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DETERMINATION OF AFLATOXIN IN THE BLOOD OF PATIENTS WITH HEPATOPATHY AND EVALUATION ITS EFFECT ON SOME IMMUNE PARAMETERS

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ABSTRACT

Background: *Aflatoxin-producing Aspergillus species, Aspergillus flavus and Aspergillus parasiticus, are a main producer of aflatoxins, which a class of toxic molds that can cause cancer and other health issues.* ***Aims of study:*** *Serum aflatoxin B1 detection, both qualitative and quantitative by using TLC and HPLC techniques; serum cytokine levels are used to assess the aflatoxin's impact on the human immune system.* ***Methods:*** *A study was carried out between November 2023 and January 2024. Blood samples were drawn from 150 Volunteer patients who were receiving care at Tikrit hospitals. Liver function tests (AST and ALT) were measure in the hospital used an automated device, ELISA test was used to detect IL-4, TNF-a, and IL-10, while detection of serum aflatoxin B1 was done both qualitatively and quantitatively using TLC and HPLC procedures.* ***Results****: Out of 150 sample, the results showed 87 (58%) patients with (3.995±0.450) level of Aflatoxin B1 compared with 63(42%) patients of non-AFB1 group which scored (0.14±0.007) level of AFB1, these analyses were performed using HPLC. The study results showed increasing serum liver enzymes of AFB1 group compared with non AFB1 group, the pro-inflammatory cytokines increased (16.01±1.31) for AFB1, while anti-inflammatory cytokines (IL-4, IL-10) decreased (9.96*±1.54, 4.90*±1.23) respectively.* ***Conclusion****: The study's findings suggest that toxins build up over time and can interfere with a variety of biological processes, any amount of toxin in the body is harmful. Aflatoxin changes pro- and anti-inflammatory cytokines and affects liver function.*

INTRODUCTION

Aspergillus flavus, a typical species of the Aspergillus section Flavi has been utilized as a model to investigate about emergence and synthesis of toxins by fungus (Cho et al., 2022). A. flavus is known produce aflatoxins, the most carcinogenic mycotoxin, and to induce aspergillosis in patients with compromised immune systems, other detrimental consequences on humans. Aflatoxin contamination due to A. flavus infections cause financial losses and crop output reduction (Baltussen et al., 2020). Mycotoxins are dangerous compounds that are naturally produced by some species of molds. Cereals, nuts, dried fruits, spices, and other foods, all can harbor mycotoxin-producing molds (Haque et al., 2020). The most commonly mycotoxins that are harmful to livestock and human health are aflatoxins, ochratoxin A, patulin, fumonisins, zearalenone, and nivalenol/deoxynivalenol (Rovina et al., 2020). Aflatoxin-producing Aspergillus species, *Aspergillus flavus* and *Aspergillus parasiticus*, are the main producers of

aflatoxins, which a toxic mold class that can cause cancer and other health issues. "They are probably the most well-known and thoroughly studied mycotoxins in the world," the USDA claims (Kumar et al., 2021). Aflatoxin exposure is especially dangerous for children since it can cause immunological suppression, delayed development, stunted growth, aflatoxicosis, spoilt food, and liver cancer. Aflatoxin exposure has been linked in certain studies to childhood stunting (Chen et al., 2018). There are currently over eighteen different types of aflatoxins known; the most prevalent and lethal types are aflatoxins B1, B2, G1, and G2. Early detection of fungal infections is critical to controlling aflatoxin exposure. Thus, a variety of approaches, such as chromatographic procedures, molecular assays, and culture are utilized to identify aflatoxin contamination in crops and food items (Shabeer et al., 2022). The most typical way that aflatoxin poisoning occurs through ingestion; however, B1, the deadliest aflatoxin molecule, can also enter the body through the skin (Su, 2019). AFB1-induced immunosuppression may be caused through a variety mechanism, such as alter cell surface receptors that are involved in T cell activation, production of inflammatory mediators and their receptors, activation of pro-inflammatory pathways, and regulation of apoptosis (Kipkoech et al., 2023). Because they regularly eat rice, corn, peanuts, and other foods high in AFs, humans are frequently exposed to low amounts of AFs. Through the food chain, AFB1 transfer from crops to humans, and has a major negative impact on health, leading to chronic liver damage and an increased risk of liver cancer. Lipid peroxidation is brought on by AFB1 metabolism in the liver and affects hepatocytes (Zhang et al., 2020).

Aims of study

Serum aflatoxin B1 detection, both qualitative and quantitative, using TLC and HPLC techniques; serum cytokine levels are used to assess the impact of aflatoxin B1 on the human immune system.

METHODOLOGY

Study design

A study involving 150 individuals, was carried out between November 2023 and January 2024. Samples were chosen from the patients who were receiving care at the Tikrit hospital.

Blood Collection and Preservation

Ten milliliters of blood were drawn from each participant's vein using sterilized syringes, gel tubes holding the blood were brought to the main lab. Serum was extracted from the samples by centrifuging them for 15 minutes at 3,000 rpm after letting them settle for 15 minutes. Before analysis, each serum sample was divided into two parts and stored in freeze that at -20 C. The following parameters were then assessed using these serum samples:

1.Use of TLC and HPLC techniques to detect serum aflatoxin B1 in a qualitative and quantitative manner
2. AST and ALT measurements of liver function tests performed in hospitals using an automated equipment.
3. By using an ELISA, IL-4, TNF-a, and IL-10 were found.

Cytokine profile

ELISA kits of interleukin (IL)-4 (RAB0301, Sigma, USA), IL-10 (catalog no. ERI3010-1, Assaypro, USA), and tumor necrosis factor-α (TNF-α) (catalog no. ERT2010-1, Assaypro, USA) were used to count cytokines amount in the serum that was collected. The color difference was

measured at 450 nm using a microplate ELISA reader (BIO-TEK, INC., ELx, 800UV, USA) in accordance with the manufacturer's instructions.

Qualitative analysis of serum AFB1 by TLC

AFB1 Extraction from Serum Samples

Twenty microliters of proteinase K solution were added to each 700 ul test tube containing serum samples. Next, for ten minutes, the test tubes were left to react. Each sample had its filtrate extracted and the precipitate left behind after the mixture was centrifuged for 15 minutes at 3000 x g. The filtrate was divided into two layers (the serum layer and the chloroform layer) then one milliliter of chloroform (twice its size) was added and the mixture was vigorously shaken in the electric shaker apparatus. Next, using a separating funnel, the chloroform layer was separated and put in a sterile glass tube where it was left to evaporate.

Using Thin Layer Chromatography (TLC) to detect AFB1

The twenty-by-20-cm TLC plates coated with silica gel were utilized; they were first activated in an electric oven for one hour at 120 °C. At a distance of 1.5 cm from the top and bottom of the plate's base, a thin, straight line was drawn. The bottom line was used to load samples, while the top line was numerically applied. Chloroform: Methanol (98:2) was the mobile phase that was utilized to extract AFB1. Using a capillary tube, the AFB1 stock standard (15 ul) was placed on the TLC plate. Next, each extracted sample was added to the plate in increments of 15 ul, spaced 2 cm apart. After that, the areas were left to dry at ambient temperature. The plate was then inserted into the separation tank, which held a mobile phase. The thin layer plate was maintained in the tank until the mobile phase was 2 cm from the upper plate edge. Following its removal from the tank, the TLC plate was left to dry outdoors. The color and relative flow (RF) of the extracted samples were then compared to a reference toxin utilizing UV light (360 nm) inspection of the plate. HPLC-Based Quantitative Analysis of AFB1 Liu et al. (2012) state that the HPLC model SYKAM (Germany) was used for the analysis and detection of thiamethoxam. (Liu et al., 2012).

Preparation of Standard AFB1 for HPLC

AFB1 was finally dissolved to a final concentration of 0.25 ng/ml in 100 milliliters of acetonitrile solution. The AFB1 standard was developed by this procedure. 3.4.2.2 Example Preparing: 500 uL of serum sample and 1 ml of hexane were carefully combined, and the mixture was then centrifuged at 2000 g for five minutes. Following centrifugation, the blood sample was once more filled with 1 milliliter of hexane, and the serum lipids were extracted from the top layer of the hexane. These serum samples were thoroughly mixed for 4 minutes with 1 ml of chloroform before being centrifuged at 2000 g for 10 minutes. After scraping off the bottom layer of chloroform, nitrogen vapor was used to dry the area. It was dissolved using a 50 ul solution of methanol, water, and acetonitrile (25:25:50).

An isocratic acetonitrile, D.W. (30:70 v/v), flowing at a rate of 0.7 mL/min, was the mobile phase. The column was C18-ODS (25 cm * 4.6 mm), and the detector was Florescent (Ex = 365 nm, Em = 445 nm). Volume of injection: 50 ul

$$\textbf{Con. Sample (ng/ml)} = \frac{Con.Standard * absorption\ sample}{absorption.Standard} * \frac{Dilution\ factor}{Volume\ sample}$$

Statistical Analysis

Data gathered from the questionnaire, each patient, and the outcomes of the control tests. Samples were entered into a data sheet. The statistical application IBM SPSS 26 was utilized to

generate the data analysis for this investigation. Descriptive statistics were performed on the participant data for every group. To perform multiple comparisons between the groups, the Duncan test and the ANOVA table were employed. There is a statistically significant difference between the groups if the P-value is less than 0.05. The association between the variables was ascertained by comparing the observed and expected findings using the chi-square test.

RESULTS AND DISCUSSION

TLC's qualitative assessment of AFB1

The findings indicated that 87 (58%), or sample serums, had been taken from patients contaminated with AFB1, whereas 63 (42%) had been taken from individuals not contaminated with AFB1. The outcomes are displayed in Table 1. figure (1).

Table 1: Distribution of sample according to AFB1 groups by using TLC

Case	No. of samples
AFB1 group	87(58%)
Non-AFB1 group	63(42%)
Total	150(100%)
* Chi-Square Tests; X^2 Table (0.05) =.005, X^2 Calculate = 41.76, AFB1 = AflatoxinB1 Frequency =.005	

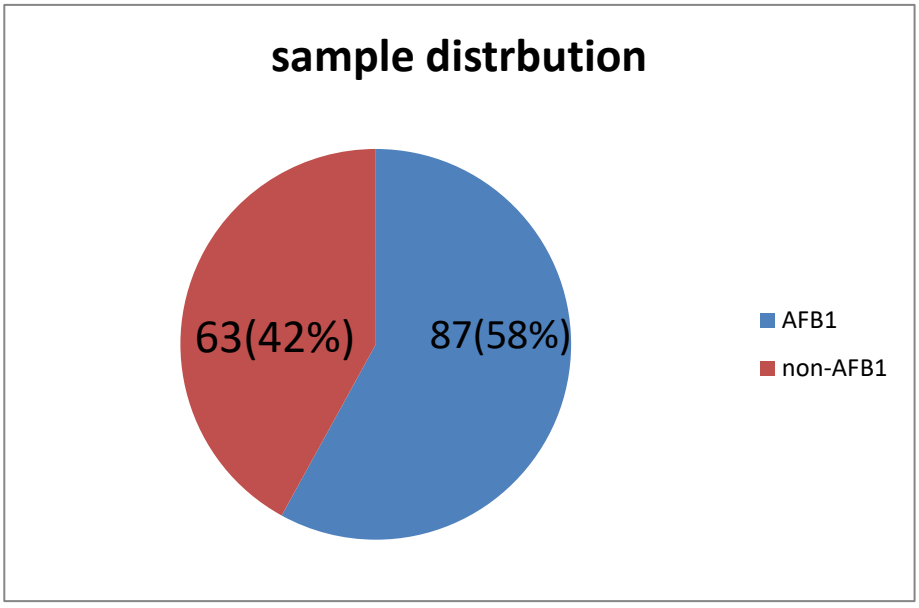

Figure (1): Sample distribution using TLC based on AFB1 groupings

Measurement Quantitative of the AFB1 by HPLC

AFB1 was evaluated quantitatively by determining the toxin concentrations in the research groups using HPLC apparatus. The statistics showed that there was a highly significant difference between the study groups, with a P-value of less than 0.001. The highest toxin levels (3.995 ng/ml) were found in the patients. Furthermore, the control group's toxin levels (0.14 ng/ml) were the highest. As seen in table (Baltussen et al., 2020), these quantities are considered high when compared to healthy individuals.

Table 2: Measurement concentration AFB1 by HPLC in patients and control groups

Groups	Mean ±SD	P-value
Patients	3.995±0.450a	0.001*

Control	0.14±0.007b	
*= significant p<0.001; A significant difference between the study groups is indicated by the difference in the letters.		

Measurement of AST and ALT enzymes in study groups

The result of liver enzyme measures in patients with AFB1 group and non- AFB1 group showed that level of AST was high in AFB-1 group (41.69 ±3.41) compared with non-AFB1 group (15.62±5.10b). While serum ALT level scored (40.20±4.31) in AFB1 group and (16.66±5.00) on non-AFB1 group, with a significant different between them, as shown in table (3).

Table 3: Liver enzyme concentration (mg/mL) in serum of studied groups

Liver enzyme	Groups	Mean± SD	P-value
AST	Patients with AFB1	41.69 ±3.41a	0.001*
	Patients without AFB1(control)	15.62±5.10b	
ALT	Patients with AFB1	40.20±4.31a	0.001*
	Patients without AFB1(control)	16.66±5.00b	

Cytokine detection by ELISA

At p<0.05, the AFB1 group was associated with a significant increase in the pro-inflammatory cytokine (TNF-α 16.01±1.31) and a significant decline in the anti-inflammatory cytokines (IL-4 and IL-10, 9.96±1.54, 4.90±1.23), relative to the levels of TNF-α, IL-4, and IL-10 in non-Aflatoxin B1 (11.99±2.11, 13.47±1.33, 9.65±1.20), respectively. Table (4) and Figure (2).

Table 4: Cytokines concentration (ng/mL) in serum of studied groups

Parameters	AFB1 group	Non-AFB1 group	P-value
TNF-α	16.01*±1.31	11.99±2.11	p<0.05
IL-4	9.96*±1.54	13.47±1.33	
IL-10	4.90*±1.23	9.65±1.20	

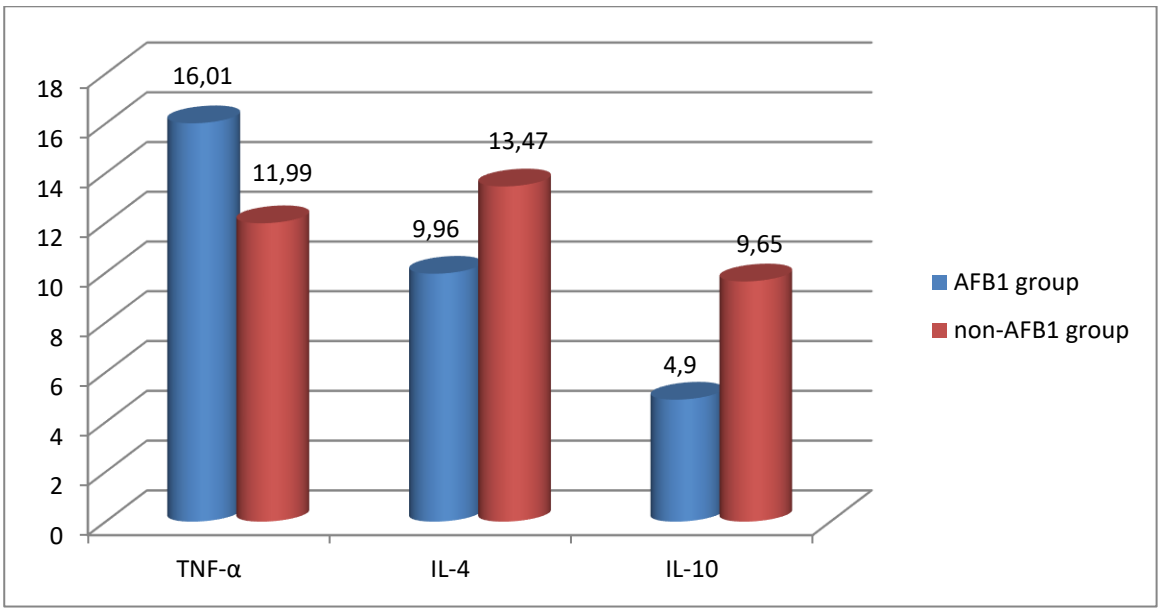

Figure 2: Cytokines concentration (ng/mL) in serum of studied groups (AFB1 and non-AFB1 groups)

Discussion

An illness known as aflatoxicosis falls within the category of hepatocellular abnormalities and is typified by the infiltration of inflammatory cells, necrosis, and apoptosis (Eom et al., 2020). Abd AL-Redha et al. (2017), who discovered a connection between toxins and both patients and controls, were consulted in the analysis of these data. The cause is attributed to AFB1's detrimental effects on patients, which may worsen their condition. It results from consuming a lot of contaminated food, undercooked meat, and vegetables, as well as ongoing exposure to infected foods. According to Hassan et al., rice in Lebanon has a notable amount of AFB1 contamination. It was calculated that eating rice every day in Lebanon will expose people to AFB1 at a dose of between 0.1 and 2 ng/kg of body weight (Hassan et al., 2022). For patients with probable chronic kidney disease (CKD), AFB1 concentrations in blood samples ranged from 0.68 to 8.33 ng/mL, for those with confirmed CKD from 1.21 to 5.6 ng/mL, and for healthy controls from 0.11 to 1.30 ng/mL (Kareem et al., 2015). Our findings supported their conclusions.

The results supported the conclusions of other studies, including those by Li et al. (Li et al., 2022), Karamkhani et al. (2020), and Navale et al. (2021), which showed that AFB1-containing groups had higher levels of ALT and AST than control groups. AST and ALT rise in response to consumption of AFB1. The circulatory and digestive systems quickly absorb it, transporting it to the liver (Lalah et al., 2019). AFB1-lysine, which is present in albumin, is one instance of an adduct created when 1-3 percent of the consumed AFs form an irreversible connection with proteins and DNA bases. Damage to DNA and protein bases results in hepatocyte malfunction and liver damage. Chronic exposure is the outcome of consuming little amounts of AFs over an extended length of time (Kowalska et al. 2017).

Significant increases in TNF-α and decreases in IL-4 and IL-10 were linked to AFB1 poisoning. Serum TNF-α was significantly reduced and IL-10 was significantly increased when HDCs were used to treat IH. According to a study by Abo-Aziza et al. (Abo-Aziza et al., 2022) there was a significant +ve association between IL-10 downregulation and TNF-α and a strong −ve correlation between IL-4 and TNF-α in the AFB1 group.

The value of IL10 (3.785ng/l) obtained in comparison to the control group (3.493ng/l) indicates that the toxicity of aflatoxin B1 considerably lowers interleukin-10 levels in liver disease patients, according to a study by AL-Kelabi et al. (Al-kelabi & Al-Obaidi, 2021). Despite this, TNF-α levels in all rats given AFB1 in the Akinrinmade et al. trial stayed mostly unaltered (Akinrinmade et al., 2016).

CONCLUSION

The study's findings suggest that since toxins build up over time and can interfere with a variety of biological processes, any amount of toxin in the body is harmful. Aflatoxin changes pro- and anti-inflammatory cytokines and affects liver function.

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