

Original Article**Effects of Aflatoxin-Contaminated Feed on Immunological Parameters of Common Carp (*Cyprinus carpio*)**

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ABSTRACT

Background: Aflatoxin contamination is a common natural phenomenon that is difficult to avoid or control and it can occur during pre and post-harvest periods under high humidity and temperature conditions, and are potentially dangerous to fish. In the present study, a feeding trial evaluated the effects of diets contaminated with aflatoxin on certain immunological parameters of common carp.

Methods: The immunotoxicity assessment of juvenile common carp was performed on 180 fish divided into five groups with triplicate: Control group received normal feed (Group I); group II was fed diets contaminated with extraction solution (methanol, acetone and diluted water) as a positive control. Group III-V was respectively fed diets contaminated with 0.5, 0.7 and 1.4 mg kg⁻¹ feed for 3 wk.

Results: Lysozyme activities, total immunoglobulin contents, complement C3 and C4 activities in plasma of common carp fed with different concentrations of aflatoxins significantly decreased when compared to that of the control fish. Although plasma ACH50 contents remained unchanged in 0.5 mg kg⁻¹ aflatoxins, ACH50 contents decreased in 0.7 and 1.4 mg kg⁻¹ groups after 21 d of aflatoxin treatment. No significant changes were observed in immunological parameters between the control positive and control groups throughout the experimental periods.

Conclusion: Oral exposure to aflatoxin (0.5 mg kg⁻¹≤) could adversely affect immunological parameters of common carp.

Keywords: Aflatoxicosis, Carp, Immunotoxicity, Mycotoxins.

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INTRODUCTION

In spite of improvements in storage and processing of agricultural products, there is still a great risk of their contamination with mycotoxins and different levels of mycotoxins can be found in foods used by humans or animals [1]. A 70% possibility of agricultural products' contamination with aflatoxins is reported [1]. Therefore, depending on environmental factors, different levels of aflatoxins can be found in grains, beans, soybean, nuts, dried fruits, and even animal products such as milk, meat and eggs [1].

Aflatoxins are secondary metabolites of certain mold strains including *Aspergillus flavus* and *A. parasiticus* which hurt humans and animals by DNA degradation [2] and epigenetic alterations [3], lipid peroxidation [4], disturbance in biochemical processes [5], tumorigenesis and hypotoxicity [6]; [4]. Disturbance in the antigen presenting capacity of dendritic cells [7], alterations in inflammatory

responses [8], a reduction in cytokinin synthesis [9], changes in the activity level of macrophages and neutrophils, lymphocytes disturbance, and reduced effectiveness of vaccination [10] are among changes observed in the immune system of organisms treated with aflatoxins.

At least 80 species of carp are identified in fresh water and many of them are successfully cultured in different countries. Common carp (*Cyprinus carpio*) is the third important cultured species across the world that yields a significant part of food needs in developing countries [11]. Grains and nuts, as the main items used in the commercial feed of carp, are potential to be contaminated with aflatoxin. Therefore, these animals are likely to be intoxicated with aflatoxins. Loss of appetite, reduced growth rate, and a decrease in the efficiency of food is reported in many fish treated with aflatoxin-contaminated diets [12]. Aflatoxin-contaminated feed makes histopathological changes

in tissues, especially the liver [13-15], releases hepatic enzymes to blood and changes blood biochemical parameters of fish. Consumption of food contaminated with aflatoxins increases the production rate of free radicals and leads to oxidative stress [16]. Moreover, aflatoxin intoxication may suppress the immune system [12], change gene expression [17], lead to blood disorders [14] and result in fish mortality [15].

Since aflatoxin toxicity can weaken the immune system of fish and increase their vulnerability to pathogens, investigating the influence of aflatoxins on non-specific immune parameters is essential. Therefore, we evaluated impacts of feeding fish with aflatoxin-contaminated diets on non-specific immune-parameters.

MATERIALS AND METHODS

Aspergillus flavus (PTCC 5006) was purchased from Persian Type Culture Collection (Iranian Research Organization for Science and Technology) and was cultured on Potato Dextrose Agar (PDA) and then placed all the test tubes in incubator at 37 °C for seventeen days [18]. Then fungal spores were transferred from inoculated test tubes on 200 g dried bread soaked in 30 ml distilled water. The material was shifted in eight 500 ml sterilized conical flasks and put on orbital shaker at 28 °C and 150 rpm for a period of one month. After 30 d, the aflatoxins were extracted from culture media with methanol, acetone (70:30 ratios) and diluted water and then used for aflatoxin analysis by HPLC method [15].

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. Three experimental diets with 0.5 mg kg⁻¹, 0.7 mg kg⁻¹, and 1.4 mg kg⁻¹ aflatoxins were prepared by adding the required quantities from the stock solution into the oil portion of the diet before blending and the alcohol and acetone were allowed to evaporate [13]. The ingredients were mixed with water, extruded and then dried.

Healthy common carp (*C. carpio*) were used in the present study according to the National Ethical Framework for Animal Research in Iran [19].

Common carp (*C. carpio*) were obtained from the culture ponds of private farm, Ahvaz, Khuzestan Province, Iran. Fishes were maintained in fiberglass tanks filled with fresh water in the laboratory

condition. The water was changed daily to maintain water quality at appropriate level. One hundred and eighty healthy fish with mean weight 30.7±4.5 gr, after a period of adaptation for two weeks, were transferred to fifteen experimental tanks and allowed to acclimatize to these tanks for a week. During this period, fishes were fed a commercial diet by Beyza Feed Mill (Shiraz, Iran) twice a day at the rate of 2% of body weight. The basic physicochemical parameters of water such as dissolved oxygen (6-7 mg L⁻¹), pH (7.2-7.4), temperature (22-26 °C), and salinity (0 g L⁻¹) were maintained constant. Three experimental groups were fed on diets containing 0.5 mg kg⁻¹, 0.7 mg kg⁻¹ and 1.4 mg kg⁻¹ of crude aflatoxin for 3 wk, while a fourth group fed on the diet containing extraction solution (methanol, acetone, and diluted water) as a positive control and a fifth group were fed on normal diet as control group. Fishes were deprived of food 24 h before sampling. After 21 d, 12 fish were randomly captured from each group and then anesthetized with clove powder solution (200 mg L⁻¹). Next, the fish blood was collected from caudal vein, and stored in heparinized sterile glass vials at 4 °C containing the anticoagulant heparin. The blood was centrifuged for 10 min at 6000 gr, 4 °C. Plasma samples were immediately stored at -25 °C until biochemical analysis.

Total immunoglobulin (Ig) was determined using [20] method. Thus, 100 µL of plasma sample (100-fold dilutions in PBS) was mixed with an equal volume of 12% (v/v) solution of polyethylene glycol and incubated for 2 h at room temperature that reduced the Ig. The Ig molecules were removed by centrifugation (5000 gr at 4 °C) and the protein content was determined like total protein determination by the Biuret reaction. This value was subtracted from the total protein level, which corresponds to the total Ig level and was expressed in mg mL⁻¹.

Alternative complement activity (ACH50) was evaluated following the procedure of [21] using sheep red blood cells (ShRBC) (Bahar Afshan Research & Development Institute, Iran). ShRBC were washed and adjusted to 2 × 10⁸ cell mL⁻¹ in ethylene glycol tetraacetic acid magnesium-gelatin veronal buffer (0.01 M). One hundred µL of the ShRBC suspension was lysed with 3.4 mL of distilled water and the hemolysate was determined at 414 nm against distilled water to reach 100% lysis. The test plasma was diluted, and different volumes ranging from 0.1 to 0.25 mL were brought up to 0.25 mL total volume before being allowed to react with 0.1 mL of RaRBC in test tubes. After

incubation at 20 °C for 90 min with occasional shaking, 3.15 mL of a 0.9% (v/v) saline solution was added to each tube with centrifugation at 1600 × G for 10 min at 4 °C. The absorbance (A) of the supernatant was measured using a spectrophotometer at 414 nm. A lysis curve was obtained by plotting the percentage of hemolysis against the volume of plasma added. The volume of plasma producing 50% hemolysis (ACH50) was determined and the number of ACH50 units/mL was obtained for each fish.

The immunoturbidimetric test (Pars Azmun, Iran) was adopted to determine the serum complement level. C3 and C4 in serum samples were mixed with the antibody provided by the kits, and then an antigen-antibody complex was produced. The optical density (OD) value was measured at 340 nm. Compared with the values of the standards from the kits, C3 and C4 contents were expressed in $\mu\text{g mL}^{-1}$ [22].

The turbidimetric assay for lysozyme activity was carried out according to [23] with minor modifications. Thus, plasma (50 μL) was added to 2 mL of a suspension of *Micrococcus luteus* (Actinobacteria: Micrococcaceae) (0.2 mg mL^{-1}) in a .05 M sodium phosphate buffer (pH 6.2). The reaction was carried out at 25 °C and absorbance was measured at 570 nm after 0.5 min and 4.5 min by a spectrophotometer. PBS was used as the blank. Hen's egg white lysozyme (Sigma) was used in sterile sodium phosphate Buffer (PBS) as a standard curve to determine lysozyme activity of the samples. The specific activity (units/ml plasma) of lysozyme was determined.

The significant difference in the immunological parameters of fish treated with different concentrations of aflatoxins was examined using one-way ANOVA. Data were checked for normality (Kolmogorov-Smirnov test). Means were compared by Duncan's test and a $P < 0.05$ was considered statistically significant. Statistical analyses were performed using IBM SPSS Statistics 19 (Chicago, IL, USA). Data are presented as mean (SD).

RESULTS

Plasma lysozyme activities of common carp fed with different concentrations of aflatoxin significantly decreased when compared to that of the control fish. However, in plasma, no significant changes were observed in lysozyme activity between the control positive and control groups throughout the experimental periods (Fig.1).

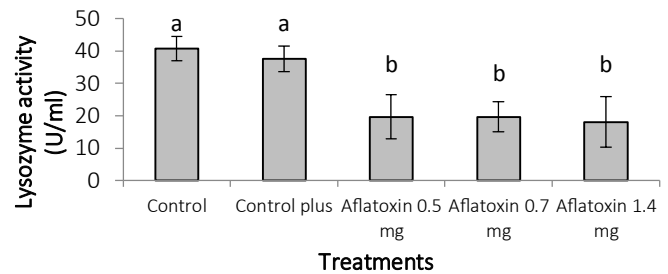


Figure 1. Plasma lysozyme activity of *Cyprinus carpio* fed diets contaminated with aflatoxin extract. Data are presented as mean \pm S.D. Different letters stand for statistically significant differences at $P < 0.5$.

In all treated groups, a decrease in Ig content was observed when compared to that of control. No significant changes were detected in Ig contents between the positive control and control groups throughout the experimental periods (Fig. 2).

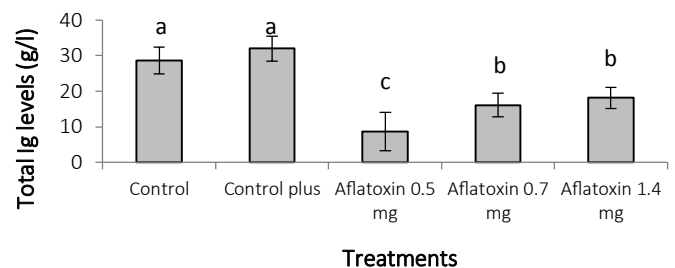


Figure 2. Plasma total immunoglobulin levels of *Cyprinus carpio* fed diets contaminated with aflatoxin extract. Data are presented as mean \pm S.D. Different letters stand for statistically significant differences at $P < 0.5$.

No significant difference in the ACH50 contents was found between the positive control and control groups throughout the experiment. Plasma ACH50 contents remained unchanged in 0.5 mg kg^{-1} aflatoxin on day 21, when compared to that of the control. However, ACH50 contents decreased in 0.7 and 1.4 mg kg^{-1} groups after 21 d of aflatoxin treatment (Fig. 3).

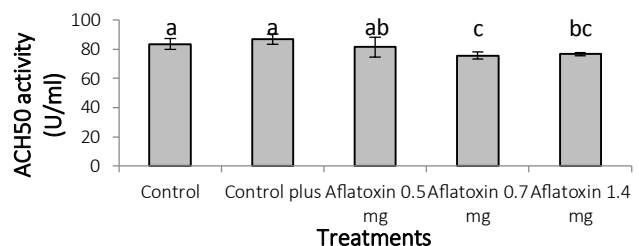


Figure 3. Plasma total complement (ACH50) activity of *Cyprinus carpio* fed diets contaminated with aflatoxin extract. Data are presented as mean \pm S.D. Different letters stand for statistically significant differences at $P < 0.5$.

No changes were observed in the contents of plasma complement C3 and C4 between the positive control and control groups. In plasma, complement C3 and C4 decreased in aflatoxin groups when compared to the control (Fig. 4, 5).

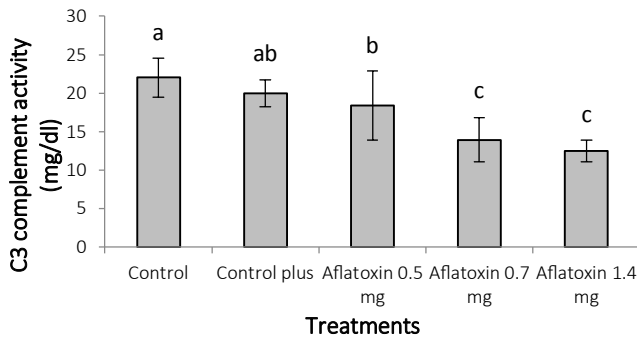


Figure 4. Plasma C3 activity of *Cyprinus carpio* fed diets contaminated with aflatoxin extract. Data are presented as mean \pm S.D. Different letters stand for statistically significant differences at $P < 0.5$.

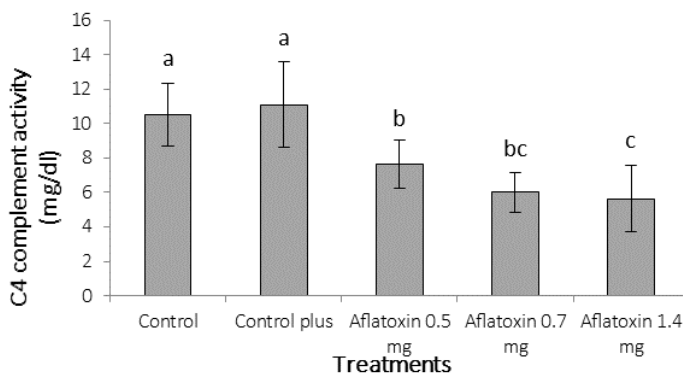


Figure 5. Plasma C4 activity of *Cyprinus carpio* fed diets contaminated with aflatoxin extract. Data are presented as mean \pm S.D. Different letters stand for statistically significant differences at $P < 0.5$.

DISCUSSION

Fish have a complex immune system including an innate immunity (e.g. Lysozymes, the complement system, immune cells, and cytokines) and an adaptive immunity (i.e. antibodies and lymphocytes [24]). Among these, Lysozyme is one of the major constituents in the innate immune system of fish with a key role in protecting the animal against infections by gram-positive and -negative bacteria [24]. Therefore, an alteration in the activity level of Lysozymes is an appropriate index to evaluate the innate immunity in the immunotoxicity of fish [24]. A decrease in the activity of Lysozyme in aflatoxin-treated fish suggests the immunotoxicity of this substance for carp. Reduced Lysozyme activity makes these fish

more vulnerable to bacterial infections. A decrease in lysozyme, C4, C3, ACH50 activities and antibody levels in birds affected by aflatoxicosis is reported [25]. Feeding Moulard duck with aflatoxin resulted in a decrease in lysozyme activity [26]. This decrease was also observed in *Oreochromis niloticus* [27] and *Labeo rohita* [28].

Total immunoglobulin (Ig) is the main element in the adaptive specific immune of blood in bony fish. The results of this study indicate that aflatoxin toxicity can significantly decrease plasma Ig in fish. Ig molecules may be the target molecules for aflatoxins. Moreover, aflatoxin can influence B-lymphocytes population, reduce the synthesis of immunoglobulin and therefore reduce Ig levels of plasma. A reduced immunoglobulin level was also found in piglets treated with aflatoxin [9]. A decrease in immunoglobulin level was reported in people intoxicated with aflatoxin [29].

The complement system has a vital role in the innate immune system and by inducing B cells to proliferate, affects the adaptive immune system as well [24,30]. The complement system is one of the first element in the innate immunity that develops. A combination of factors such as molecular diversity, high titer, and the high efficiency of the complement system in different environmental conditions has turned this system into one of the most effective parameters involved in the immune system of fish [31].

The successive activation of complements occurs from three routes which have an overlap to some extent [31]. In all these three routes, complement C3 and C4 are the main proteins [30]. Therefore, any changes in the activity level of total complement (ACH50) can affect the immunity of fish in removing pathogens [32]. In experimental animals, effects of aflatoxin B1 in disturbing thymus function, a reduction in the number and disturbance in the function of T lymphocytes, reduced activity of phagocytosis and a decrease in the complement activity are reported [29].

Complement C3 is a glycoprotein built by hepatocytes and monocytes. However, new cellular and molecular methods show that complement proteins are synthesized in different parts [30]. Complement C3 is one of the major proteins in the complement system of fish, which usually initiates both classic and alternative routes [31]. In diploid or triploid fish such as bony fish, several complement components are coded by several genes which encode different structural and functional subunits [30]. In common carp (*C. carpio*), 5 varieties of C3 are detected [30], while it

consists of 3 subunits in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) [30]. Activation of C3 leads to the production of a penetrating complex capable of lysis of the pathogen's membrane and removing pathogens through inflammation, producing anaphylatoxin and opsonins. Complement C3 is of the family alpha 2-macroglobulins and consists of two chains of alpha and beta linked in a disulphide bond [30]. Aflatoxin intoxication may reduce gene expression or damage protein structure of complement C3 and so reduce its level in plasma of fish. Complement C3 is a flexible molecule associated with other proteins of the complement system. Therefore, any changes in C3's function can affect the activity of other complement components [33]. Complement component 4 (C4) is a glycoprotein produced by macrophages and monocytes. Complement C4 identifies some inflammatory agents with the help of other proteins of the immune system, then this complement makes them interact and removes the inflammatory agents [34]. A decrease in C4 in aflatoxin-treated fish may be due to a decrease in macrophages and monocytes or a reduced synthesis level of C4 in such cells. A reduced level of C4 can account for a suppressed immune system in fish treated with aflatoxins. Suppression of a cell-dependent immune response and disturbance in the phagocytosis performance of monocytes and reduced number of monocytes were observed in different species of animals treated with aflatoxins [29].

Aflatoxin reduces protein synthesis in cells which produce lysozyme, C4, C3, ACH50 and total immunoglobulin [25] and thus may decrease their activity in plasma of fish.

CONCLUSION

The presence of aflatoxin in diets of common carp is highly toxic. Furthermore, aflatoxin (0.7 and 1.4 mg per kg diet) reduces the innate immune system of common carp by decreasing the activity of lysozyme, total complement (ACH50), C3, C4 and Ig in plasma. Therefore, aflatoxin has toxic effects on the immune system of fish even in low concentrations.

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