for 30 min.
2. The extract was filtered through filter paper No.1.
3. fifty ml of filtrate was placed in 250 ml separatory funnel and was defated by shaking with 50 ml of hexan for many times then leaved given 2 separate layers. The upper layer was discarded and the process was repeated by adding another 50 ml of hexan to discard the fatty layer.
4. Fifty ml of chloroform was added to the collected lower layer in other separatory funnel and mixed well, then leaved to separate the 2 layers. The lower layer was passed through glass funnel that had filter paper which contains unhydrous Sod. sulfate to be discarded from water and the filtrate was collected in rounded bottle. (The process repeated 2 times) to be concentrated by rotary evaporator at 60˚C.
5. The concentrated extract was transferred to small glass vial mixed with 1 ml of chloroform then kept in cool and dry place.

B. Production of Aflatoxin in liquid culture (Method C): According to (15) which have been modified by (16) to separate aflatoxin from liquid culture.

- Dense conidial suspension (1ml of $1x10^7$ spore/ml) were inoculated into 100 ml of YES broth (2% yeast extract, 15% sucrose) contained in 250 ml flask and incubated at 25˚C for 2 weeks with a daily shaking. The extract of aflatoxin was prepaired through the following steps.

1. The fungal growth was mixed well, then filtrated through filter paper Whatman No.1through funnel.
2. Pooled culture filtrates were extracted twice with chloroform (1:2,v/v) in separating funnel with shaking to discard gases.
3. The mixture was leaved for 15 min to separate the 2 layers; the lower layer was passed through filter paper contains anhydrous Sod. sulfate.
4. The chloroform extract was subsequently evaporated to dry and the residue was dissolved in 1ml of chloroform. Samples were stored at vials.

- **Detection of Aflatoxins:** The production of aflatoxins was determined by thin-layer chromatography (TLC) by using aluminium sheets (20 by 20 cm) of silica gel G60. According to (17). This silica coated was activated by ovine at 105˚C for 2 hrs. then spotted with 20 µl of extract and standerd aflatoxin and developed in a solvent consisting of chloroform and methanol (90:10). The air-dried plates were observed under long-wave UV light (365 nm) for aflatoxins**.**
- **Quantification of aflatoxins:** Extracts of aflatoxins were identified by high-performance liquid chromatography (HPLC). The apparatus used was a model Cecil 1100 which is available in Ministry of Science and Technology that had a loop of 50 µl and was equipped with spectroflurescence detector and small a C18 (250 by 4.6 mm, 5µm particle size). The mobile phase was pumped at 1ml/ min and consisted of H2O:CH3CN (70 ml:30 ml).
- **Experimental Design:** Twenty-five Swiss male albino mice were divided randomly into 5 groups (5 animals for each group) and caged separately according to (18).

Group 1 (Untreated control): Animals were maintained without any treatment. Animals of the 4 groups were orally administered with aflatoxins in 25µg/ 0.2 ml olive oil/ animal/ day of A and C method respectively for 21 and 30 days. After 35 days treatment, the animals were sacrified and some internal organs of treated groups were kept in 10% formalin buffer solution to study the histopathological changes. Also blood was collected to utilize the biochemical analysis. The data were statistically analyzed using SAS (2004) (19). The levels of significance was accepted with $p<0.05$ by L.S.D.

Results

1. **Testing the ability of *Aspergillus flavus* isolates for aflatoxins production**.

The results of aflatoxins production from the fungal isolates on cracked maize showed that there was a variation in the ratio of coloration from blue and yellow green coloration of these strains after exposure to UV light at 365 nm but the highest and stronger brightness was selected from 2 isolates which were choose for further study of detection of aflatoxin and study of aflatoxins effect in mice.

2. Determination of aflatoxins production.

HPLC analysis of extracts (method A and C) showed 2 peaks at retention time identical to that of standard aflatoxins. The identity of the first peak was confirmed by spikign the extract A with standard aflatoxin G1and the second peak was confirmed by spikign the extract C with standard aflatoxin B1. Aflatoxins were quantified on the basis of HPLC fluorometric response compared with that of aflatoxin standard. In corn culture (method A) the fungus produced 5.25 ppm of aflatoxin G1, while in YES culture, the aflatoxin B1concentration found were 2 ppm.

3. Histopathological changes:

A. Aflatoxin B1-

1. **Kidney-**Histopathological sections showed fibrous connective tissue proliferation with mononuclear cell infiltration around glomeruli as well as cystic dilatation of renal tubules with acute cellular degeneration characterized by vacculation and desquamation of epithelial lining cells of renal tubules (Fig.1).
2. **Brain**- The brain showed hemorrhage in the parenchyma with congestion of blood vessels (Fig.2).
3. **Intestine-**The main lesions characterized by hyperplasia of lymphoid tissues in the sub mucosal.
4. **Lung-** The lung showed hyperplasia of epithelial lining cells with mononuclear infiltration in the interstitial tissue (Fig.6).

B. Aflatoxin G1-

1. **Liver-** Histopathological lesions characterized by vaccular degeneration in the cytoplasm of hepatocytes with single cell showed apoptosis (Fig.3); also multiple granulomatous lesions consist from aggregation of macrophage scattered in the liver paranchyma (Fig.4), and sever mononuclear cells aggregation around the central vein. Other section the liver showed severs fatty changes in the cytoplasm of hepatocytes.
2. **Kidney-**Dilatation of renal tubules with acute cellular degeneration (Fig.5), hypertrophy and congested capillary blood vessels and inflammatory cells perivascular cuffing with dilatation of renal tubules which contained pinkish proteinous material in their lumen (Fig.7).
3. **Brain**- Gliosis, perineural edema around nerve cells and congested blood vessels.
4. **Lung-** Hypertrophy of smooth muscles of air ways was seen, increased thickness of intra alveolar septa due to congestion of capillary blood vessels.
5. **Heart-** Inflammatory cell infiltration mainly macrophage and lymphocyte in the epicardium, with congested blood vessels between muscle fibers.

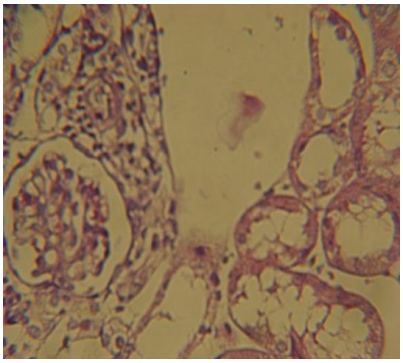

Fig (1) Histopathological section in kidneyof animal 30 days of infection with aflatoxin B1showed connective tissue mononuclear cell infiltrationaround glomeruli, cystic dilatation of renal tubules vacculation and desquamation of epithelial lining degenerative cells of renal tubules (H&EX400).

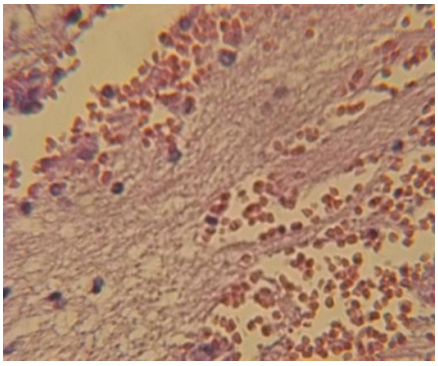

Fig (2) Histopathological section in brainof animal 30 days of infection with aflatoxinB1showed hemorrhage in the parenchyma with congestion of blood vessels (H&EX400).

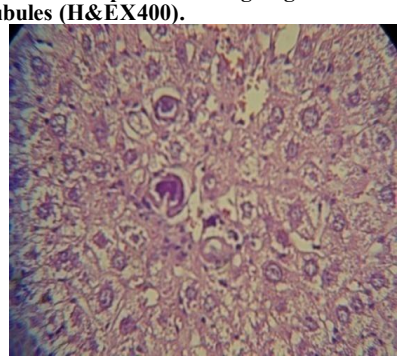

Fig (3) Histopathological section in liver of animal 30 days of infection with aflatoxinG1showedvaccular (hydropic) degeneration in the cytoplasm of hepatocytes with single cell apoptosis (H&EX400).

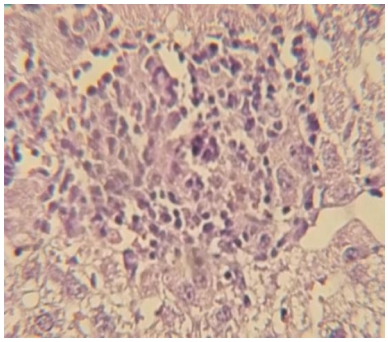

Fig (4) Histopathological section in liver of animal 30 days of infection with aflatoxinG1 showedmultiple granulomatous lesions consist from aggregation of macrophage scatter in the liverparanchyma (H&EX400).

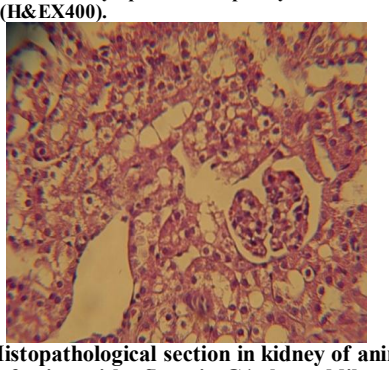

Fig (5) Histopathological section in kidney of animal 30 days of infection with aflatoxin G1 showeddilatation of renal tubules with acute cellular degeneration (H&EX400)

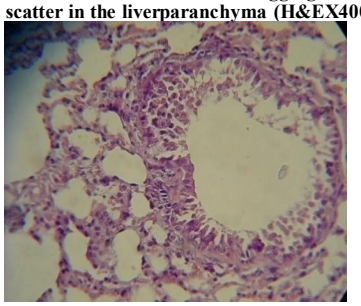

Fig (6) Histopathological section in lung of animal 21 days of infection with aflatoxin B1showedhyperplasia of epithelial lining cells with mononuclear infiltration in the interstitial tissue (H&EX400).

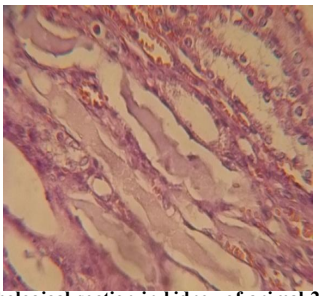

Fig (7) Histopathological section in kidney of animal 21 days of infection with aflatoxin G1 showeddilatation of renal tubules which contained pinkish proteinous material in their lumen (hyaline degeneration) (H&EX400).

4. Biochemical tests:

The results of the effect of Aflatoxins (B1 and G1) on enzymes of liver and kidney showed highly significant differences (P<0.05) between the groups. The results revealed highly significant differences (P<0.05) in enzyme activity of GOT, GPT and ALP in G1 treated group at 30 days of experiment as compared with other groups; while the biochemical tests of the kidney showed highly significant differences (P<0.05) in urea, creatinin and uric acid in B1 treated group at 30 days of experiment as compared with other groups. (Table 1,2).

Table (1): Effect of aflatoxins (B1 and G1) on enzymes of liver. GOT, GPT and ALP in serum of mice at 21 and 30 days of experiment

	GOT	GPT	ALP
Control	7.3	6	1020
G1 (21 days)	8.4	7.3	1192
G1 (30 days)	9	7.9	1204
B1 (21 days)	7.5	7	1165
B1 (30 days)	7.8	7.6	1180
L.S.D	* 0.873	* 0.915	* 69.382

L.S.D=P<0.05

Table (2): Effect of aflatoxins (B1 and G1) on enzymes of kidney. urea, creatinine and uric acid in serum of mice at 21 and 30 days of experiment

	urea	creatinin	Uric acid
Control	45	1.2	4.7
G1(21 days)	69	7.5	7.2
G1(30 days)	94	7.6	8.0
B1(21 days)	78	2.2	5.2
B1(30 days)	142	9.5	8.5
L.S.D	* 23.59	* 3.38	* 2.09

L.S.D=P<0.05

Discussion

Mycotoxins are secondary fungal metabolites that are harmful to animals and humans as they may cause serious diseases (20). At least 13 types of Aflatoxins are produced in nature by two ***Aspergillus*** species that are ubiquitous in areas of the world with hot, humid climates. Whether exposure is predominantly to aflatoxin B1 or to mixed B1 and G1 depends on the geographical distribution of the Aspergillus strains. *Aspergillus flavus*, which produces aflatoxins B1 and B2, occurs worldwide; while *A. parasiticus*, produces aflatoxins B1, B2, G1 and G2. (21). However, many studies revealed that not all *A. flavus* and *A. parasiticus* strains could secrete Aflatoxins. On the other hand, there may be one strain pocesses the ability to secrete all Aflatoxin types (B1, B2, G1 and G2) and other strain can secrete Aflatoxin B without Aflatoxin G; while all strains which secrete Aflatoxin G should produce Aflatoxin B (22) which is compatible to result obtained from this study showed that *A. flavus* can secrete Aflatoxin B&G. Recent methods of analysis for Aflatoxins are TLC and HPLC. Thin layer chromatography (TLC), also known as flat bed chromatography or planer chromatography is one of the most widely used separation techniques in Aflatoxin analysis (23). This method is also used to verify findings by newer, more rapid techniques. So we used this type which is considered the method of choice to identify the quality and quantity of Aflatoxins. Also, Aflatoxins were analysed by using HPLC is quite similar to TLC. The major advantages of HPLC over TLC are more quick, automation, more exact, and precision. Both normal-phase and reverse-phase HPLC

separations have been developed for aflatoxin analyses. Other studies revealed that aflatoxins could be separated on nominal-phase columns and detected with either a UV detector or a fluorescence detector (2, 23, and 24). The result of experiment showed that the effect of Aflatoxins in mice, that Aflatoxin G1had higher effect from B1 on the liver than kidney, while Aflatoxin B1had higher effect from G1 on the kidney than liver, and this agree with (25) who pointed out that oral administration of Aflatoxin B1 in different strains of mice, rats and hamsters caused renal cell tumors with a low incidence of tumors at other sites including liver and colon; While oral administration of aflatoxin G1 induced altered hepatocytes, hepatocellular adenomas and carcinomas and renal-cell tumours. Considerable emphasis has been placed on the hepatic tumours induced by these mycotoxins. However, there is evidence that although the liver may be the principal site of induction of neoplasia other tumours may be induced in the gastrointestinal tract. These are principally of the alimentary tract (oesophagus, glandular stomach, duodenum and colon in the rat) and also possibly salivary gland (26). Kidney-epithelial tumours may also be induced by aflatoxin G1. In their experiments aflatoxin G1 produces a high incidence of such tumours, while they are rarely seen following aflatoxin B1.This may return to that aflatoxins have induced oxidative damage and caused to generate free radicals which reacted with cellular componentand led to pathological changes in liver and kidney functions (27). Morever, another study attained to the same of our results when mice were exposed to aflatoxins B1 and G1 via their feed (4.8 ng AFG1, 0.8 ng AFB1 or both/kg body wt./day). At six months of age, hepatorenal studies were carried out. The AFG1 caused significant accumulation of only neutral fat in the liver, a slight rise in serum triglyceride and intensified hepatocellular inflammation, necrosis and bile duct proliferation. The AFB1, caused cytotoxic to the kidneyand liver. Hematological indices, serum total protein and albumin levels were not affected by the aflatoxins (28) and this may referred to the action of cytochrome P450 which is secreted from liver cells, renal tubular cells and lung cells. This enzyme caused detoxification of toxin complex compound and this will lead to degeneration of organ cells and may cause cancer. We found that B1 toxin had an effect on the (CP450) enzyme of the kidney and lung, whereas G1 toxin had an effect on the (CP450) enzyme of the liver. The hazards of Aflatoxins may be either those of acute toxicity or those of long-term chronic toxicity and carcinogenicity. These toxicity had relationship with the route of administration,exposure period and dosage. This elucidated the histopathological changes in our study that did not reach to carcinogenic level because of the shortness of exposure period (21 and 30 days) , used in oral administration route without injection and smallest dose (25µg/0.2 ml olive oil/animal/day) which coincide with (18) who carried out his experiment on Aflatoxin by oral administration for 30 days and noticed significant increase in lipid peroxidation in the kidney ,consequently, this explain increasing urea, uric acid and creatinin of blood serum level in Aflatoxins treated mice when compared with control animals. Biochemical tests of liver enzyme level in blood serum of mice treated with Aflatoxin showed increasing of GOT, GPT and ALP levels in 30 days of treatment as comparing with 21 days of treatment, and this may be due to the time of exposure to the aflatoxin that agreed with (29) who said the treatment with aflatoxin B1 significantly increased the liver enzymes (GGT, ALT and AST), kidney function markers (uric acid and creatinine). So, other studies must be performed on the effect of Aflatoxins in all internal organs for longer period of exposure and using other tests for detection in the field like ELIZA.

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