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Research Article

Genotoxic and Toxicopathological Effect of Aflatoxin B_1 in Grass Carp *(Ctenopharyngodon idella)*

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Abstract

The current study was designed to evaluate genotoxic and toxicopathological effect of aflatoxin B_1 (AFB_1) in fresh water fish also known as Grass carp *(Ctenopharyngodon idella)*. For the *in vitro* production of AFB_1, *Aspergillus flavus* spores were grown on potato dextrose agar (PDA) and then rice was used as a substrate. The quantity of AFB_1 was found 50ppb/g of rice using high performance liquid chromatography (HPLC) technique. A total of n=150 Grass carp (42±5 g) were divided into 5 groups (A, B, C, D, E). Group A (kept as a control), while groups B, C, D and E were exposed to 25 ppb, 50 ppb, 75 ppb and 100 ppb AFB1 per kg of diet, respectively, for 49 days. The average weight gain (WG) and specific growth rate (SGR) of fish was significantly higher in group A (WG-15.35 g and SGR-0.63%) compared to group E (WG-6.55 g and SGR-0.28%). The Feed Conversion Ratio (FCR) was significantly (P<0. 05) different between control group and groups D and E. No mortality was notice in any group of fish because of AFB_1. However, increasing concentrations of AFB_1 significantly lowered the red blood cells (RBC) count, hematocrit, hemoglobin concentration, mean corpuscular volume (MCV), white blood cells (WBC) count and lymphocytes as compared to the control group. Additionally, significant increase of aspartate amino transferase (AST), alanine amino transferase (ALT), glucose, urea and creatinine was determined in AFB_1 treated groups by serum biochemistry. Total serum proteins and albumin level was found significantly higher in control group (6.05 and 4.2 g/dL), compared to AFB_1 exposed groups including; group B (5.8 and 3.9 g/dL), group C (5.4 and 3.7 g/dL), group D (4.2 and 2.7 g/dL) and group E (3.8 and 2.06 g/dL). The genotoxicity of AFB_1 was only recorded in fish of group D and E exhibiting micronuclei frequency percentage of 0.85 and 2.15% respectively. The histopathological study revealed that higher concentrations of AFB_1 were causing pathological changes in liver, kidney, intestine and gills tissue. The present study concluded that AFB1 was responsible for reduction in fish production performance, alteration in fish hematological and serum biochemical profiles, structural and functional alteration in tissues and DNA of tissues of fish.

Keywords: *Toxicology, Aflatoxin B_1, Grass carp, Ctenopharyngodon Idella, Hematology, Histopathology, Genotoxicity*

Ot Sazanı *(Ctenopharyngodon idella)*’nda Aflatoksin B_1’in Genotoksik ve Toksikopatolojik Etkileri

Öz

Bu çalışma, aflatoksin B_1’in (AFB_1) ot sazanı *(Ctenopharyngodon idella)* olarak da bilinen tatlı su balıklarında genotoksik ve toksikopatolojik etkilerini değerlendirmek amacıyla planlandı. AFB_1’in *in vitro* üretimi için, *Aspergillus flavus* sporları patates dekstroz agar (PDA) üzerinde büyütüldü ve daha sonra substrat olarak pirinç kullanıldı. Yüksek performanslı likit kromatografi (HPLC) tekniği kullanılarak yapılan analizlerde, pirinçteki AFB_1 miktarı 50 ppb/g bulundu. Toplam 150 adet Ot sazanı (42±5 g) 5 gruba (A, B, C, D, E) ayrıldı. A (kontrol), B, C, D ve E grupları 49 gün boyunca sırasıyla kg başına 25 ppb, 50 ppb, 75 ppb ve 100 ppb AFB1’e maruz bırakıldılar. Balıkların ortalama ağırlık artışı (WG) ve spesifik büyüme hızı (SGR), A grubunda (WG-15.35 g ve SGR-0.63), E grubuna kıyasla (WG-6.55 g ve SGR-% 0.28) anlamlı olarak daha yüksekti. Yemden yararlanma oranı (FCR), kontrol grubu ile D ve E grupları arasında anlamlı derecede farklıydı (P<0.05). Balık gruplarının hiçbirinde AFB1 nedeniyle ölüm gözlenmedi. Bununla birlikte, artan AFB_1 konsantrasyonları, kırmızı kan hücreleri (RBC) sayısını, hematokrit, hemoglobin konsantrasyonu, ortalama eritrosit hacmi (MCV), beyaz kan hücreleri (WBC) sayısı ve lenfositleri kontrol grubuna göre önemli ölçüde düşürdü. Ek olarak, serum biyokimyasal analizlerinde AFB_1’e maruz kalan gruplarda aspartat amino transferaz (AST), alanin amino transferaz (ALT), glukoz, üre ve kreatinin düzeylerinde anlamlı artış tespit edildi. Toplam serum proteinleri ve albümin seviyesi kontrol grubunda (6.05 ve 4.2 g/dL) AFB_1’e maruz kalan gruplara göre anlamlı derecede yüksek bulundu; B grubu (5.8 ve 3.9 g/dL), C grubu (5.4 ve 3.7 g/dL), D grubu (4.2 ve 2.7 g/dL) ve E grubu (3.8 ve 2.06 g/dL). AFB1’in genotoksisitesi sadece D ve E grubundaki balıklarda kaydedildi ve mikronükleus frekans yüzdesi sırasıyla %0.85 ve 2.15 olarak belirlendi. Histopatolojik bulgular, yüksek AFB_1 konsantrasyonlarının karaciğer, böbrek, bağırsak ve solungaç dokusunda patolojik değişikliklere neden olduğunu ortaya koydu. Bu çalışmada AFB_1’in balık üretim performansında düşüş, hematolojik ve serum biyokimyasal profillerinde farklılaşma, dokularda ve DNA’da yapısal ve fonksiyonel değişikliklerden sorumlu olduğu sonucuna varıldı.

Anahtar sözcükler: *Toksikoloji, Aflatoksin B_1, Ot sazanı, Ctenopharyngodon Idella, Hematoloji, Histopatoloji, Genotoksisite*

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INTRODUCTION

An *Aspergillus flavus*, an opportunistic fungus, is one of the major cause of mycotoxins contamination in crop such as cotton, maize and peanuts around the world [1]. The important mycotoxin is aflatoxin in which AFB_1 is the most toxic, teratogenic, carcinogenic and mutagenic [2]. A high dose of AFB_1 could cause mortality from aflatoxicosis [3].

Aflatoxin contamination in feed has adverse effects on health in fish such as reduced growth rate and presence of gross and microscopic lesions which leads to economic losses due to low production, mortalities and poor quality of fish and fish products [4]. Importantly, AFB_1 tends to induce oxidative stress in the host which may leads to functional and structural alteration in the living body including AFB_1-induced cell injury, lipid peroxidation, misfolded protein formation and DNA damage, which may ultimately lead to neoplasia [5]. The toxic and metabolic effects of AFB_1 and AFM_1 are principally observed in the liver [6]. Evidences have been accumulated that chromosomal damage could occurs due to free radicals formed under the influence of AFB_1 [7]. The reactive oxygen species may be partly involved in the carcinogenic activity of AFB_1 [8]. The minimum permitted levels of aflatoxin recognized by many countries ranges from 1-25 µg/kg of total aflatoxin. The minimum permissive level of aflatoxin for food commodities in global trade is 15 µg/kg [9]. In freshwater aquatic species aflatoxicosis has been studied mainly in rainbow trout [10], American channel catfish [11], Nile tilapia [12], Indian carp [13].

The first aflatoxicosis case in aquaculture was reported in May 1960, when an epidemic of hepatomas occurs in *Oncorhynchus mykiss* hatcheries farm in Idaho, United State. The epidemic of hepatomas was mainly characterized by the presence of carcinoma of primary hepatocytes, with multinodular hepatomegaly by postmortem examination [10].

The present study was designed keeping in view the potential of AFB_1 in the generation of genotoxic and toxico-pathological effects directly in the animal tissues. Therefore we evaluated growth performance, hematological, serological, histopathological and genotoxic effects of various concentrations of AFB_1 in grass carp.

MATERIAL and METHODS

The experiments were carried out according to the Rules and Regulations of the Animal Ethics Committee FAHVS, The University of Agriculture Peshawar. Healthy grass carp (n=150) of similar age (<1 year), weight (40 g-45 g), length (10-12 cm) and of ether sexes were obtained from Government Fish Hatchery, Mardan and were divided into five groups each having three replicates. Group A receiving no treatment while group B, C, D and E were exposed to different concentrations of AFB_1 25 ppb, 50 ppb, 75 ppb and 100 ppb/kg of diet respectively for 7 weeks. Fish were acclimatized for one week in glass aquaria before starting the experiment.

Aflatoxin Production, Extraction and Quantification

Aspergillus flavus spores were cultured on rice for the production of AFB_1 [14]. HPLC and fluorescent detection method was used for the quantification of AFB_1 [15].

Specific Growth Rate (SGR) and Feed Conversion Ratio (FCR)

All fish in each tank were weighed and counted on 7th week. The SGR was calculated as follows:

$$\text{Specific growth rate (\%/day)} = \ln(\text{final weight}) - \ln(\text{initial weight}) \times 100/t$$

Where t = Number of days. Feed conversion ratio = Total feed intake/weight gain

Hematological and Blood Biochemical Profile

At the end of the experiment the fish were euthanized using clove oil 3-4 drops per liter of water. The blood from the caudal vein of each fish was collected for hematological parameters including RBCs count, Hb, MCV, MCHC, MCH, PCV, TLC and DLC using hematology analyzer (Celly 70). Liver and kidney function tests were determined using the serum. These tests includes glucose, AST, ALT, total protein, albumin, globulin, urea and creatinine estimation using semi-automatic chemistry analyzer (Convergys 100).

Genotoxicity

The genotoxic potential of the AFB_1 was measured by micronucleus (MN) [16]. For micronucleus assay, a thin smear of the freshly collected blood was made on the pre-cleaned slide. Slides were fixed with methanol for 20 min and then allowed to air dry. The slides were stained with Giemsa solution for 25 min. From each slide a total of 2.000 RBCs were examined at 1000X magnification under couple charged device (CCD) attached microscope (Olympus CX41). Micronucleus was scored as ovoid or circular, non-refractive, small chromatin body displaying the same staining and focusing pattern as the normal nucleus. The percentage of micronuclei was calculated by the following formula.

$$\text{Micronucleus \%} = \frac{\text{Number of erythrocytes containing micronucleus}}{\text{Total number of erythrocytes scored}} \times 100$$

Histopathology

The tissue samples of kidney, gills, liver and intestine were collected from freshly euthanized fish and directly put in 10% formalin for fixation to avoid tissue deterioration. The samples were then subjected to histopathology as reported elsewhere [17]. Briefly, these samples were washed overnight with running tape water in order to remove formalin. Then tissues were dehydrated gradually with 30, 50, 70, 80, 90, 100% alcohol respectively. Fish tissues were then cleared from alcohol with xylene. Infiltration of tissues was done with liquid paraffin. After impregnation of tissues the blocks were prepared by using automatic tissue embedding

assembly (Tissue-Tek® TEC™ Sakura). Blocks were made by pouring carefully melted paraffin over the placed tissue in plastic cassettes. Blocks were then shifted and placed in cold chamber of Tissue Tek® and were allowed to solidify. Paraffin tissue blocks were sectioned with thickness of 5 µm by using microtome (Accu-Cut® SRM™ 200 Sakura, Japan). The cut fine sections were placed in water bath (M-1450 Sakura) at 56ºC so that it floats over the surface of water and folds were removed. For proper sticking of sections albumin was applied on clean glass slides. Section was mounted over the slide and placed on slide drying hot plate (Mod. 1452, Sakura) for 30-40 min for drying followed by placing in hot air oven (Mod. LDO-060E, Daihan Lab Tech. Co. Ltd, Korea) for 2-3 h for drying and removal of extra paraffin. Slides with sections of tissue were placed for staining after final drying. For staining of slide section, Hematoxylin (H) and Eosin (E) stain was used. Automatic slide staining machine (Tissue-Tek® DRS™ 2000 Sakura, Japan) was used for staining process [17].

Histopathological Lesion Scoring

A semi quantitative lesion scoring method *(Table 1)* was used which involved the application of severity grades and it is a defined numerical severity score of specific lesions [18]. Severity grading relies on estimates of severity rather than actual measurements [19].

Statistical Analysis

The data obtain was compiled in Microsoft Excel and was analyzed through Statistix 8.1 software using one way ANOVA (CRD).

Table 1. *The illustration of severity grading*

Numerical Score	Description	Definition
0	Normal/Minimal 0-25%	The tissue considered being normal, under the conditions of study and considering sex, strain and age strain of the animal. Changes may be present which, under other circumstances, would be considered abnormal
1	Mild 26-50%	The lesion is easily recognized but of limited severity
2	Moderate 51-75%	The lesion is prominent but there is significant potential for increased severity
3	Severe 76-100%	The degree of change is as complete as possible (Most part of the organ is involved)

RESULTS

Our analyses revealed that average weight gain and SGR was significantly affected (P<0.05) by increasing concentrations of AFB_1 in the feed. The higher weight gain and SGR was recorded in control group followed by group B, C, D and E *(Table 2)*.

There was no significant difference in FCR of control group and group B and C (P>0.05) but FCR of control group was significantly high (P<0.05) than group D and E. No mortalities were recorded in all groups and the survival rate was 100% *(Table 2)*.

The total RBC count of group E was significantly (P<0.05) decreased as compared to control group but there was no significant difference between control group and group B and C. For PCV there was no significant difference (P>0.05) in group A, B and C while the PCV of group A was significantly different (P<0.05) from group D and group E. The hemoglobin concentration of control group was significantly different from group C, group D and group E but no significant difference (P>0.05) from group B. The MCV in group A, B, C and D has no significant difference (P>0.05) but the MCV of group A was significantly decreased (P<0.05) from the group receiving higher dose of AFB_1 in the diet. Among all the groups the concentration of MCH and MCHC show no significant difference (P>0.05). On the basis of erythrocyte indices the anemia was classified as normocytic normochromic in group A, B, C and D respectively while microcytic normochromic anemia was found in group E *(Table 3)*.

The TLC of all the groups significantly (P<0.05) decreases with the increase in concentration of AFB_1. The lymphocyte percentage of group A was significantly higher as compared to the groups fed with higher concentration of AFB_1 in the diet but there was no significant difference (P>0.05) between group A and B. There was significant increase (P<0.05) in monocyte and neutrophil in group E as compared to control group. The percentage of basophil and eosinophil have no significant difference *(Table 4)*.

The ALT and AST showing significant increase (P<0.05) in

Table 2. *The average weight gain, specific growth rate, FCR, survival rate and micronuclei of Grass carp fed with different concentrations of AFB_1 added diet for 7 weeks*

Group	Average Final Weight (g)	Average Initial Weight (g)	Average Weight Gain (g)	SGR (%)	FCR	Survival Rate (%)	Micronucleus Frequency (%)
A	57.86	42.51	15.35±0.10a	0.63±0.07a	2.09±0.05c	100	0
B	53.12	43.28	9.84±0.04b	0.42±0.07b	2.14±0.03bc	100	0
C	52.14	43.36	8.78±0.03b	0.36±0.09c	2.18±0.03bc	100	0
D	49.69	42.86	6.83±0.05bc	0.30±0.05d	2.23±0.04b	100	0.85^a±0.02
E	48.73	42.18	6.55±0.09c	0.28 ±0.08d	2.34±0.04a	100	2.15^b±0.01

Values (Mean±SE) in the column shown by different letters are significantly different (P≤0.05)

Table 3. *Hematological parameters of Grass carp fed with different concentrations of AFB_1 added diet for seven weeks*

Group	RBC*10/µL	PCV (%)	Hb (gm/dL)	MCV (fL)	MCH (pg)	MCHC (gm/dL)
A	1.60±0.01a	32.6±0.88a	8.2±0.14a	203.28ᵃ±0.34a	51.2±0.11a	25.2±0.64b
B	1.50±0.03ab	32.0±0.52a	8.0±0.14ab	201.14±0.8a	50.5±0.94a	25.1±0.73b
C	1.53±0.02bc	30.3±0.66ab	7.6±0.11c	198.23±0.59a	49.7± 0.49a	25.0±0.92b
D	1.47±0.01c	28.6±0.66b	7.6±0.17bc	194.09±0.98ab	51.9± 0.77a	26.7±0.21ab
E	1.40±0.12d	25.6±0.33c	7.0±0.06d	182.45± 0.35b	50.2± 0.31a	27.5±0.3a

Values (Mean±SE) in the column shown by different letters are significantly different (P≤0.05)

Table 4. *TLC and DLC of Grass carp fed with different concentrations of AFB_1 added diet for seven weeks*

Group	WBC*10³/µL	Lymphocyte (%)	Monocyte (%)	Neutrophil (%)	Basophil (%)	Eosinophil (%)
A	12.4±0.55a	71.3±0.88a	1.6±0.33b	24.0±0.15c	1.3 ±0.33a	1.6±0.66a
B	12.7±0.2a	69.0±0.15a	1.6±0.66b	25.3±0.66bc	1.6±0.33a	2.3±0.33a
C	10.5±0.37b	64.6±0.85b	3.3±0.88b	28.0±0.52b	2.0±0.57a	2.0±0.57a
D	9.3 ±0.12c	62.0±0.15b	6.0±0.78a	28.6±0.88b	1.6±0.66a	1.6±0.33a
E	8.1±0.13d	53.0±0.15c	7.6±0.33a	35.0±0.15a	1.6±0.33a	2.6±0.66a

Values (Mean±SE) in the column shown by different letters are significantly different (P≤0.05)

Table 5. *Hepatotoxic and Nephrotoxic effect in Grass carp fed with different concentrations of AFB_1 added diet for seven weeks*

Group	ALT (IU/L)	AST (IU/L)	Glucose (mg/dL)	Total Protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Creatinine (mg/dL)	Urea (mg/dL)
A	15.8±0.42e	26.6±0.23e	62.3±0.45d	6.05±0.19a	4.2±0.12a	1.8±0.07a	0.19±0.013d	10.1±0.15c
B	21.08±0.77d	33.2±0.17d	68.6±0.45c	5.8±0.05ab	3.9±0.13b	1.9±0.07a	0.24±0.012c	12.3±0.63b
C	25.6±0.73c	40.5±0.07c	75.0±0.73b	5.4±0.24b	3.7±0.10b	1.6±0.33a	0.28±0.011b	12.6±0.84b
D	31.7±0.78b	47.7±0.84b	80.6±0.20a	4.2±0.08c	2.7±0.11c	1.5±0.17a	0.30±0.011ab	13.1±0.48ab
E	38.3±0.60a	55.2±0.34a	83.6±0.45a	3.8±0.04d	2.06±0.03d	1.7±0.07a	0.32±0.012a	14.6±0.21a

Values (Mean±SE) in the column shown by different letters are significantly different (P≤0.05)

the level with increase in AFB_1 concentration. Group E shows significant raise (P<0.05) in level of ALT and AST as compared to the control group A and other treatment groups (B, C and D). The significant increase (P<0.05) was also demonstrated among the different treatment groups such as group B, C, D and E with rise in AFB_1 concentration. Glucose concentration shows significant rise (P<0.05) in the level with the increasing concentration of AFB_1. Among group E and D there was no significant variance (P>0.05) but there was significant increase (P<0.05) in glucose level with increase in AFB_1 concentration. Total protein and albumin shows the drastic decrease in concentration with the increasing level of AFB_1, which show significant (P<0.05) inverse relationship. Globulin concentrations showing no significant increase (P>0.05) in varying levels of AFB_1 treatment in grass carp. The urea and creatinine levels significantly increase (P<0.05) with increasing level of AFB_1 concentration in the feed *(Table 5)*.

The micronucleus frequency percentage of grass carp of group A, B and C were zero percent but there was significant increased (P<0.05) in group E followed by group D *(Fig. 1, Table 2)*.

The liver tissue of grass carps exposed to different levels of AFB_1 showed hydrophic degeneration, fatty change, necrosis and leukocytic infiltration and necrotic cells with pyknotic nuclei *(Fig. 2)*. The fatty change was more severe in group E followed by group D. Mild leukocytic infiltration and pyknotic nuclei were also observed. Microscopically the kidney of grass carp show nephrosis, leukocytic infiltration, vacoulation of epithelial cell, necrotic cells with pyknotic nuclei and glomerular shrinkage in dose dependent manner *(Fig. 3)*. The increase in the level of AFB_1 in the diet causes severe changes. The histomorphology of intestine of grass carp show hypoplasia of goblet cells, villi sloughing, leukocytic infiltration with the increase in concentrations of AFB_1 in diet *(Fig. 4)*. The gills of grass carp were also affected. In group D and E there was sloughing of respiratory epithelium, degeneration of the lamellae, congestion, leukocytic infiltration and necrosis *(Fig. 5)*. The lesion scoring of liver, kidney, intestine and gills are presented in *Tables 6, 7, 8* and *9* respectively.

DISCUSSION

The present study revealed that weight gain and specific growth rate of grass carp fed with different concentrations of AFB_1 in the diet was significantly lower than control group (P<0.05) with no mortality. The results of current study were agreed with the study reported previously [12,20,21]. They

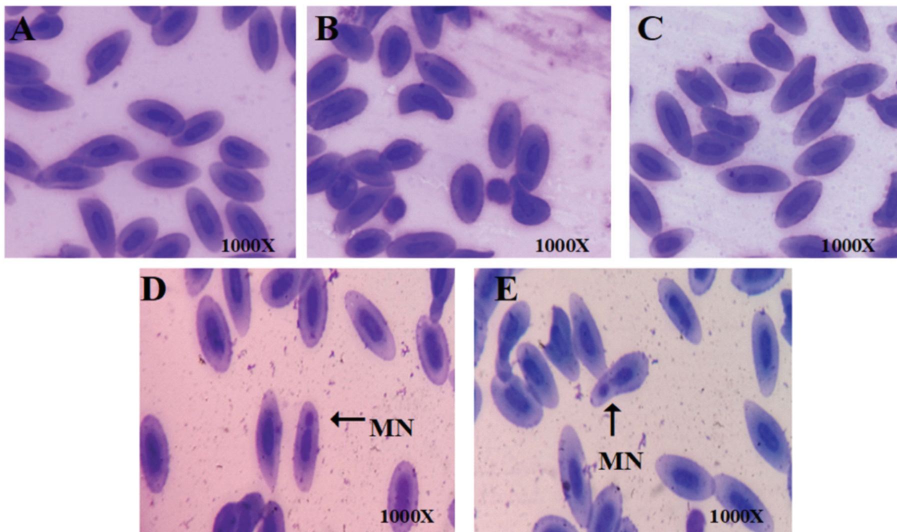

Fig 1. Erythrocytes of grass carp of Group A, and Group B are showing normal morphology. Group D and Group E showing micronuclei (MN), Geimsa stained (1000X)

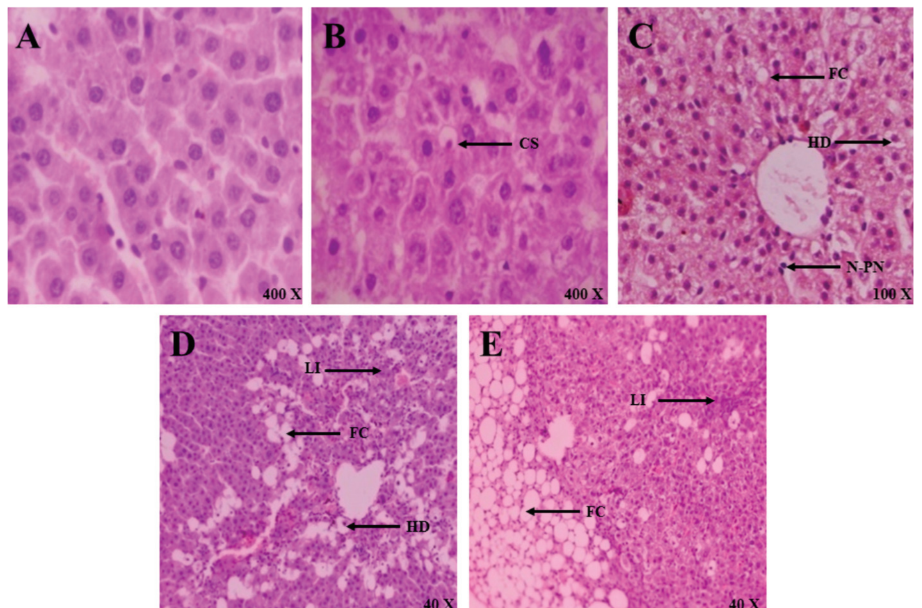

Fig 2. Photomicrograph of liver of grass carp exposed to different levels of AFB_1. **A)** Liver of control group; **B)** Liver of grass carp given 25 µg AFB_1 showing mild cloudy swelling of hepatocyte; **C)** Liver showing fatty change (FC), hydrophic degeneration (HD) and necrotic cell showing pyknotic nuclei (N-PN); **D)** Severe fatty change (FC), hydrophic degeneration (HD), leukocytic infiltration (LI); **E)** Showing severe fatty change (FC), leukocytic infiltration (LI). H&E stained, 40X, 100X, 400X

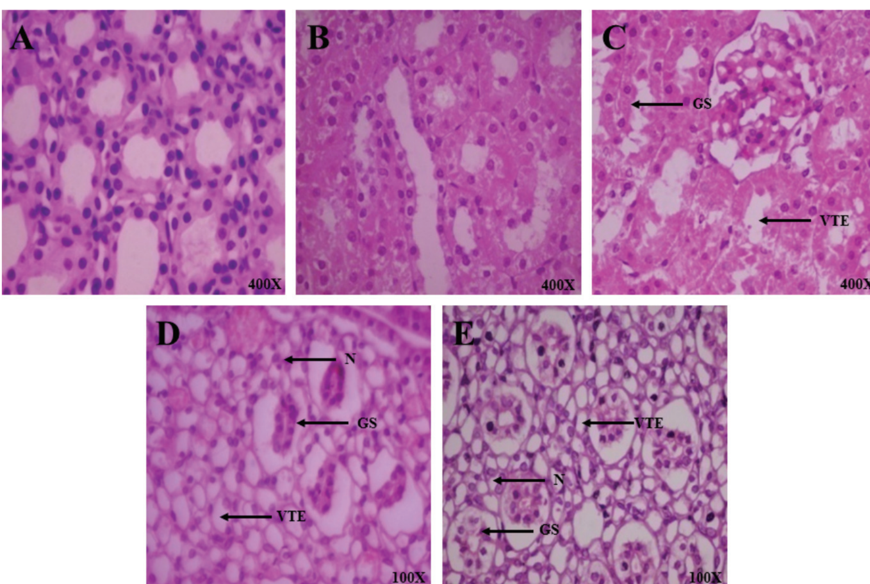

Fig 3. Photomicrograph of kidney of grass carp exposed to AFB_1. **A)** Kidney of normal group; **B)** Kidney of grass carp given 25 µg AFB_1 showing normal histomorphology; **C)** Kidney showing vacoulation of tubular epithelial cell (VTE) and glomeruli shrinkage (GS); **D)** Vacoulation of tubular epithelial cell (VTE), nephrosis (N) and glomeruli shrinkage (GS); **E)** Showing vacoulation of tubular epithelial cell (VTE), nephrosis (N) and Necrotic cells showing pyknotic nuclei (N-PN). H&E stained, 100X, 400X

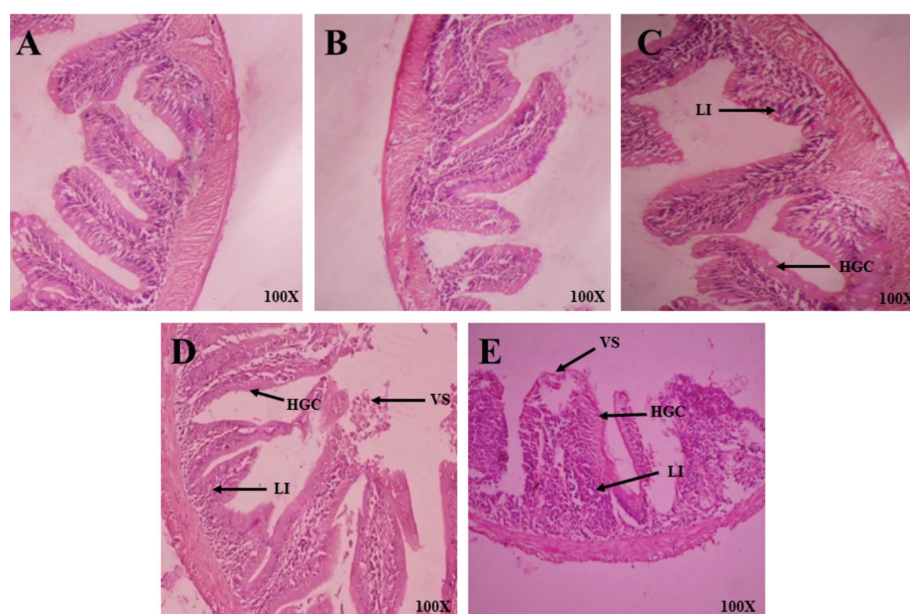

Fig 4. Photomicrograph of intestine of grass carp exposed to AFB_1. **A)** Normal intestine of control group; **B)** Normal intestine of grass carp exposed to 25 µg AFB_1; **C)** Showing hypoplasia of goblet cells (HGC), leukocytic infiltration (LI); **D)** Hypoplasia of goblet cells (HGC), villi sloughing (VS); **E)** Hypoplasia of goblet cells (HGC), villi sloughing (VS), leukocytic infiltration (LI). H&E stained (100X)

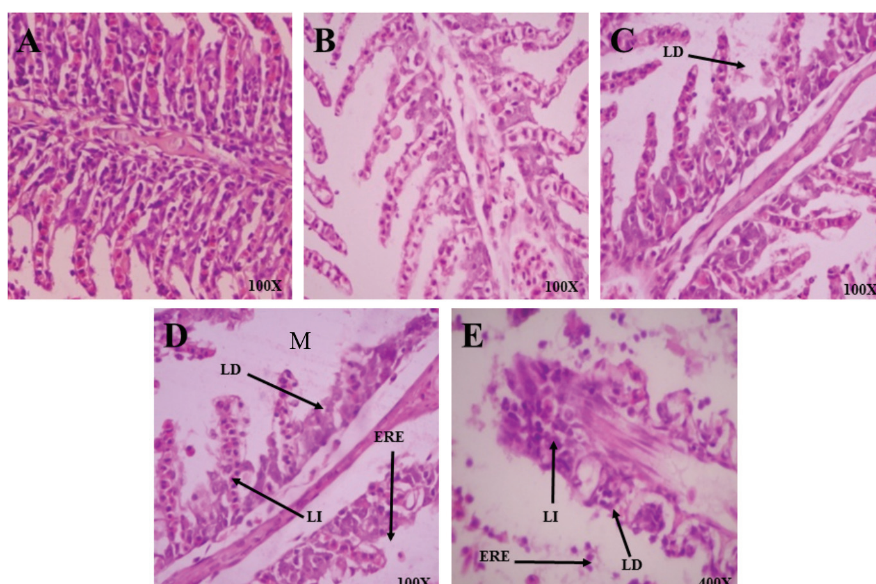

Fig 5. Photomicrograph of gills of grass carp exposed to AFB_1. **A)** Normal gills of control group; **B)** Normal histomicrograph of grass carp exposed to 25 µg AFB_1; **C)** Showing lamellae degeneration (LD); **(D-E)** Showing lamellae degeneration (LD), exfoliated respiratory epithelium (ERE), leukocytic infiltration (LI). H&E stained 100X and 400X

Table 6. *Histopathological scoring of liver of Grass carp fed with different concentrations of AFB_1 added diet for seven weeks*

Group (AFB_1 µg/kg)	Lesion Score							Severity Percentage
	Hydrophic Degeneration	Fatty Change	Pyknotic Nuclei	Leukocytic Infiltration	Necrosis	Total Score	Max. Possible Score	
A	2	0	0	0	0	2	150	1
B	8	2	1	4	0	15	150	10
C	21	12	7	9	4	53	150	35.33
D	27	25	13	14	6	85	150	56.66
E	31	35	18	26	9	119	150	79.33

investigated that feeding of low to high concentrations of AFB_1 for a longer duration produces a decrease in feed intake and weight gain efficacy. Andleeb et al.[22] conducted study on fry Catlacatla and found the highest weight gain in control group as compared to aflatoxin treated groups. The survival rate was more than 90% in all AFB_1 treated group without any significant difference [23]. Sepahdari et al.[24] investigated that there was a decrease in weight gain and SGR in fish treated with 75 ppb and 100 ppb AFB_1 per Kg of diet as compared to control group after two months. The similar results were observed by Wang et al.[25] in Yellow catfish *(Pelteobagrus fulvidraco)* fed with

Table 7. *Histopathological scoring of kidney of Grass carp fed with different concentrations of AFB_1 added diet for seven weeks*

Group (AFB_1 µg/kg)	Lesion Score								Severity Percentage
	Congestion	Vacoulation in Tubular Epithelial Cells	Pyknotic Nuclei	Glomeruli Shrinkage	Leukocytic Infiltration	Necrosis	Total Score	Max. Possible Score	
A	0	0	0	0	0	0	0	180	0
B	3	4	2	3	3	0	15	180	8.3
C	4	11	12	10	5	3	45	180	25
D	9	14	13	18	15	8	77	180	42.7
E	14	17	20	27	19	13	110	180	61

Table 8. *Histopathological scoring of intestine of Grass carp fed with different concentrations of AFB_1 added diet for seven weeks*

Group (AFB_1 µg/kg)	Lesion Score						Severity Percentage
	Reduction in Goblet Cells	Leukocytic Infiltration	Sloughing of Epithelial Cells	Necrosis and Degeneration	Total Score	Max. Possible Score	
A	1	0	0	0	1	120	0.83
B	2	2	1	3	8	120	6.66
C	8	4	7	6	25	120	20.83
D	12	9	12	11	44	120	36.66
E	19	16	22	12	69	120	57.5

Table 9. *Histopathological scoring of gills of Grass carp fed with different concentrations of AFB_1 added diet for seven weeks*

Group (AFB_1 µg/kg)	Lesion Score							Severity Percentage
	Lifting of Respiratory Epithelium	Degeneration of Lamellae	Congestion	Leukocytic Infiltration	Necrosis	Total Score	Max. Possible Score	
A	0	0	0	0	0	0	150	0
B	4	2	3	2	0	11	150	7.33
C	4	9	6	3	5	27	150	18
D	8	12	7	6	8	41	150	27.33
E	15	18	8	11	12	64	150	42.66

200, 500 and 1.000 µg kg^{-1} of AFB_1 in feed which showed decrease weight gain and growth rate and altered feed conversion ratio.

The hematological and blood biochemical profile in the current study was significantly affected by different concentrations of AFB_1. The hematocrit and hemoglobin concentration was reduced because of the decreased in total erythrocyte count. Due to damage to the lymphoid follicle the lymphocyte percentage declined. The effect of AFB_1 in low concentrations for long duration in Nile tilapia which revealed marked anemia and leucopenia [26]. Similar results were observed in channel catfish and common carp [22,24]. The results showed that AFB_1 causes increase in the serum AST, ALT, glucose, urea and creatinine level. The increase in AST, ALT and glucose is due to the damage to liver. AFB_1 causes injury to the hepatocytes which contain preformed AST and ALT released into the blood. The higher level of glucose in the blood indicates liver damage because the damage liver cannot respond to insulin. The findings of current study were agreed with the results of [12,27] which showed elevated levels of urea and creatinine in the blood of Nile tilapia exposed to different concentrations of AFB_1. The serum total protein and albumin levels decreased significantly in a dose dependent manner. This is due to the binding of AFB_1 metabolites with the macromolecules of cell and damage to the hepatocytes. Liver is mainly involved in the synthesis of different proteins. AFB_1 hepatotoxicity interfere the synthesis of proteins by bond formation of AFB_1 adducts to the macromolecules of cell [28].

The histopathological examination showed that higher concentrations of the AFB_1 can cause changes in the liver, including hydrophic degeneration, leukocytic infiltration, necrosis and progressive fat deposition particularly at 75 ppb and 100 ppb AFB_1/kg. The results are similar with the study of Evalvn et al.[29] who reported AFB_1 contamination in trout fish which showed microscopically basophilic hepatic cells with hyperchromatic nuclei in irregular cords. The gills showed degeneration of lamellae and lifting of respiratory epithelium. In kidney tissue nephrosis, glomeruli shrinkage and cell vacoulation were observed. The intestine showed hypoplasia of goblet cells, sloughing of epithelial cells and leukocytic infiltration. Histopathological studies revealed liver cell degeneration and necrosis and progressive fat deposition at a level of 75 ppb and 100 ppb AFB_1/kg of diets after 2 months [25]. The results are also in agreement with the study of [22], who reported that AFB_1 in *Catla catla*

fish causing histopathological changes in liver, kidneys and intestine. The higher concentrations of AFB_1 caused degenerative changes, pyknotic nuclei and increased vacoulation of liver hepatocytes. In kidneys necrotic changes in nucleus were quite prominent, the Bowman's space increased which was indicative of glomerular atrophy and there were eosinophilic proteinaceous material in the lumen of tubules. The intestine showed higher leukocytic infiltration, sloughing of epithelium and vacoulation in enterocytes.

The genotoxic effect of AFB_1 in grass carp was studied by micronucleus assay. The genotoxicity was only recorded at 75 ppb and 100 ppb AFB_1/kg, having micronucleus frequency percentage of 0.85 and 2.15% respectively. Abdullah et al.[30] investigated the DNA damage of AFB_1 in rainbow trout (sensitive) and channel catfish (resistant) using comet assay. Through intra peritoneal route 0.5 mg AFB_1/mL DMSO/kg body weight was administered to fish. The Comet assay was performed on total blood, kidney cells and hepatocytes of both channel catfish and rainbow trout after 4 and 24 h. Significant ($P<0.05$) and high genotoxicity was exhibited by trout kidney tissue and blood tested after 4 h which then reduced by 24 h. DNA damage gradually increased with time in liver cells.

The present study concluded that the production performance of fish is reduced in AFB_1 treated groups lead to economic loss. AFB_1 in higher concentrations affect the hematological and blood biochemical profile. The microscopic examination of tissues showed that AFB_1 causes pathological changes. AFB_1 is genotoxic and induced DNA damage in fish at higher concentrations (75 and 100 µg/kg of diet).

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