Original Article

Histopathological study of common carp (*Cyprinus carpio*) fed aflatoxin-contaminated diets

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Abstract: This study aimed to evaluate the effects of aflatoxin-contaminated diet on histopathological alterations of the gill, liver, kidney and intestine tissues in common carp. Fish were randomly distributed into 15 tanks, i.e. in five experimental groups; (I) control fed with normal diet without solvent and aflatoxin, (II) positive control received feed with only solvent, and (III-V) fed on diets containing 0.5, 0.7 and 1.4 mg kg^{-1} of aflatoxin, respectively. After 21-days, 12 fish per treatment were randomly caught, anesthetized and euthanized. Then, histological sections of the tissues were prepared. The main aflatoxicosis symptoms in the gills were fusion and disorganisation of the secondary gill lamellae, shortening of the secondary lamellae, inflammation of mucous membranes, and exfoliation of the gill epithelium. Liver of the infected fish indicated cloudy swelling of hepatocytes, cellular hypertrophy, formation of vacuoles in the cytoplasm, and necrosis of liver parenchyma. Expansion of Bowman's space, necrosis of urinary tract, exfoliation and degeneration of the urinary tract epithelium, expansion of the urinary lumen and dilation of the urinary space were observed symptoms in the kidney. Changes in the intestine of the aflatoxin-treated fish were; expansion of goblet cells, necrosis of mucous layers, exfoliation of the mucous epithelium, and bleeding in the intestinal wall. The results indicates that feeding common carp with diets contaminated with aflatoxin, even in low concentrations (≤ 1.4 mg kg^{-1} feed) can cause histopathological damages and disturb their physiological balance.

Introduction

Aflatoxin produced by *Aspergillus flavus* was discovered about 50 years ago after an outbreak of turkey X disease in England (Kensler et al., 2011). Aflatoxins are recognized as the most toxic and carcinogenic compounds among mycotoxins. It is the secondary metabolite of *A. parasiticus* and *A. flavus* that are found in corns, peanuts, oilseed crops, almonds, and pistachios in tropical and subtropical regions (Taheri et al., 2012; Almeida et al., 2011; Ruby et al., 2013). Cereals, including corns, are the main materials in making feed in aquaculture and its contamination with aflatoxin can increase chances of toxicity in fish. Aflatoxin can be produced in different phases of food production, during the harvesting stage, when crops are stored in warehouses or even after the aquatic feed production. Feeding fish with aflatoxin-contaminated diet also increases the risk of aflatoxin-induced effects to consumers of the aquatic food (Huang et al., 2011).

Four main groups of aflatoxins are B1, B2, G1 and G2 which contaminate agricultural products and have a potential risk for humans and farmed species (Kensler et al., 2011; Wacoo et al., 2014). Aflatoxins, specifically aflatoxin B1, converts to exo-8,9-epoxide (AFB1-8,9-epoxide) by CYP3A4 in the liver of fish and other organisms (Deng et al., 2010; Chawanthayatham et al., 2015). Exo-8, 9-epoxide is very reactive and acts as the main mediator of cell damage. Moreover, they have an important role in DNA-adducts formation and hepatocarcinogenesis (Kensler et al., 2011). Although other enzymes of the cytochrome P450 are involved in degradation of aflatoxins and their metabolites (Mary et al., 2012),

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hydrogen peroxide and hydroxyl radicals produced during aflatoxin process (Matur et al., 2011; Adedara et al., 2010) may lead to peroxidation of cell membrane and cytological alterations (Mary et al., 2012; Bernabucci et al., 2011). Alflatoxin B1 and its metabolites hinder DNA and RNA synthesis and reduce protein synthesis (McKean et al., 2006).

Aflatoxin is reported to cause immune-suppression (Sahoo and Mukherjee, 2001; Almeida et al., 2011), growth disorder (Tuan et al., 2002; Almeida et al., 2011), gene expression alterations (Mahfouz, 2015), changes in hematological parameters (Mohapatra et al., 2011; Vaziryan et al., 2017), tissue damage (McKean et al., 2006; Mohapatra et al., 2011; Raghavan et al., 2011), and mortality in fish (Russo and Yanong, 2010; Spring and Fegan, 2010; Raghavan et al., 2011). Also, aflatoxins are capable of disturbing DNA replication, inhibiting RNA polymerase and disturbing mRNA transcription, hindering amino acid transport and protein synthesis (Denli et al., 2009). Therefore, its toxicity may prevent renewal of damaged tissues. That is why reversible and irreversible damage to tissues and cells can cause severe changes in biochemical parameters (El-Sayed and Khalil, 2009; Arafa et al., 2014; Vaziryan et al., 2017), cause oxidative stress and finally disturb biochemical and physiological balances of fish.

Pathological methods are quick, sensitive, relatively reliable and inexpensive tools to evaluate damages in vital tissues of fish exposed to toxic compounds. Therefore, studying alterations and histopathological changes may provide direct evidence of the toxic effects of aflatoxins on fish. Hence, this study investigated pathological alterations in gills, liver, kidney, and intestines of common carp, *Cyprinus carpio*, that were treated with sublethal concentrations of aflatoxin in a 21-day period.

Materials and Methods

This study was performed in Aquaculture and Biology Laboratory of Behbahan Khatam Alanbia University of Technology, Iran in 2015. *Aspergillus flavus* (PTCC 5006) purchased from Persian Type Culture Collection (Iranian Research Organization for Science

and Technology), and was cultured on Potato Dextrose Agar (PDA), and placed all the test tubes in incubator at 37°C for 17 day (Shotwell et al., 1996). Then, the fungal spores were transferred from inoculated test tubes on 200 gr-dried bread soaked in 30 ml distilled water. The material was shifted in eight 500 ml sterilized conical flasks and put in an orbital shaker at 28°C and 150 rpm for a period of 30 days. Then, the aflatoxins were extracted from culture media with methanol, acetone (70:30 ratios) and diluted water and used for aflatoxin analysis by HPLC method (Varior, 2003).

All the ingredients of commercial feed were powdered, sieved, blend and extruded through a kitchen noodle maker with a 3 mm die, dried at 55°C overnight and stored in freezer. The experiment diet had the same composition as that the control diet to which varying concentrations of the aflatoxin was added from the stock solution. For the feeding, aflatoxin solution (aflatoxin dissolved in ethanol and acetone as solvent) were added into the oil portion of the diet before blending. Since acetone and ethanol mixture is toxic to fish if used as in diet, therefore it was allowed to evaporate. Then the ingredients were mixed with water, extruded and then dried.

Healthy common carp were used in the present study according to the National Ethical Framework for Animal Research in Iran (Mobasher et al., 2008). The common carp were obtained from commercial suppliers (Ahvaz, Iran) and transported to the laboratory facilities. Before the assay, fish were acclimated for two weeks in fiberglass 1000 L tank with de-chlorinated tap water, at a pH of 7.4±0.2, temperature 24±2°C, photoperiod: 16 h light: 8 h dark and with continues aeration enough for keeping the dissolved oxygen always higher than 6 mg L^{-1}.

After acclimation period, fish (n: 180; 30±5 g) were randomly distributed into 80 L polystyrene tanks, in five experimental groups (12 fish per tank); Group I as control group fed with normal diet without solvent and aflatoxin. Group II received feed with solvent but without any aflatoxin. This group served as a positive control for the solvent in which the aflatoxin was dissolved. Group III-V was fed on diets containing
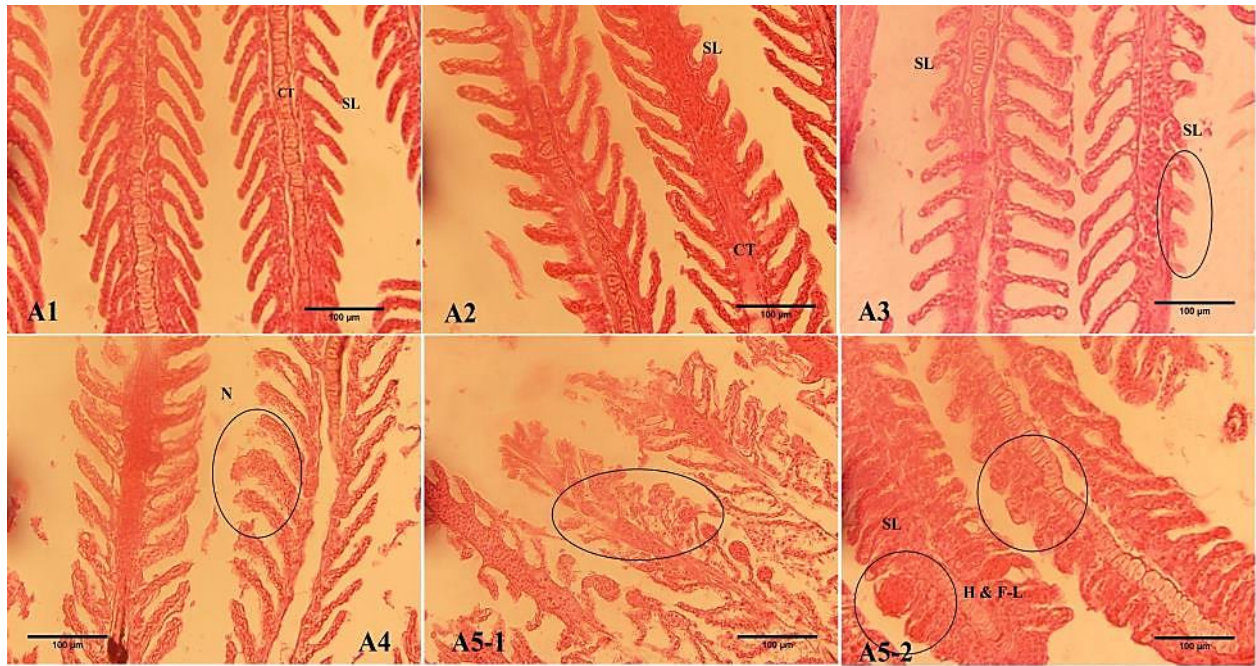
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Figure 1. Histopathology of gills in fish treated with different concentrations of aflatoxin. (A1) control group, (A2) positive control, (A3) 0.5 mg aflatoxin, (A4) 0.7 mg aflatoxin and (A5) 1.4 mg aflatoxin. Primary lamellae (PL), secondary lamellae (SL), hypertrophy (H), and cartilage tissue (CT).

0.5, 0.7 and 1.4 mg kg^{-1} of aflatoxin for 21 days, respectively. The water was renewed (50% rate) every 24 hrs and experiment was performed for 21 days. Fishes were deprived of food 24 hrs before sampling.

After 21 days, 12 fish from each treatment were randomly captured. Then, fish were euthanized after being anesthetized with a solution of clove powder (1: 5000) (Banaee et al., 2013). Following autopsy, the liver, kidney, intestine and gill tissue of the control and aflatoxin-treated fish were removed to evaluate lesions and tissue damages. Then, the tissues were fixed in Buin's solution. Then 5 μm histological slides of the tissues were prepared based on Banaee et al. (2013) and Eagderi et al. (2013).

Results

Histopathological alternations observed in the gills, liver, kidney, and intestine of the exposed fish to different concentrations of aflatoxin are illustrated in Figures 1-4. Bleeding and necrosis of gills, curling and clubbing of the lamellae, shortening of the secondary lamellae and their fusion, inflammation of mucous cells and gill epithelium necrosis are the major histopathological alterations found in the gill of fish treated with different concentrations of aflatoxin (Fig. 1).

Liver in the control group and the positive control which received no aflatoxin had normal structures. Hexagonal hepatocytes had round nuclei and a uniform cytoplasm. Obvious histological changes were not found in fish treated with the extract (ethanol and acetone mixture) (Fig. 2).

The relative increase of bleeding and liver discoloration were the main apparent changes in aflatoxin-treated fish. Deformation of hepatocytes, as well as disarrangement of cells, cloudy swelling of hepatocytes, cells hypertrophy, vacuole formation in cytoplasm (Fig. 2: B2, B3, B4, B5), necrosis of liver parenchyma and histopathological alterations in pancreas were detected in treated fish. Degenerated cells had big and dark nuclei (Fig. 2: B3, B4, B5). Great changes were observed in fat surrounding degenerated cells (Fig. 2).

Destruction of the renal tract, glomerular atrophy and necrosis, enlargement of Bowman's space (Fig. 3: C3, C4), urinary tract necrosis, exfoliation and degeneration of the urinary tract epithelium (Fig. 3: C3, C5), increased urinary lumen space, dilation of urinary space (Fig. 3: C3-5) and increase in melanomacrophage centers (Fig. 3: C3-4) were
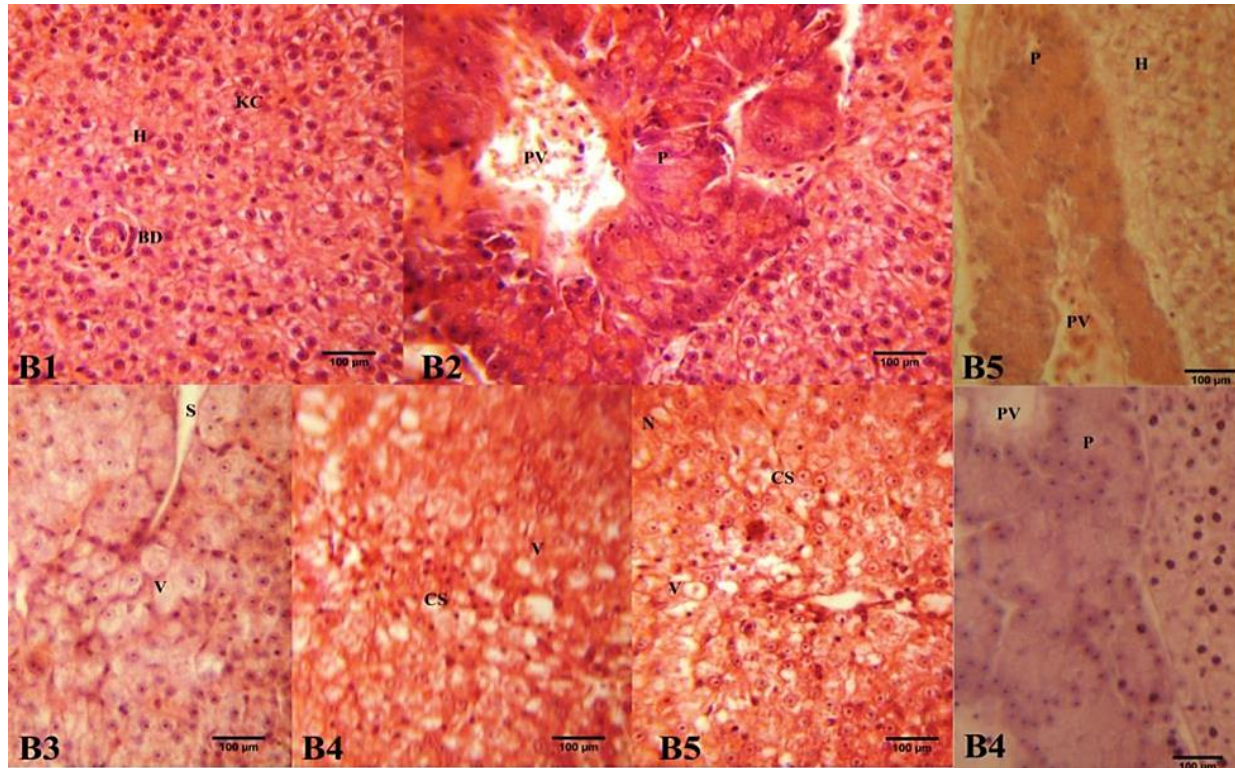
Figure 2. Liver histopathology of fish treated with different levels of aflatoxin; (B1) the control, (B2) positive control, (B3) 0.5 mg aflatoxin, (B4) 0.7 mg aflatoxin and (B5) 1.4 mg aflatoxin. Kupffer cells (KC), liver sinusoid (S), hepatocytes (H), pancreas (P), portal veins (PV), cloudy swelling (CS), degeneration and formation of cytoplasmic vacuoles (V).
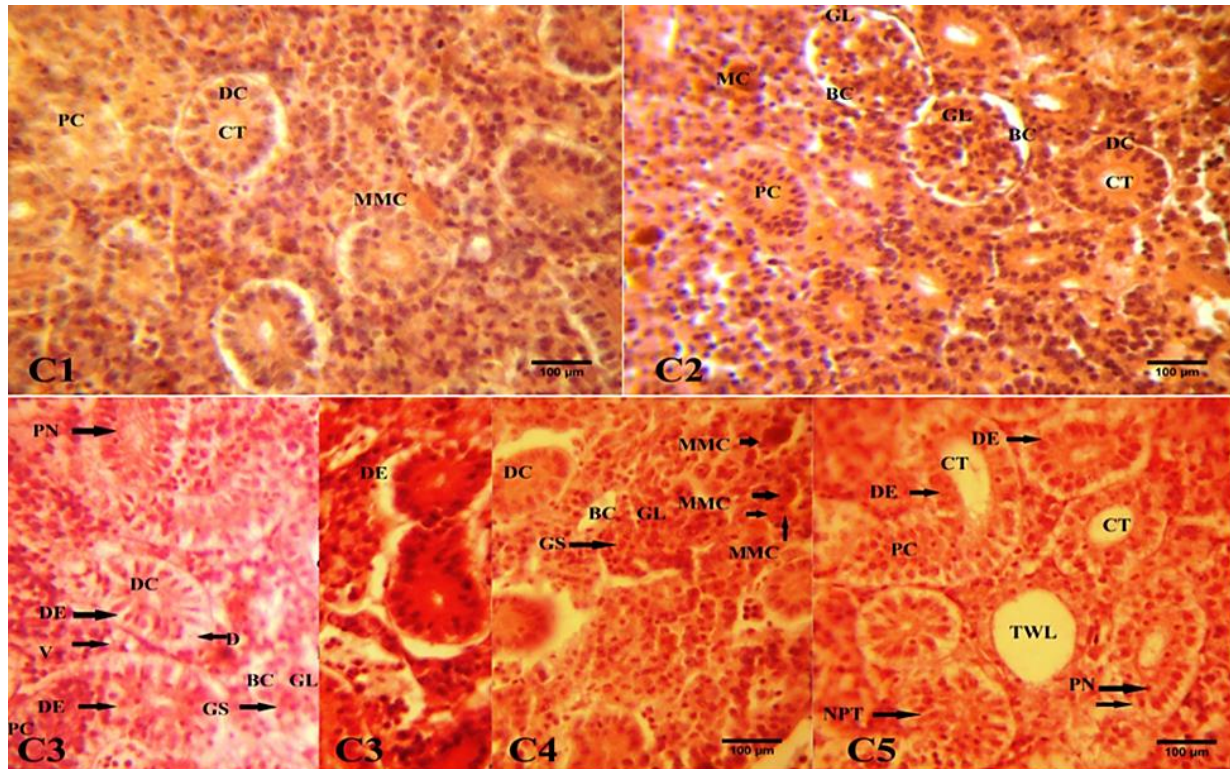

Figure 3. Kidney histopathology of fish treated with different levels of aflatoxin; (C1) the control, (C2) positive control, (C3) 0.5 mg aflatoxin, (C4) 0.7 mg aflatoxin, and (C5) 1.4 mg aflatoxin; glomeruli (GL), Bowman's capsule (BC), collecting tubule (CT), distal convoluted tubule (DCT), proximal convoluted tubule (PCT), tubules with widened lumen (TWL), degenerated epithelium (DE), desquamation (D), glomerular shrinkage (GS), urinary tract dilation (TD), pyknotic nuclei (PN), melano-macrophage centers (MMC), necrosis of distal convoluted tubule (NPT).
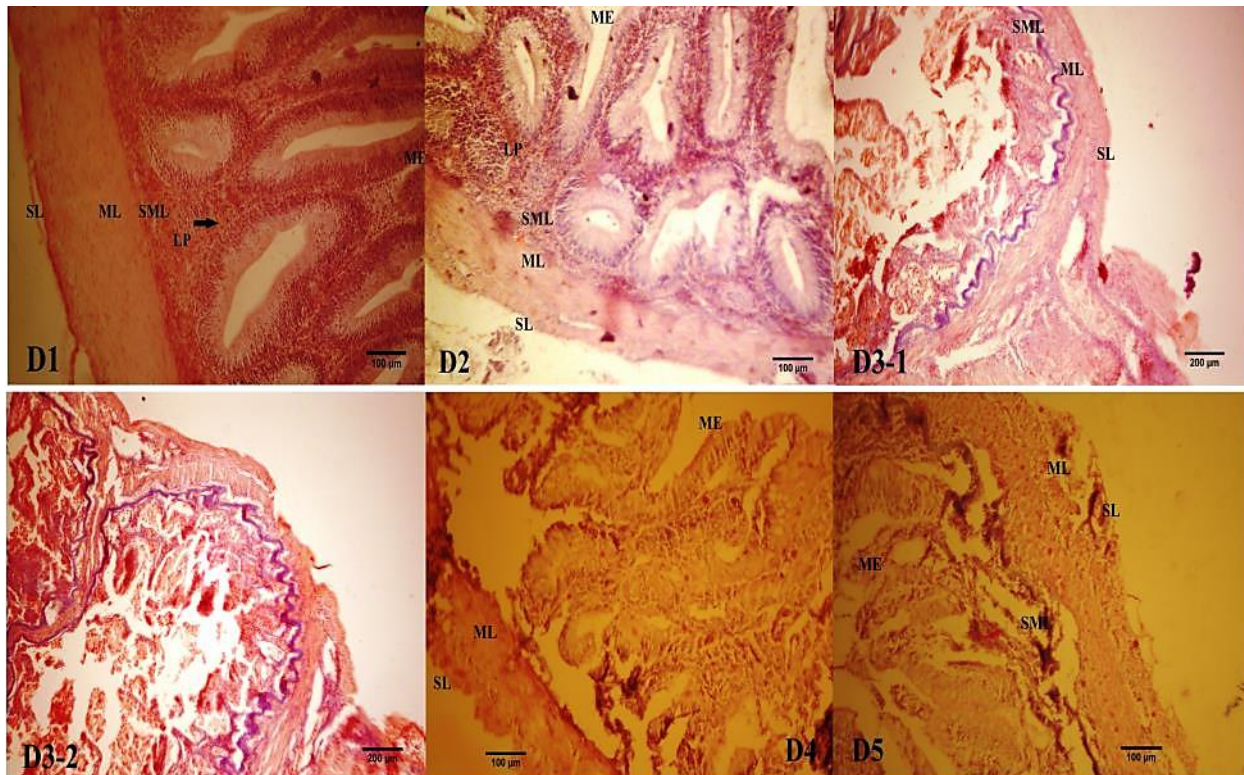
Figure 4. Intestine histopathology of fish treated with different levels of aflatoxin; (D1) the control, (D2) positive control, (D3) 0.5 mg aflatoxin, (D4) 0.7 mg aflatoxin, and (D5-1, 2) 1.4 mg aflatoxin; serous layer (SL), muscle layer (ML), mucosal epithelium (ME), lamina propria (LP), submucosal layer (SML).

observed in aflatoxin-treated fish.

In control and positive control fish, the intestine was normal. Necrosis of mucous cells and mucous layer epithelium (Fig. 4: D3-5), and bleeding in the intestinal wall were observed in the intestine of fish treated with aflatoxin.

Discussion

Aflatoxicosis can have an economically significant effect on the production of farmed fish. Liver, gills, intestine and kidney are the most important target organs in fish that are susceptible to aflatoxicosis (Zychowski et al., 2013). Therefore, we evaluated their damages in common carp that were fed sublethal levels of aflatoxin (0.5, 0.7 and 1.4 mg kg^{-1} feed) for 21 days.

Bleeding in tissues indicates aflatoxins effects on the endothelial cells of the circulatory system. Endothelial cells are apparently sensitive to aflatoxin and most pathologic damages were observed in these cells (Mehrim et al., 2006). Similar changes were observed in rohu (*Labeo rohita*) (Sahoo et al., 2001).

Gills are sensitive organs of teleost when exposed to toxic compounds (Robert, 2001). Epithelium hyperplasia, mucous cells of gill filaments, fusion of gill lamellae and bleeding were observed in fish fed aflatoxin-contaminated diets. Damage to gills in aflatoxin-treated fish can disturb osmotic balance, gas exchange and ammonia excretion in the long term (Mehrim et al., 2006). Cell necrosis and bleeding are reported in gill lamellae of rohu with aflatoxicosis (Sahoo et al., 2001, 2003).

In the present study, fat changes in hepatocytes, cloudy swelling, formation of cytoplasmic vacuoles, necrosis of liver cells, tissue fibrosis of portal veins, excessive proliferation of bile duct cells and necrosis of pancreatic cells were found in liver of aflatoxin-treated fish. Hepatocytes necrosis in fish fed aflatoxin-contaminated diet indicates aflatoxin's effect in destruction of cell membranes and necrosis of tissues. Nucleus hypertrophy, hyper chromosome, widespread biliary hyperplasia, liver focal necrosis and cellular inflammation are reported in hybrid sturgeons treated with aflatoxin-contaminated feed (Raghavan et al.,

2011). Aflatoxin-induced damage to liver can disturb homeostasis and physiological balance of fish. Similar histopathological damages are reported in liver of *Oreochromis niloticus* (Mahfouz and Sherif, 2015; Chávez-Sánchez et al., 1994), rainbow trout, *Oncorhynchus mykiss*, (Arana et al., 2014) and *Sciaenops ocellatu* (Zychowski et al., 2013) fed aflatoxin-contaminated diets.

In this study, microscopic examinations showed that aflatoxin has a significant effect on the kidney's structure. Increased urinary space, loss of renal tract epithelium, atrophy and necrosis of glomeruli and enlargement of the Bowman's space in fish treated with aflatoxin could be attributed to lipid peroxidation of cell membranes in kidney (Mehrim et al., 2006). Necrosis and atrophy of glomeruli, expansion of Bowman's capsule and increased urinary space lead to increased kidney filtration and disposal of large quantities of amino acids, proteins, glucose, electrolytes and water from the body of fish (Singh, 2012; El-Greisy and El-Gamal, 2015; Taheri et al., 2017). Degenerated urinary tracts and necrosis of urinary tract epithelial cells are reported in aflatoxin-treated rohu (*Labeo rohita*) (Sahoo et al., 2001, 2003). Blood clots, necrosis and atrophy of glomeruli, as well as melanosis coli are other alterations found in *Oreochromis niloticus* fed aflatoxin-contaminated diet (Chávez-Sánchez et al., 1994).

Major pathologic intestinal changes in fish fed diets contaminated with different levels of aflatoxin were atrophy and necrosis of mucous cells, exfoliation of the mucous layer epithelium, as well as bleeding and rupture of intestinal capillaries. Damage to the intestine can negatively affect absorption of nutrients and lead to malnutrition, reduced growth rate and reduced physiological regeneration in aflatoxin-treated fish (Mehrim et al., 2006). Exfoliation of the mucous layer epithelium and hemorrhage in the intestine were reported in rohu treated with aflatoxin (Sahoo et al., 2001). Pathological changes, reduced digestion and absorption of food in the intestine, decreased enzyme activity and malnutrition are all aflatoxins' effects on the digestive system of fish (Applegate et al., 2009).

Although the median lethal dose (LD_{50}) of aflatoxin for warm water fish is 12.6 mg kg^{-1} (Sahoo et al., 2001), the results of this study indicates the sensitivity of common carp to doses lower than LD_{50} ($\leq$ 1.4 mg kg^{-1} feed) of aflatoxin in the long term. Also, feeding fish aflatoxin-contaminated diets can provide the grounds for aflatoxicosis. However, the severity of tissue damage in fish treated with different levels of aflatoxin rises with an increase in dosage. Tissue damage in fish treated with different levels of aflatoxin can be used as a clinical device in diagnosing aflatoxicosis.

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چکیده فارسی

مطالعه آسیب‌شناسی بافت‌های مختلف در ماهی کپور معمولی (*Cyprinus carpio*) تغذیه شده با جیره غذایی آلوده به آفلاتوکسین

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چکیده:

هدف از این مطالعه ارزیابی اثر آفلاتوکسین موجود در جیره غذایی بر تغییرات آسیب‌شناسی بافت‌های مختلف ماهی کپور معمولی است. در این مطالعه ماهی‌ها بطور تصادفی در ۱۵ مخزن در ۵ گروه آزمایشی توزیع شد. گروه I، به‌عنوان گروه کنترل با جیره نرمال و گروه II به‌عنوان کنترل مثبت با جیره حاوی حلال عصاره‌گیری تغذیه شدند. گروه‌های III-V به‌ترتیب با جیره حاوی ۰/۵، ۰/۷ و ۱/۴ میلی‌گرم آفلاتوکسین بر کیلوگرم غذا تغذیه شدند. پس از گذشت ۲۱ روز از آغاز آزمایش، ۱۲ ماهی از هر تیمار به‌صورت تصادفی صید و بیهوش و آسان‌کشی شد. سپس از بافت‌های آبشش، کبد، کلیه و روده نمونه‌برداری و بافت‌های مورد نظر تثبیت و جهت بررسی میکروسکوپی آماده شد. مهمترین علائم بالینی آفلاتوکسیوزیس در آبشش شامل گرزی شدن انتهای لاملاها و بهم چسبیدگی آنها، بهم‌ریختن آرایش لاملاهای ثانویه، کوتاه شدن لاملای ثانویه، تورم سلول‌های موکوسی و نکروز اپیتلیوم آبششی بود. کبد ماهیان مبتلا، تورم ابری هپاتوسیت‌ها، هیپرتروفی سلول‌ها، پیدایش واکوئل در سیتوپلاسم سلول‌ها، نکروز پارانشیم کبدی، تغییرات هیستوپاتولوژیک در پانکراس نشان داد. علاوه بر این، افزایش فضای بومن، نکروز مجاری ادراری، پیکنوز هسته، نکروز و دژنره شدن اپیتلیوم مجاری ادراری، افزایش فضای لومن ادراری از علائم مشاهده شده در کلیه بود. از دیگر تغییرات می‌توان به افزایش اندازه سلول‌های جامی، نکروز سلول‌های مخاطی، پوسته پوسته شدن اپیتلیوم لایه مخاطی، خون‌ریزی در دیواره روده ماهیان است. نتایج این مطالعه نشان داد که تغذیه ماهی کپور معمولی با جیره غذایی آلوده به آفلاتوکسین در غلظت‌های پایین (۱/۴≥ میلی‌گرم به ازای هر کیلوگرم غذا) نیز می‌تواند موجب بروز آسیب‌های بافت‌شناسی و برهم خوردن تعادل فیزیولوژیکی آنها گردد.

کلمات کلیدی: آفلاتوکسیوزیس، مسمومیت غذایی، ماهی کپور، آسیب بافتی.