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BIOLOGICAL AND PHYSIOLOGICAL EFFECTS OF AFLATOXIN ON MICE LIVER FUNCTION ENZYMES

Mayssam Ali Hamada Hassan
Al-Nahrain University College of Biotechnology Department of molecular and medical biotechnology

Ahmed Abd al-Hussain Abd al-Sadah Atban
Al-Qasim Green University College biotechnology Department biotechnology

Assel Ammer Ali Mohammed
University of Fallujah College of applied science Department of Biotechnology

Amir Abd al-Hussain Abd al-Sadah Atban
Al-Qasim Green University College biotechnology Department biotechnology

Abstract: In this study, the biological and physiological effect of aflatoxin was evaluated by detecting the mouse serum level of liver functional enzymes (AST, ALT and ALP). Three groups of animals (mice) were taken. First and second groups treated with aflatoxin at concentrations 0.75 and 1.5 ppm respectively for 14 successive days, while the third group treated with distilled water as negative control. Results indicated that the aflatoxin caused a significant dose dependent, increase in serum liver functional enzymes levels with maximum level following 1.5 ppm administration 236.00, 162.44, 97.56 U/L for AST, ALT and ALP, respectively.

1.1 Introduction

Aflatoxin is toxic metabolite produced by different species of toxigenic fungi, called mycotoxins. By the periodic consumption of contaminated food, humans can be exposed to aflatoxins. The exposure to aflatoxin participates in increase in nutritional deficiencies, immunosuppression and hepatocellular carcinoma (Wagacha and Muthomi, 2008).

Aflatoxins (AFs) have a wide occurrence in different kinds of food and feeds such as spices, cereals, oils, fruits, vegetables, milk, meat, etc. There are 18 different types of aflatoxins identified; the major members are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2), M1 (AFM1) and M2 (AFM2) which are produced by *Aspergillus flavus* and/or *Aspergillus parasiticus*. Strains of *A. flavus* can vary from non- toxic to highly toxigenic and are more likely to produce AFB1 than AFG1 (Coppock and Christian, 2007).

Aflatoxin B1 is the most common (Hussein and Brasel, 2001) in the world which accounts 75% of all aflatoxins contamination of food and feeds (Ayub and Sachan, 1997). The hydroxylated products of aflatoxins B1 and B2 are aflatoxins M1 and M2, respectively, and are associated with cow, milk; aflatoxins M1 and M2 where formed from aflatoxins B1 and B2 remain stable during milk processing (Stroka and Anklam, 2002).

Aflatoxins biosynthesis as all secondary metabolites which strongly dependent on growth conditions such as substrate composition or physical factors such as pH, water activity, and temperature or modified atmospheres (Giorni et al, 2008; Schmidt-Heydt et al, 2009).

1.2 Literature Review

1.2.1 Aflatoxin

1.2.2 Major Human Diseases Caused by Aflatoxins Consumption

It has been reported that more than 5 billion people in developing countries are at risk of chronic exposure to aflatoxins through contaminated foods (Shephard, 2003; William et al, 2004). The adverse effects of aflatoxins in humans and animals have been categorized in two general forms:

a. Acute Aflatoxicosis

It is produced when consumed aflatoxin at a moderate to high levels. Disease cause hemorrhage, acute liver damage which cause hepatotoxicity with a case fatality rate of about 25%, edema, absorption and/or metabolism of nutrients and alteration in digestion. The symptoms of hepatotoxicity from aflatoxicosis include anorexia, malaise, and low-grade fever. Acute high-level exposure can leads to potentially lethal hepatitis with vomiting, abdominal pain, jaundice, fulminant hepatic failure and death (Cullen and Newberne, 1994; Strosnider *et al*, 2006).

b. Chronic Aflatoxicosis

It is produced from consuming of low to moderate levels of aflatoxins. The common symptoms are decline food conversion and slower rates of growth with or without the occurrence of an overt aflatoxin syndrome (Walderhaug, 1992). Aflatoxicosis is not only caused by inhalation, but also caused by aflatoxin ingestion. In 1976 the International Agency for Research on Cancer (IARC) recognized aflatoxins as carcinogenic because the chronic exposure to aflatoxin causes Hepatocellular Carcinoma (HCC), generally in association with hepatitis B virus (HBV) or other risk factors (Henry *et al*, 2002; Omer *et al*, 2004; Wang *et al*, 1996).

The effects of aflatoxin consumption are the same in all animals, but the susceptibility differs according to the species, age, and individual variation. Acute aflatoxicosis symptoms include depression, anorexia, weight loss, disease, gastrointestinal bleeding, pulmonary edema and liver damage. The liver shows total changes caused by central lobular crowding and hemorrhage and fatty changes of surviving hepatocytes. Death of the animal may occur within hours or a few days. Symptoms of prolonged exposure to moderate to aflatoxins may be reflected in a reduction in feed consumption and production (growth and production of eggs and milk). It also affect the quality and products of milk, which represent the risk of the presence of AFM1 as it derived from AFB1 consumed by lactating females(Pier, 1992; Denli and Pérez, 2006).

1.2.3 Types of Aflatoxin

There are 18 different types of aflatoxins has been identified, the most important members are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), G2(AFG2), M1 (AFM1) and M2 (AFM2) which are produced by *Aspergillus flavus* and/or *Aspergillus parasiticus*. *Aspergillus flavus* strain will be ether non- toxic or highly toxigenic and they produce AFB1 more than AFG1, while *A. parasiticus* strains produce AFB1 and different amounts of AFB2, AFG1 and AFG2 and have less toxigenic variation in (Coppock and Christian, 2007).

Acute and chronic toxicity is arranged as AFB1 > AFG1 > AFB2 > AFG2, according to epoxidation of the 8,9-double bond and the high potency of the cyclopentenone ring of the B series as compared with the six-membered lactone ring of the G series. The hydroxylated forms of AFB1 and AFB2 are AFM1 and AFM2 (Mclean and Dutton; 1995, Wogan, 1966).

Whereas the B-group aflatoxins exhibit blue fluorescence, the G-group exhibits yellow-green fluorescence under ultraviolet (UV) light, thus making the use of fluorescence important in identifying and differentiating between the B and G groups (Hussein and Brasel, 2001).

Aflatoxin B1 (AFB) is prevalent and considers the most hazardous, which is listed by the International Agency for Research on Cancer as Group I carcinogen (Lee *et al*, 2004). Various methods have been developed for the detection of AFB (Li *et al*, 2011), such liquid chromatography (Bacaloni *et al*, 2008), high- performance liquid chromatography (Khayoon *et al*, 2011), immunochromatography (Ren *et al*, 2011), enzyme-linked immunosorbent assay (ELISA) (Li *et al*, 2013) and electrochemical immunoanalysis (Tang *et al*, 2009).

1.2.4 Aflatoxin B1

Aspergillus flavus and *A. parasiticus* produce aflatoxin B1, which is the most potent carcinogen known. Aflatoxin B1 considered as a common contaminant in foods including peanuts, cottonseed meal, corn, and other grains (Galvano *et al*, 2005) and animal feeds (Rania *et al*, 2005). Aflatoxin B1 is known as the most toxic aflatoxin and it is probably the main cause of hepatocellular carcinoma (HCC) in humans (Ilic *et al*, 2010), while in animals, it shown to be mutagenic and cause immunosuppression (Meissonnier *et al*, 2008). Several methods have been used to test aflatoxin B1 including thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), mass spectroscopy, and enzyme-linked immunosorbent assay (ELISA) (Alex and Mims 1979).

In particular environmental conditions dermal exposure to aflatoxin leads to major health risks (Boonen, 2012). The most susceptible organ to aflatoxin B1 toxicity is the liver. In animal studies, pathological lesions caused by aflatoxin B1 intoxication include lose the liver (Fernández *et al*, 1996), hepatocytes vacuolation(Espada *et al*, 1992) and hepatic carcinoma (Larsson *et al*, 1994). Liver lesions include hepatic cells enlargement, fatty infiltration, necrosis, hemorrhage, fibrosis and bile duct proliferation/hyperplasia (Patterson, 1977).

1.2.5 Biotransformation of Aflatoxin

Biotransformation (metabolism) is the process in which a chemical substance is changed from one chemical form to another by a series of enzymatic or chemical reactions within the body and eventually the byproducts or metabolites excreted mainly through renal excretion. In toxicology biotransformation plays an important defense mechanism in the elimination of the toxic xenobiotics and body wastes in which they are converted into less harmful and substances that can easily be excreted (Monosson, 2012; Vondracek *et al*, 2001). The process of metabolism and excretion of chemical substances from the body involves two main phases:

1. Phase I Metabolism of Aflatoxin:

In phase I metabolism, aflatoxins undergo oxidation reactions including epoxidation, hydration, hydroxylation and O-demethylation reactions involving the CYP 450 mainly in the liver to produce AFB1-exo-8,9 epoxide (AFBO), AFB2a, AFM1, AFQ1 and AFP1 (Monosson, 2012, Dhanasekaran *et al*, 2011, Omar, 2013)

Cytochrome P450s (CYP450s) are a large superfamily of heme-binding enzymes involved in the synthesis and metabolism of endogenous substrates as well as in the biotransformation of xenobiotics like aflatoxins (Monosson, 2012; Dhanasekaran *et al*, 2011; Code *et al*, 1997). In the liver, aflatoxins are metabolized mainly by CYP1A2 and CYP3A4, the CYP3A4 metabolizes AFB1 to its reactive intermediates AFB1-exo-8, 9 epoxide and then to less toxic metabolite aflatoxin Q1 (AFQ1).

The AFB1- exo-8, 9- epoxide reacts with the N7 atom of guanine to form a DNA adducts (aflatoxin-N7-guanine). The aflatoxin-DNA adduct is unstable and undergoes depurination, leading to its urinary excretion. Some of the DNA adducts are fairly resistant to DNA repair processes so this causes gene mutation and hence the development of cancer. CYP3A4 have relatively low affinity for AFB1 epoxidation and is primarily involved in AFB detoxification by AFQ1 formation (Eaton *et al*, 1995). AFB1 has been metabolized to AFM1 and the AFB1-exo-8,9-epoxide by CYP1A2, which is considered as the main effective route of metabolism of aflatoxins (Eaton *et al*, 1995; Lampe *et al*, 2000).

AFB metabolism by CYP1A2 leads to formation of some exo-epoxide and a high proportion of endo-epoxide and AFM1. In aflatoxins metabolism the expression of CYP1A2 and CYP3A4 depends on affinity and level of expression in the liver. The CYP1A2 has high-affinity for the bioactivation of AFB1 at low substrate concentrations following dietary exposure (Wild and Turner, 2002; Lampe *et al*, 2000).

Carcinogenicity is caused by AFBO and AFB1-dihydroxide intermediates, while AFB2a cause acute toxicity (e.g. liver necrosis and cellular metabolizing enzyme inhibition). Some of the AFB intermediates undergo further metabolism in Phase II by conjugation with glutathione to produce less toxic metabolites that are easily excreted in urine and bile (Dhanasekaran, 2011).

2. Phase II Metabolism of Aflatoxins:

Phas II includes the conjugation of the phase I metabolites with glutathione or glucuronic acid (Massey *et al*, 1995). Epoxide conjugated with glutathione in the presence glutathione-S-transfrase (Wang *et al*, 2001). Glutathione-S-transfrase is a necessary enzyme for reduction and preventation of aflatoxin B1caused cancer, the conjugated epoxide and hydroxylated AFB1 metabolites are secreted via bile to the intestinal tract (Kord *et al*, 2001).

1.2.6 Correlation Between Aflatoxin & Liver Function Enzyme

Aflatoxin is associated with both toxicity and carcinogenicity in human and animal populations. In developed countries, sufficient amounts of food combined with regulations that monitor aflatoxin levels in these foods protect human populations from significant aflatoxin ingestion. However, in countries where populations are facing starvation or where regulations are either not enforced or nonexistent, routine ingestion of aflatoxin may occur (Cotty *et al*., 1994 Abdelhamid, 2010). Worldwide, liver cancer incidence rates are 2 to 10 times higher in developing countries than in developed countries (Henry *et al*., 1999).

In clinical diagnosis, determination of transaminases is of great importance. The activity of ALT and AST is a sensitive indicator of acute hepatic necrosis and hepatobiliary disease (Kaplan, 1987). It was indicated that afla toxin treatment caused a significant, dose and time-dependent elevation in ALT, AST and ALP activities in serum of rats. The liver is considered to be the principle target organ for AFS and the increase of ALT, AST and ALP activities indicated acute hepatic necrosis (Abdel-Wahhab and Soher, 2003). In addition, Liu *et al.*, (2001) also reported that afla toxin resulting in elevation of serum AST and ALT activities.

2.1 Materials

2.1.1 Equipment and Apparatus

The following equipments and apparatus were used in this study:

Equipment	Company (Origin)
Centrifuge	Eppendrof (Germany)
Distallator	GFL (Germany)
Micropippet	Eppendrof (Germany)
Spectrophotometer	Shimadzu (Japan)

2.1.2 Kits

Chemical	Company (Origin)
AST and ALT	Biolabo (France)
Alkaline Phosphatase	Biolabo (France)

2.1.3 Afla Toxin Preparation

Afla toxin concentrate was previously preparaed and kindly provided from Al-Nahrain University – College of Biotechnology. Two different concentrations were supplied 1.5 and 0.75 ppm.

2.1.4 Experimental Animals

A group of Swiss albino BALB/c mice, which were obtained from the Al-Nahrain Biotechnology Research Center, were used in this study.

The group consists of 15 mice (males). They were used for the determination of liver function enzymes. Their ages ranged between (8-12) weeks and weighting (25-30) gram. They were divided into subgroups and each group was kept in a separate plastic cage. The cages were kept in animal house with 23-25ºC temperature. The mice were fed with suitable quantity of water and complete diet.

Three groups of mice were used in this experiment and treated as follows:

Group I: Control (5 mice): treated with 0.1ml of distilled water.

Group II: Afla toxin treatment (5 mice), treated with 0.1ml of afla toxin (1.5 ppm).

Group III: Afla toxin treatment (5 mice), treated with 0.1ml of endotoxin (0.75 ppm).

The distilled water and afla toxin were administrated orally for 14 successive days, and the mice were sacrificed by the end of the 14th day and blood samples were collected from all of animals from the heart for biochemical analysis.

2.2 Methods

2.2.1 AST and ALT Tests

According to Reitman and Frankel (1957), blood samples were collected from the mice by heart puncture. Blood left to clot and serum was separated by centrifugation at 5000 rpm for 10min. The serum was tested as follows:

Two test tubes were used for each sample, the first contained the blank reagent and the second contained the sample. These samples were tested as in the following:

	ALT	AST
Reagent 1	1 ml	---------
Reagent 2	---------	1 ml
Incubation for 5min at 37ºC		
Serum	0.2 ml	0.2 ml
Mixing and incubation at 37ºC	1 hour	30 min
Reagent 3	1 ml	1 ml
Mixing and let to stand for 20min at room tempreature		
NaOH 0.4N	10 ml	10 ml
Mixing and waiting for 5min and measure under conditions identical to those used for the standard curve.		

Wave length: 505nm (490-520nm).

Activities of these two enzymes in the serum were estimated from the activity table attached with the kit of each enzyme.

2.2.2 ALP Test

Samples used in this test were the same serum samples used for ALT and AST tests. To estimate the activity of ALP enzymes, procedure of Kind and King (1954) was used.

Four test tubes for each sample were prepared, the first contained the sample, the second contained the blank sample, the third contained the standard and the fourth contained the blank reagent, as shown below:

	Serum Sample	Serum Blank	Standard	Reagent Blank
Reagent 1	2 ml	2 ml	2 ml	2 ml
Incubation for 5min at 37ºC				
Serum	50 µl	--------	--------	--------
Reagent 2	--------	--------	50 µl	--------
Incubation for 15min at 37ºC				
Reagent 3	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Mixing very well				
Reagent 4	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Serum	--------	50 µl	--------	--------
D.W	50 µl	--------	--------	--------
Mixing and incubating at room temperature for 10min and away from light. The absorbance of the serum blank, standard and serum sample were measured at 510nm against reagent blank.				

ALP Activity = Serum Sample – Serum Blank / Standard x 20 (Kind and King Units/ 100 ml)**.**

3. Results and Discussion

3.1 Effect of Aflatoxin on Liver Functional Enzymes (AST, ALT and ALP) Level in Mice

The effect of Aflatoxin on liver functional enzymes has been investigated throughout this study by measuring serum markers of liver injury: AST (aspartate amiontransferase), ALT (alanine aminotransferase) and ALP (alkaline phosphatase).

Results in Table (3-1) and Fig. (3-1) showed that a significant difference in AST level was observed after treatment with higher concentrations 1.5 ppm (compared with the negative control 176.32 U/L, p=0.00043) and the recorded levels was (236.00 U/L). While the lower concentration showed no significant differences in AST levels as compared with the control.

Table (3-1): Aflatoxin effect on liver functional enzymes(AST, ALT and ALP)

Concentration (ppm)	Liver Function Enzyme		
	AST (U/L) Mean±SD	ALT (U/L) Mean±SD	ALP (U/L) Mean±SD
Control	176.32±10.26	84.11±7.61	66.64±5.99
0.75	193.33±5.51	86.44±2.94	82.09±5.47
1.5	236.00±8.32	162.44±6.25	97.56±3.00

Different letters: significant (p≤0.05) between the means of column.

At the same time, the influence of endotoxin on ALT level showed no significant changes with the lower concentration treatment compared with negative control (84.11 U/L). However an increase in ALT level was obtained when afla toxin dose increased, and reached its significant level (p=<0.0001) at 1.5 ppm

(162.44 U/L). Regarding ALP levels, aflatoxin treatment caused significant differences as compared with the negative control 66.66U/L) for both treatments with *p* value of 0.0161 and 0.0005, for 0.75 and 1.5 ppm, respectively.

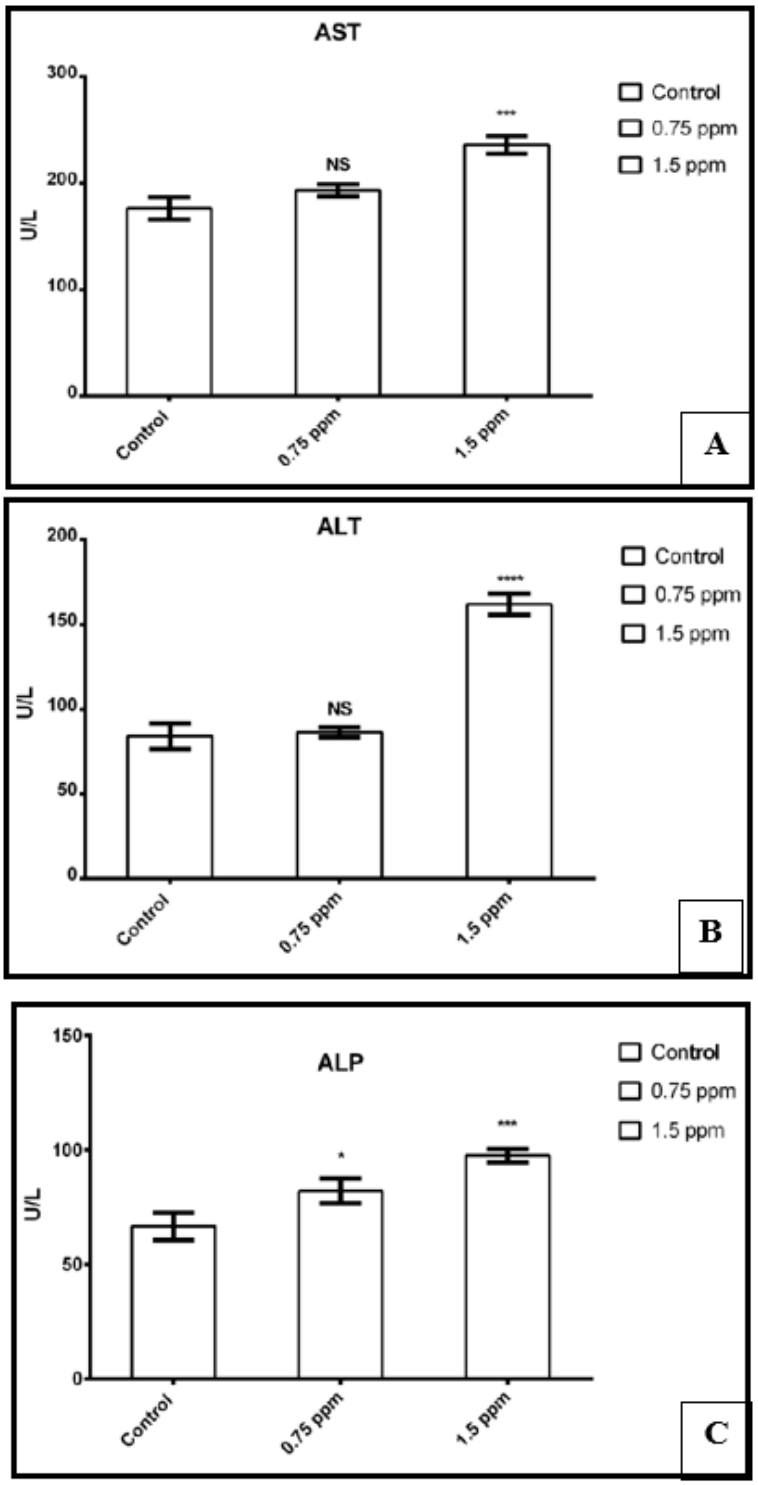

Fig. (3-1): Effect of Aflatoxin concentrations (0.75 and 1.5 ppm) on mouse liver function enzymes, (A) AST, (B) ALT and (C) ALP.

Results indicated that aflatoxin did affect the liver functional enzymes in which an increase in the levels of AST, ALT and ALP enzymes is significantly apparent following the administration of aflatoxin and the effect appeared to be dose dependent, only higher doses showed higher levels for each enzyme except for ALP in which 0.75 ppm also showed significant differences from that of the control.

Reports indicated that feeding rats with diet contaminated with AFS resulting in significant increase in AST, ALT and ALP in serum (Abdel- Wahhab *et al.*, 2002). Also Karakilcik *et al*., (2004) fed rabbits on diet AFB1 for 10 week noticed that, the levels of AST, ALT and ALP in serum were significantly elevated. Moreover, Preetha *et al*., (2006) mentioned that oral administration of rats by AFB1 resulting in a significant increase in ALP, AST and ALT activity in serum and decreased it in liver after 72h of treatment. The results also showed that the AFS oral administration significantly resulted in decrease of serum level of TP and albumin concentrations with increase of AF dose and time of treatment.

4. Conclusions and Recommendations

4.1 Conclusions

This study showed that aflatoxin caused significant changes in mouse serum liver functional enzymes (ALT, AST and ALP) in a dose dependent activity.

4.2 Recommendation

1) Examine the different classes of Aflatoxin and their exposure effect in vivo. 2) 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

Resources

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