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Original Research Article
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EFFICACY OF LYCOPENE ON AFLATOXIN B1- INDUCES OXIDATIVE STRESS, HEPA-TOTOXICITY, APOPTOSIS AND IMMUNODEFICIENCY IN JAPANESE QUAIL

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Abstract: Mycotoxins are harmful auxiliary metabolites delivered by species of filamentous organisms developing on grains some time recently collect and in capacity. The study was planned to evaluate the protective role of lycopene (LYC) against Aflatoxin B_1 (AFB_1) induces oxidative stress, hepatic toxicity, and apoptosis in Japanese quail. Sixty Japanese quail chicks (three-week-old) were randomly allocated into four groups; Negative control group; Aflatoxin -B_1 (AFB_1) group (1 mg/kg feed) as positive control; lycopene supplemented group (200 mg/kg feed) and AFB_1 (1 mg/kg feed) with lycopene supplemented group (200 mg/kg). The growth performance parameters, serum biochemical indices, and liver antioxidant activities as well as histopathological studies, and immunohistochemical pictures were performed. The results showed a significant increase on growth performance and a significant decrease on feed conversion ratio (FCR) in aflatoxin inclusion with lycopene supplemented group. Moreover, the liver enzymes (ALT, AST, and ALP) were significantly ($P < 0.05$) decreased. In addition, malondialdehyde (MDA) levels significantly decreased, while superoxide dismutase (SOD), Glutathione peroxidase (GSH-Px) and catalase (CAT) activities increased in liver tissues. Furthermore, when compared to the aflatoxin-inclusion with the lycopene supplemented group had improved liver tissue and lower levels of cytokine production (IL-6 and TNF-). In conclusion, these findings implied that dietary administration of lycopene has significantly alleviated AFB_1-triggered oxidative stress, inflammatory response and liver apoptosis in Japanese quail.

Key words: Lycopene; Aflatoxin B_1; hepatotoxicity; cytokine; liver apoptosis; Japanese quail

Introduction

Mycotoxins are poisonous compounds that are normally created by certain sorts of moulds (organisms). Moluds that can deliver mycotoxins develop on various foodstuffs, such as cereals, dried natural products, nuts, and flavors. Most mycotoxins are chemically stable and survive nutrient preparation. About a fourth of the cereals are globally estimated to be contaminated by fungal mycotoxins (1). Exposure to the danger of mycotoxins can occur either through contaminated cereals or through residues found in meat and eggs, such as aflatoxins, which are the end products of two fungal species named (*Aspergillus flavus* and *Aspergillus parasiticus*) that predominantly spoil feed ingredients during storage, especially in warm and humid climates (2). Aflatoxin B_1 (AFB_1) has been stated to induce various health problems, including hepatotoxicity, teratogenicity, mutagenicity, and carcinogenicity (3). AFB_1 has been categorized as a Group I carcinogen for humans via the "International Agency for Research on Cancer (IARC)" (4). The reactive metabolite (AFB1-exo-8,9-epoxide) produced from AFB_1 biotransformation by cytochrome P450. This metabolite robustly binds to DNA adducts, generating reactive oxygen species and leading to the AFB_1 carcinogenic effect

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(5). Indeed, aflatoxin poisoning has been linked to liver cancer in a number of countries, particularly in Asia and Africa, where grains are more likely to be contaminated with aflatoxin due to favourable humid conditions (6). Previous studies provided evidence that oxidative stress, inflammation, and cell apoptosis play a significant role in AFB_1-induced toxicity, suggesting that antioxidants could be used as a convenient approach to limit the progress of the toxic impacts associated with acute AFB_1 toxicity (7). Therefore, there is a dire need for finding antioxidant substances that might be effective in alleviating the deleterious effects of AFB_1 exposure. Lycopene (LYC) is a naturally occurring carotenoid that is mainly found in tomatoes, tomato by-products, grapefruit, guava, papayas, and red peppers (8). Lycopene proves to have anti-inflammatory, anticancer, and antioxidant activities as well as anti-cardiovascular disease capability and detoxification activity (9). A previous study reported that lycopene supplementation at a 5 mg/kg dose could relieve liver damage in mice (10). LYC has potent free radical scavenging efficacy, protecting the liver, kidney, mucous membrane, and immune system against AFB1-triggered oxidative stress (11). Based on the prior studies, we assume that Lycopene has a protective effect against AFB_1-triggered hepatotoxicity. Hence, the aim of this study is to investigate whether LYC supplementation exerts anti-inflammatory, antioxidant, and antiapoptotic effects on the damaged liver and kidneys in the Japanese quail chicks fed AFB1-contaminated diets.

Material and methods

Feed additives

Aflatoxin B1 was obtained from the Animal Health Research Institute, Dokki, Giza, Egypt. It was added to the diet at a dose of 1 mg/kg diet (12). Lycopene extract powder was purchased from Giftlover natural herbal company, China, and added with a dose of 200 mg/kg diet (13).

Birds

A total of sixty (21days-old) unsexed Japanese quail chicks (Coturnix japonica) with initial body weight of 128.56 ± 1.75g were used in this study. Birds were randomly divided into four treatments (n = 15), and each group included three replicates (n = 5) and were placed into galvanized wire cages (95×65×160 cm) and reared up to day 56 of age.

Experimental design

The experimental groups were as follow: 1) control group; fed basal diet, 2) AFB_1group; received AFB_1-supplemented diet (1 mg/kg diet), 3) Lycopene group; fed Lycopene supplemented diet (200 mg/kg diet), 4) AFB_1+ LYC group; fed 1mg AFB1 + 200 mg LYC /kg supplemented diet. Quail chicks were housed in battery cages rather than litter floor. Initial temperature was adjusted at 22°C. The nutritional requirements were prepared according to the recommendations of National Research Council (14). Free access for feed and water was provided on lightening schedule of 18 h of daylight and 6 h of darkness. During the experiment, body weight (BW) and feed intake (FI) were recorded weekly. The data were used to calculate average weekly gain (AWG), average weekly feed intake (AWFI) and feed conversion ratio (FCR) weekly and cumulatively. All experimental procedures of this study were conducted according to the guidelines of the "Local Experimental Animal Care Committee and the ethics of the institutional committee of Department of Pharmacology, Faculty of Veterinary Medicine, Zagazig University, Egypt".

Sampling

At 56 day of age, nine birds were chosen randomly (n = 3 birds per replicate). Sera were obtained by blood samples centrifugation at 2,500 rpm for 10 min at 4°C and stored at -20 °C until performing the biochemical analyses. Liver samples were rapidly dissected out and rinsed with NaCl 0.9% and divided into 2 parts; the 1st part was snap frozen then stored at -80 °C until used for hepatic antioxidant enzyme assay analysis and the 2nd part was immediately fixed in 10% buffered formalin for histopathological examination.

Liver and kidney functions

For assessment of liver function parameters, the concentrations of aspartic aminotransferase (AST), alkaline phosphatase (ALP), and alanine aminotransferase (ALT) were estimated. Creatinine

and uric acids were determined. These parameters were spectrophotometrically assayed by using semi-automated spectrophotometer (Erba-Chem7, Germany) and using commercial kits as mentioned by(15) following the manufacturer's instructions ("CUSABIO BIOTECH CO. Ltd., Houston, TX 77054, USA").

Serum inflammatory markers

The levels of cytokines including tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) were estimated in serum using commercial ELISA kits ("CUSABIO BIOTECH CO. Ltd., Houston, TX 77054, USA").

Hepatic antioxidant enzyme assay

The activities of hepatic antioxidant enzymes were assayed according to (16). Malondialdehyde content (MDA), catalase (CAT) Glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities were assessed using specific kits from ("CUSABIO BIOTECH CO. Ltd., Houston, TX 77054, USA") according to the manufacturer's instructions. These parameters were spectrophotometrically assayed by using semi-automated spectrophotometer (Erba-Chem7, Germany).

Histopathological study

Excised liver specimens were fixed in 10% buffered formalin. Fixed specimens were dehydrated through graded concentrations of ethanol, cleared in xylene and then embedded in paraffin wax. Afterwards, paraffin blocks were cut into sections of 5 µm in thickness and stained with hematoxylin-eosin (H&E).The method was carried out as previously explained by (17).

Histochemical study for caspase-3 expression in liver:

The procedure was performed according to (18). Briefly, for immunohistochemical (IHC) staining, sections from the selected paraffin blocks were cut into 4 µm thick sections. The endogenous peroxidase was blocked by immersing slides in 3% H_2O_2 for 10 min at room temperature. After washing, slides were incubated with primary anti-caspase 3 antibodies. Afterwards, incubation with the appropriate secondary antibody was performed. Prior to dehydration and mounting, all slides were lightly counterstained with hematoxylin for 30 sec. Apoptotic cells were seen by microscope as cells with brown nuclei.

Immunohistochemical scoring

Cytoplasm of hepatocytes reacting to Caspase 3 was considered positive. Stained tissue sections undergo semi-quantitative analysis which accomplished through modified Allred scoring system guidelines (19). The average number of positive cells was estimated after counting them in three different high-power fields (HPF) (400 xs). Individual percentages of positive cells (0–5) and cytoplasm staining intensity (0–3) were summed to get the final grades. The percentage of positive cells was determined as follows: 1) less than 10 positive cells; 2) between 10-20 positive cells; 3) between 20-50 positive cells; 4) between 50-70 positive cells; and 5) for more than 70 positive cells. The staining intensity was scored as: 1- weak; 2- medium; and 3-strong.

Statistical analysis

Data were analyzed using The Graph Pad Prism software (version 9, San Diego, USA). Kruskal Wallis was used to determine the statistical significance of the various groups. Dunn's multiple comparisons test, post hoc test was performed for in-between groups' comparison. ($P < 0.05$) was selected as the significance level. The data is displayed as mean ± standard error.

Results

Effect of Lycopene and aflatoxin on growth performance

(Table 1), Non-significant ($P > 0.05$) increase was detected in FI throughout the experimental trial. Nonetheless, birds fed dietary AFB_1 showed a reduction in AWG ($P < 0.05$) and increase in FCR as compared to the group fed the basal diet. Japanese quail group fed a combination of AFB_1 and LYC exhibited an improvement in AWG compared to the group fed AFB_1 only. Besides, a decrease ($P < 0.05$) in FCR was observed in AFB_1 + LYC group compared to AFB_1 group. Moreover, feeding AFB_1 supplemented with LYC improved BW (+14.72%) and BWG (+25.66%) compared to the AFB_1 treated group.

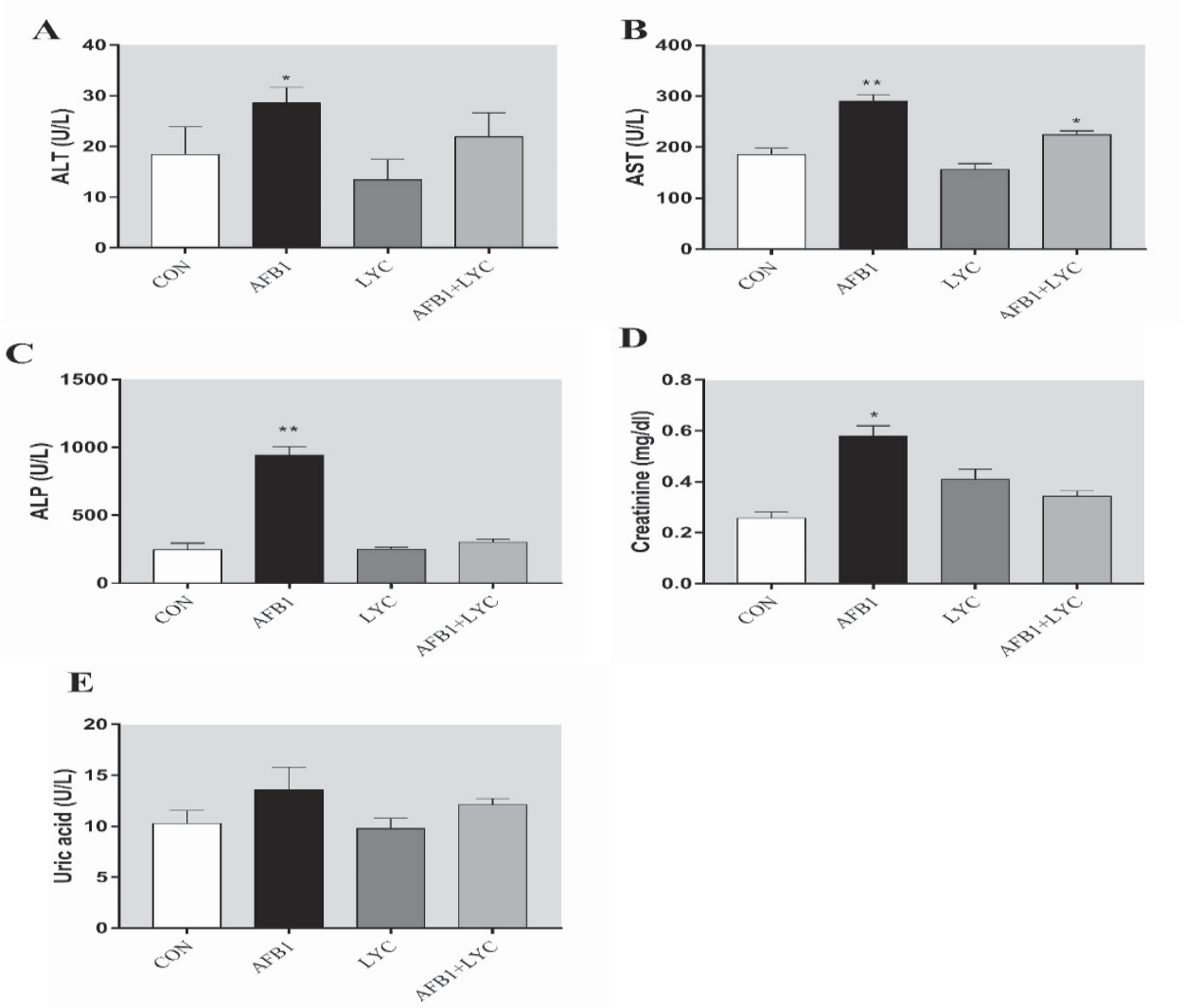

Figure 1: Effect of LYC on quails' liver and kidney functions of fed diets contaminated with AFB1. Values are represented as the mean ± SE (n = 9). Means within the column with different stars are significantly different, $p< 0.05$

Table 1: Effect of dietary supplementation of LYC and AFB1 on growth performance

Diets	Final weight(g)	Weight gain (g)	Feed intake(g)	FCR (%)
CON	297.43±8.17	177.14±11.07	270.35±5.91	1.53±0.086
AFB1	279.43±7.19 *	151.43± 5.38*	266.31±8.12	1.77±0.064*
LYC	330.57±4.81	191.71±3.508	271.51±4.93	1.41±0.024
AFB1+LYC	320.57±3.26	190.29±4.58	282.03±6.33	1.49±0.036

Values are represented as the mean ± SE (n = 15 per group). Mean values within a column with (*) were significantly different (P < 0.05). CON: control; AFB_1: Aflatoxin B1; LYC: Lycopene; FCR: feed conversion ratio

Ability of Lycopene to restore abnormal liver and kidney function enzymes caused by AFB1

As summarized in Fig. 1, AFB_1 exposure for 35 days elicited a noticeable increase in the levels of serum ALT, AST and ALP compared to the control group. Conversely, LYC co-administration decreased (P <0.05) the levels of these biomarkers in the group received dietary AFB_1. No remarkable (P > 0.05) alterations were detected in serum ALT, AST and ALP levels between the control and Lycopene groups. Whereas, AFB_1 inclusion significantly ($P< 0.05$) elevated creatinine level compared to that of the control group. Thus, LYC addition improved($P<0.05$) kidney function, as evidenced by the decreased creatinine level when compared to the AFB_1 group.

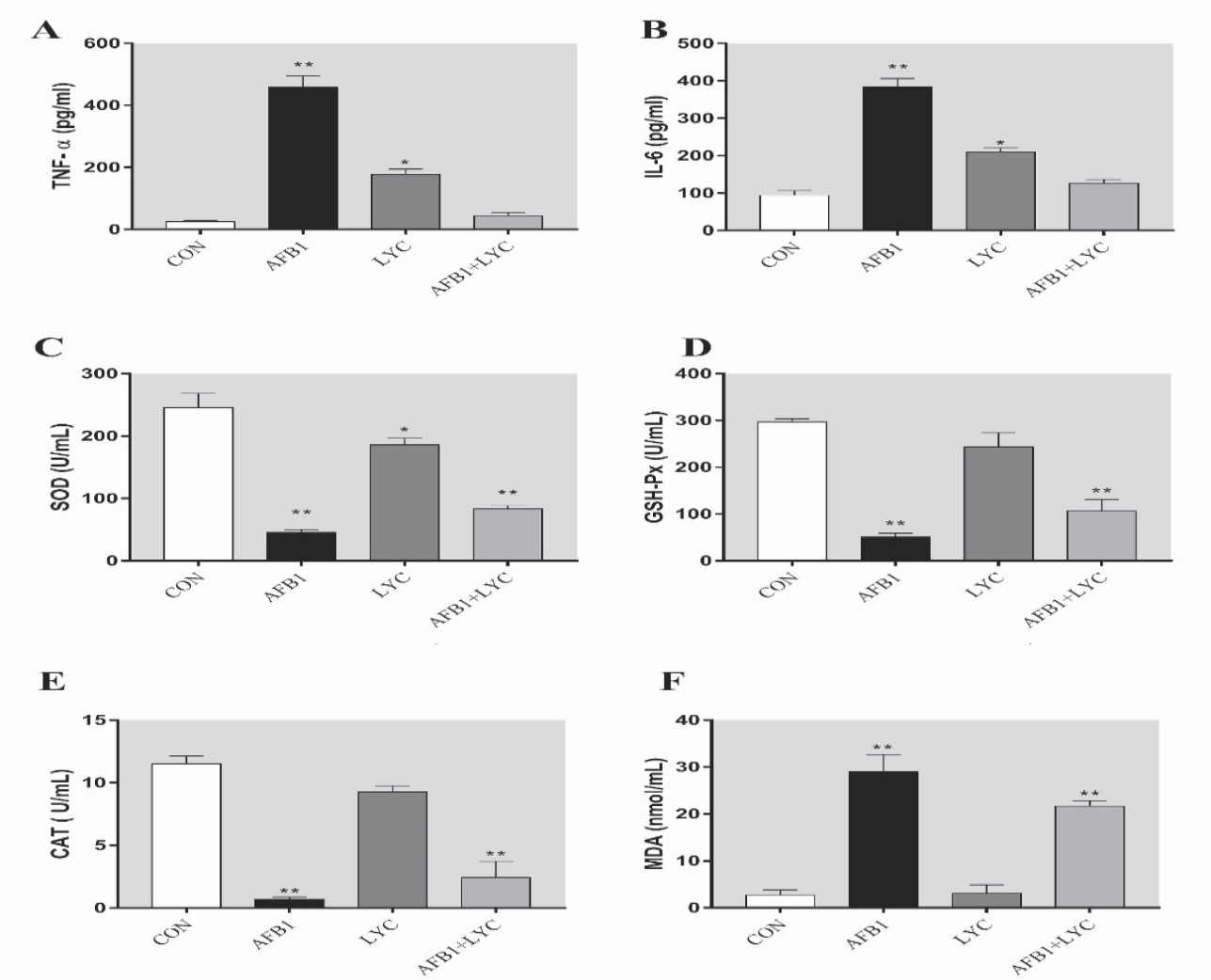

Figure 2: Effects on AFB1-induced liver oxidative stress markers and serum inflammatory cytokines. Values are repre-sented as the mean ± SE (n = 9). *column with different superscript letters were significantly different ($p < 0.05$). AFB1: aflatoxin B1); LYC: Lycopene; SOD: total superoxide dismutase; MDA: Malondialdehyde; GSH-Px: glutathione peroxidase; CAT: catalase, IL6: interleukin-6; and TNF-*a*: Tumor necrosis factor-alpha

Lycopene reversed AFB1-induced Inflammatory Response

As depicted in Fig. 2, birds exposed to AFB_1 showed a marked increase ($P < 0.05$) in the serum levels of IL-6 and TNF-α compared to the control group. Remarkably, supplementation of LYC reduced the elevation of IL-6 and TNF-α triggered by AFB_1. Hence, the results indicated that LYC could alleviate the negative influences evoked by AFB_1 on the inflammatory response.

Lycopene inhibited AFB1-induced oxidative injury

In order to assess the extent of oxidative damage, MDA,CAT,GSH-Px and SOD were measured in liver tissues. Compared to the control group, the activities of CAT, GSH-Px and SOD enzymes were significantly reduced in groups received the AFB_1-contaminated diet ($P< 0.05$), whereas the MDA levels increased ($P< 0.05$) in the AFB_1-fed group compared to those in birds fed the control diet. Notwithstanding, feeding a diet containing LYC (200 mg/kg) to AFB_1-fed quail could significantly mitigate the reduction in CAT, GSH-Px, and SOD levels and the increase in MDA level ($P< 0.05$). The results confirmed that LYC could prevent the oxidative injury of liver tissues provoked by AFB_1 as displayed in Fig. 2.

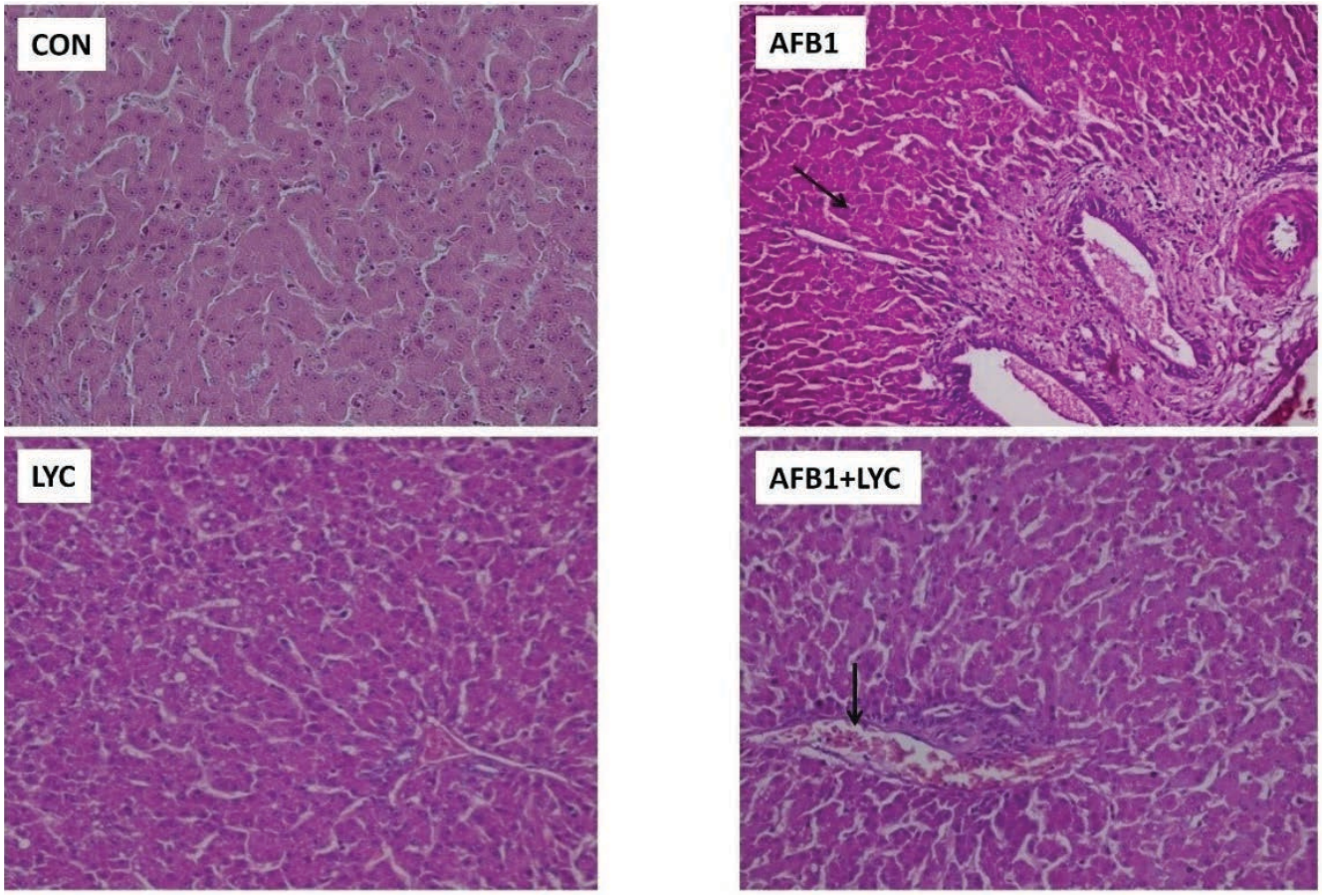

Figure 3: H&E-stained liver sections of CON Control quails showing normal histological appearance of the liver, AFB1 Hepatic tissues of quails treated with aflatoxin revealing marked fibrosis of portal traid area associated with focal dilation of hepatic sinusoids (arrow) and LYC+AFB1 aflatoxin -induced quails treated with Lycopene revealing almost normal portal traid area associated with moderate dilation of hepatic sinusoids (arrow). (H&E, X400)

Table 2: Effect of dietary supplementation of LYC and AFB1 on immunohistochemical staining of the liver sections

Item	Control	Lycopene	AFB1	AFB1+ Lycopene
Average number of positive cells ± SD	23 ± 4.5 3	27 ± 8.5 3	109± 20.5 5	53.3± 7.6 4
Intensity of staining	Weak 1	Weak 1	Strong 3	Moderate 2
Total score	3	3	> 5	4

Histopathological observation

As depicted in Fig. 3, the control and LYC (200 mg/kg) treated group showed no marked histopathological lesions in the liver of Japanese quail. Conversely, the liver sections from the AFB_1 group, showed severe histopathological alterations ($P< 0.05$), including marked fibrosis of the portal triad area associated with focal dilatation of hepatic sinusoids compared with those from quail fed the control diet. Noteworthy, the supplementation of LYC (200 mg/kg) to AFB_1 diet counteracted the hepatic injury evoked by AFB_1. Moreover, histological findings assured the beneficial effect of LYC on ameliorating the toxic impacts triggered by AFB_1 in Japanese quail.

Effect of Lycopene treatment on caspase-3 expression

To elucidate how LYC protects the liver from AFB_1 damage, the proapoptotic protein expression level of caspase-3 was estimated by the im-

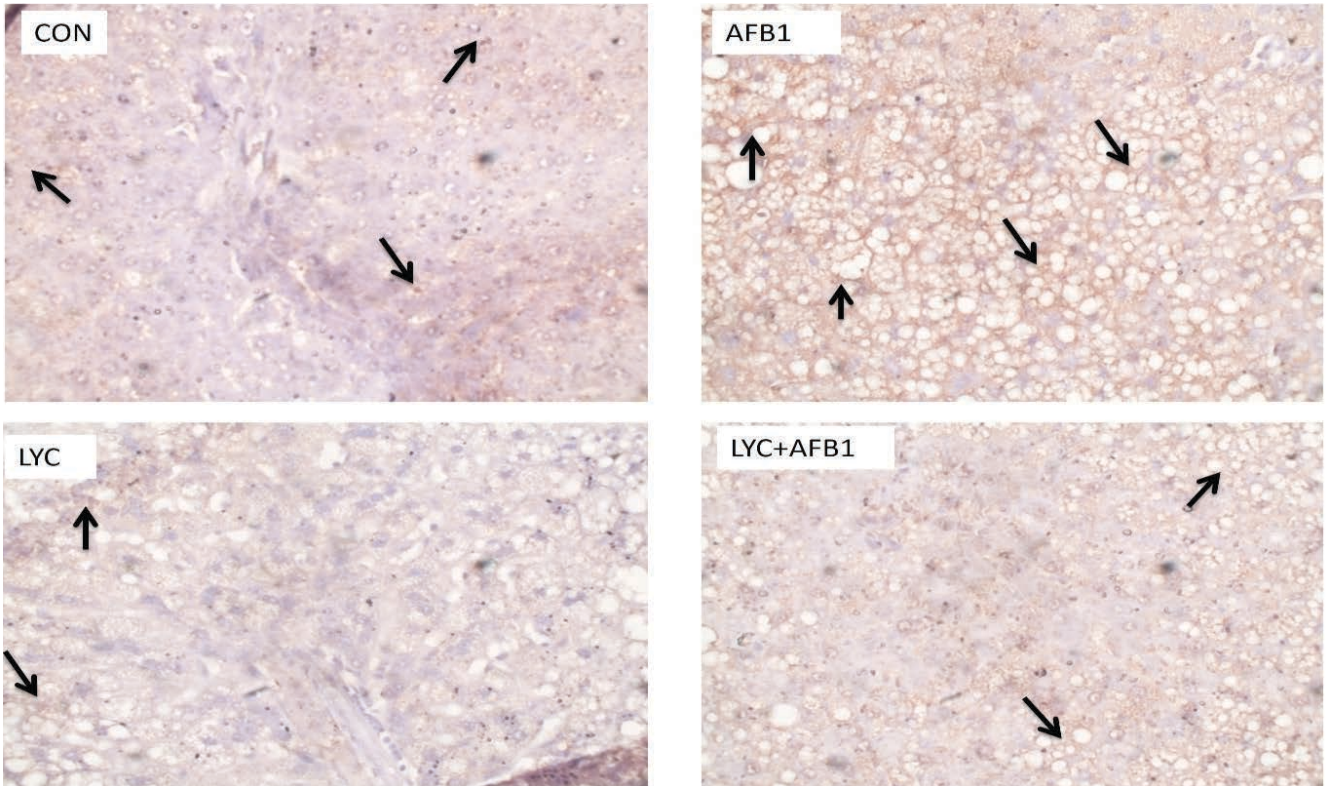

Figure 4: The immunohistochemical staining of the liver sections, positive cells appeared as brown cells (Black arrows). In CON group Scattered uniform hepatocytes show weak positive reaction to caspase 3 antibody, In AFB1 group hepatocytes show weak positive reaction to caspase 3 antibody, In LYC group hepatocytes show strong positive reaction to caspase 3 antibody and in LYC+AFB1 showed Significant reduction in number of cells and intensity of reaction to caspase 3

munohistochemical method. The results revealed a marked increase ($P < 0.05$) in caspase-3 expression level in the liver tissues in the AFB_1 group compared to that in the control group as shown in Fig. 4 and Table 2.

On the other hand, co-treatment of LYC with AFB_1 decreased the expression of caspase-3 than that in the AFB_1diet (Fig. 4).

Discussion

The dangers of mycotoxin contamination in feed resources are still an unavoidable problem, which should be taken into account to avoid its adverse effects on both humans and animals. The present study evidently demonstrated the influence of LYC on hepatotoxic and nephrotoxic implications of AFB_1, which were expressed as impaired growth rate, increased the activities of AST, ALP, and ALT; while the activities of CAT, GSH-Px, SOD as well as the activities of apoptotic protein caspase-3 were enhanced. Our data recorded a significant reduction in body weight (-6.05%) and BWG (-14.51%) in AFB_1-challenged quail against to the control group. The negative impact of AFB_1 on growth performance could be ascribed to the changes induced by AFB_1. AFB_1 could contribute to altering the intestinal morphology, absorbing function, decreasing the activity of digestive enzymes as (pancrelipase, amylase, and trypsin) and modifying the cellular energy metabolism by disturbing the gluconeogenesis and fatty acid synthesis (20). These findings match those observed in the previously published results of (21), which revealed that AFB_1 consumption has detrimental influences on the growth rate, evidenced by reduced WG and increased FCR in mice. Children exposed to aflatoxin toxicity not only suffered from serious health problems as growth impairment and stunted growth, but also died from infectious diseases, lower school achievements, decreased life-time earnings, and productivity (22).In our study, the inclusion of Lycopene (200 mg/kg) into the AFB_1 contaminated diets improved body weight (+14.72%) and BWG

(+25.66%) when compared with those in the AFB_1 group. Substantially, some authors have observed that dietary administration of LYC in chicken diets not only increased ADG but also could relieve the deleterious effects due to stress conditions on growth performance(23).The study of (24) showed that chronic aflatoxin exposure in African children is closely related to low birth weight, stunted growth, immunosuppression and impaired liver function. Moreover, LYC co-treatment in quail's diets improved the weight gain and FCR even under stress conditions (25). The observations of the current work are similar to those of (20) who reported that dietary LYC administration (200 and 400 mg/kg) significantly improved the intestinal digestive enzymes (amylase and lipase) activities in broiler chicks received AFB_1 diet. The possible explanation for the increased enzyme activities could be attributed to improving the intestinal morphology and integrity by dietary LYC supplementation, thereby, improving the growth performance of birds challenged with AFB_1. The increased activities of AST, ALT and ALP in serum have been known as crucial pointers for the diagnosis of hepatocellular damage or dysfunction, or bile duct obstruction (26). Therefore, in the current study, quail chicks fed AFB_1 contaminated diet had elevated levels of AST, ALP, and ALT, indicating liver injury. This could be attributed to the fact that AST and ALT, which are normally found in the cytoplasm, are only released into the bloodstream when the structural integrity of the liver is altered, resulting in enzyme leakage (27). In our study, the histopathological picture of the liver in AFB_1 challenged quail showed destroyed hepatic structure and hydropic/fatty degeneration which confirmed the increase in ALT, AST and ALP activities. However, these histopathological and biochemical alterations were reversed by the addition of LYC to the quail diet. These findings agree with the findings reported by (28) indicating that LYC supplementation provides significant protection against AFB_1-induced liver injury. Moreover, our findings revealed an increment in the levels of creatinine and uric acid, which act as indices of impaired renal functions. This result is similar to previous studies conducted on broilers (29)and quail (30)following ingestion of AFB_1 diets. Prolonged exposure to toxins poses potential risks to the body's health. AFB_1 toxicity causes hepatic injury, which in turn leads to liver cirrhosis and even cancer in severe cases (31). On the other hand, our data showed that co-supplementation of LYC to the AFB_1-treated diet significantly improved the negative impacts of AFB_1 on the liver and kidney. Supporting these results, the previous study of (34) stated that LYC inclusion substantially alleviated aflatoxin (AF) induced nephrotoxicity and hepatotoxicity. The CAT, GSH-Px and SOD enzymes have an essential role in the antioxidant defense system due to acting as scavengers for free radicals produced by oxidative stress, reducing oxidative damage, and maintaining cell structure (33). The MDA content acts as a key indicator for assessing the potential antioxidant capacity of the body (34). Therefore, the degree of oxidative damage can be evaluated by estimating the activity of antioxidant enzymes (CAT,GSH-Px and SOD) and the content of MDA. Our data revealed that ingestion of AFB_1 decreased the activities of CAT,GSH-Px and SOD, but elevated the MDA content. Our results are in line with those of previous study of(35) who concluded that AFB_1 administration provoked oxidative stress and prohibited the antioxidant enzymes activities in broilers. Our data recorded that LYC inclusion in the AFB_1-treated diet significantly lowered MDA concentration and increased the CAT,GSH-Px and SOD activities, demonstrating that AFB_1-induced oxidative stress could be overcome by LYC administration. In this regard, several studies strengthen the protective efficacy of LYC against oxidative stress caused by different toxic agents mainly through augmenting the antioxidant capacity of SOD and CAT, rather than the inhibiting ROS generation (11).

AFB_1 up regulated the activities and mRNA expression of caspase-3 and caspase-9. These findings implied that $AFB_1$is implicated in excessive oxidative damage to the liver in mice (36). However, these negative impacts could be alleviated by LYC supplementation. Therefore, one of the pivotal mechanisms of LYC against AFB_1 immunotoxicity is the anti-apoptotic effect (37). Consequently, Lycopene up regulated anti-apoptotic Bcl-2, lowered the expression of proapoptotic Bax, and suppressed the activation of caspase-3 (38).From the present study, it is interesting to note that AFB_1 treatment showed a significant increment in tumor necrosis factor-α (TNF- α) and serum IL-6 levels as previously mentioned by (39).Conversely, the enhancement of the serum pro-inflammatory cytokines was significantly relieved by co-administration with

Lycopene as compared with the AFB_1-treated group (39). Our data confirm previous findings suggested by (37)proving that LYC revealed the potent immunoenhancing activity and ability to decrease TNF-α and IL-6 expression. Furthermore, the ameliorative effect of LYC against AFB_1-induced immunotoxicity may be partially ascribed to the direct capturing of free radicals and stimulating the antioxidant capacity.

Conclusion

This study revealed that Lycopene supplementation could protect livers from AFB_1-triggered injury. Moreover, LYC counteracted hepatotoxicity by diminishing hepatic oxidative damage and suppressing apoptosis. Thus, Lycopene may be a promising candidate for alleviating the toxic effects of aflatoxin.

Acknowledgement

The authors declare that there is no conflict of interest.

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