

Original Article**Evaluation of Nephrotoxic Effects of Aflatoxins on Common Carp (*Cyprinus carpio*)**

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ABSTRACT

Background: We investigated the effects of different dose of aflatoxins, secondary toxic metabolites produced by *Aspergillus flavus*, on some biochemical parameters in kidney of common carp (*Cyprinus carpio*).

Methods: This study was done in Aquaculture and Biology Laboratory of Behbahan Khatam Alanbia University of Technology, Behbahan, Iran in 2015. Fishes were distributed into five groups: Group I-III was fed contaminated diets with 0.5, 0.7 and 1.4 mg kg⁻¹ feed, respectively. Group IV was fed contaminated diets with extraction solution (methanol, acetone, and diluted water) as a positive control. Control group received normal feed (Group V). After 21 d of experiment, activities of cellular enzymes and oxidative stress biomarker were evaluated.

Results: Aflatoxins (0.7 and 1.4 mg kg⁻¹) caused a significant increase in ALT activity. Although, significant increase of LDH activity ($P<0.05$) were found in kidney of fish fed diet contaminated with 0.5 mg kg⁻¹ of aflatoxins, LDH activity was significantly decreased in kidney of fish fed diet contaminated with 0.7 and 1.4 mg kg⁻¹ of aflatoxins. A significant increase ($P<0.05$) were observed in MDA levels and CAT activity in kidney of fish fed diet contaminated with different concentrations of aflatoxins for 21 d. The total antioxidant levels, AST and ALP activities in kidney of fish were significantly reduced ($P<0.05$) on the 21st day following aflatoxins administration.

Conclusion: Diets containing certain concentrations of aflatoxins (0.5, 0.7 and 1.4 mg kg⁻¹ feed) made oxidative damage to kidney tissue, including changes in oxidative stress biomarker and biochemical parameters.

Keywords: Aflatoxins, Biochemical Parameters, Common Carp, Nephrotoxicity, Oxidative Stress.

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INTRODUCTION

The increasing amount of herbal compounds in fish feed has enhanced chances of feed contamination with toxins, fungi metabolites and mycotoxins [1, 2]. The same problem has been reported in varied species of farmed aquaculture [3]. Aflatoxins are polyketide secondary-derived metabolites produced by strains of *Aspergillus flavus* and *Aspergillus parasiticus* [1]. The carcinogenic, mutagenic and immunosuppressive potential of aflatoxins is fairly well known in fish.

Depending on the aflatoxin level in feed, the major effects of poisoning with aflatoxin in trout are a decrease in protein synthesis, changes in lipid metabolism, disturbance in mitochondrial respiration, liver cancer, inhibition of nucleic acids, and an increase in lipid accumulation in liver [4]. However, the toxic effects of mycotoxins may differ depending on the fish species and age.

Younger fish are usually more vulnerable to aflatoxins. Symptoms of acute aflatoxin toxicity are pale gills, disturbance in blood clotting, anemia, generalized weakness and reduced growth rate [4]. Long-term exposure to low concentrations of aflatoxin B1 may lead to liver tumors (in the form of yellow nodules even found in the kidney), neoplasia, and an increase in mortality rate of fish [5-7]. A decrease in plasma total protein, globulin and antibody titer in rohu (*Labeo rohita*) [1], a decrease in growth rate, specific growth rate (SGR) and an increase in feed conversion rate (FCR) in beluga, *Huso huso* [8], as well as immunosuppression and increased sensitivity of fish to bacterial, viral and parasitic infections are other consequences of aflatoxicosis [4].

Mycotoxins can cause cytotoxicity; however, aflatoxins' exact mechanism is not fully recognized [9]. One of the probable mechanisms

of aflatoxins in cellular toxicity is causing oxidative stress attributed to the imbalance between oxidants and antioxidants [9-12]. The increased generation of free radicals and an increase in lipid peroxidation may enhance aflatoxin production by toxigenic strains [13].

Cytochrome P450 enzymes induce aflatoxin degradation [10]. Hydrogen peroxide and hydroxyl radicals produced in aflatoxins' detoxification [11, 12] are the main factors of proteins, lipids, and DNA peroxidation in spleen of rats treated with aflatoxin B1 [10]. By inducing the production of reactive oxygen species (ROS) following proteins, lipids, and DNA oxidation, aflatoxin can cause oxidative stress [10]. Moreover, aflatoxin B1 may, directly or indirectly initiate genotoxicity and apoptosis [14].

The aim of this study was a better understanding of aflatoxins toxic effects and the influence of oxidative stress in pathogenesis. For this reason, changes in biochemical parameters and indices of oxidative stress in kidney of common carp treated with aflatoxin were evaluated.

MATERIALS AND METHODS

This study was done in Aquaculture and Biology Laboratory of Behbahan Khatam Alanbia University of Technology, Behbahan, Iran in 2015. *Aspergillus flavus* (PTCC 5006) purchased from Persian Type Culture Collection (Iranian Research Organization for Science & Technology), was cultured on Potato Dextrose Agar (PDA), and placed all the test tubes in incubator at 37 °C for 17 d [15]. Then, 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 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 [16].

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 experiment diets with 0.5 mg kg⁻¹, 0.7 mg

kg⁻¹, and 1.4 mg kg⁻¹ 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. The ingredients were mixed with water, extruded and then dried.

Healthy common carp (*Cyprinus carpio*) were used in the present study according to the National Ethical Framework for Animal Research in Iran [17]. The common carp were obtained from commercial suppliers (Ahvaz, Iran) and transported to the laboratory facilities for a period of acclimation before the exposure assays. Before the assay, species were acclimated for two weeks in fiberglass tank (1000 L) 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 the acclimation period, *C. carpio* (n: 180; 30±5 g) were randomly distributed into 80 L capacity polystyrene tanks, in groups of 12 fish per tank. The water was renewed (50% rate) every 24 h and assay had duration of 21 d. During experiment 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, while a fourth group fed the diet containing extraction solution (methanol, acetone, and diluted water) as a positive control and a fifth group were fed on normal diet formed the control group. Fishes were deprived of food 24 h before sampling. After 21 d, 12 fish were captured randomly from each group and then anesthetized with clove powder solution (200 mg L⁻¹). Fish were sacrificed by decapitation and dissected to remove the liver, kidney and gills. For enzymatic and biochemical analyses, tissue samples from the target organs were homogenized on ice in cold buffer 100 mM potassium phosphate (Sigma-Aldrich, Germany) pH 7.0 containing 2 mM of EDTA (Riedel-Haën, Germany). Tissue homogenates were centrifuged at 12000x gr for 15 min at 4 °C. The supernatant was removed and freeze at -25 °C for further analysis.

Biochemical Parameters Analysis

Aspartate aminotransferase (AST) was assayed in a coupled reaction with malate dehydrogenase in the presence of NADH. In alanine aminotransferase (ALT) assay, the enzyme reacts with alanine and α-ketoglutarate to

form glutamate and pyruvate. Lactate dehydrogenase converts pyruvate to lactate and NAD^+ . AST and ALT activities were recorded by measuring changes in absorbance during 3 min at 340 nm. Lactate dehydrogenase (LDH) activity was measured based on the conversion of pyruvate to L-lactate by monitoring the oxidation of NADH. LDH activity was monitored during 3 min at 340 nm Alkaline phosphatase (ALP) assay is based on the enzyme-mediated conversion of p-nitrophenol phosphate to nitrophenol in an alkaline buffer at 405 nm [18]. Protein levels in tissues were determined by standard procedures used in clinical biochemistry laboratories according to the biochemical kits user manuals (ParsAzmun Co, Iran) [19]. CAT activity was determined [20], although, with some modifications. Catalase activity was measured by hydrogen peroxidase assay based on the formation of its stable complex with ammonium molybdate. Two hundred μL of the supernatant was incubated in working solution including 1000 μL hydrogen peroxide and 500 μL phosphate buffer (pH: 7.4) at 25 °C for 60 sec. Then 1000 μL of 32.4 $\text{mmol}\cdot\text{L}^{-1}$ ammonium molybdate was added to the reaction solution and the concentration of the yellow complex of molybdate and hydrogen peroxide was measured at 405 nm wavelengths.

$$\text{Catalase activity (kU}\cdot\text{L}^{-1}) = \frac{A(\text{sample}) - A(\text{blank 1})}{A(\text{blank 2}) - A(\text{blank 3})} \times 271$$

Blank one contained 1.0 mL substrate, 1.0 mL molybdate and 0.2 mL distilled water; blank two contained 1.0 mL substrate, 1.0 mL molybdate and 0.2 mL buffer; blank 3 contained 1.0 mL buffer, 1.0 mL molybdate and 0.2 mL buffer.

Total antioxidant capacity was estimated according to the ferric reducing ability of plasma (FRAP). Briefly, the FRAP reagent contained 5 mL of a (10 mmol/L) TPTZ (2,4,6- tripyridyl- s- triazine) solution in 40 mmol/L HCL plus 5 mL of FeCl_3 (20 mmol/L) and 50 mL of acetate buffer, (0.3 mol/L, pH=3.6) and was prepared freshly. Totally, 100 μL aliquots of the supernatant were mixed with 3 mL FRAP reagent. The conversion rate of ferric tripyridyl-s-triazine (Fe^{3+} -TPTZ) complex to ferrous tripyridyl-s-triazine (Fe^{2+} -TPTZ) at pH 3.6 and 25 °C is directly proportional to the concentration of total antioxidant in the sample. Fe^{2+} -TPTZ has an intense blue color monitored for up to 5 min at

593 nm by a UV/VIS spectrophotometer. Calculations were performed using a calibration curve of $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$ (100 to 1000 $\mu\text{M}/\text{L}$) [21].

Malondialdehyde (MDA) content was assessed by modified thiobarbituric acid assay and was expressed as $\mu\text{mol}/\text{g}$ tissue [22]. Briefly, 500 μL of the supernatant was transferred to a Pyrex tube and mixed with 2500 μL trichloroacetic acid (20%) and 1000 μL thiobarbituric acid (67%). The tubes were placed in boiling water (100 °C) for 15 min. After cooling, the chromogenic substrate was extracted into the organic phase with 1000 μL of distilled water and 5000 μL *n*-butanol: pyridine (15: 1). The mixture was then centrifuged at 2000 g for 15 min at 4 °C. The pink color produced by these reactions was measured spectrophotometrically at 532 nm to measure MDA levels. MDA concentration was calculated using MDA standard. Tetraethoxypropane and absolute ethanol were used to prepare the MDA standards. Concentrations of MDA in tissue samples are expressed in μM per g protein. All biochemical parameters were measured by UV/VIS spectrophotometer (model Biochrom Libra S22). Statistical analysis of the results was carried out by one-way ANOVA, after the data had been checked for assumptions of normality and homogeneity (Shapiro-Wilk test) and, if necessary, appropriately transformed. The Duncan test was used to compare pairs of means and detect significant differences ($P<0.05$). The statistical analysis was performed at the significance level of 5%, using the SPSS (IBM, Release 19) software. Data are presented as mean \pm S.D.

RESULTS

Concerning to kidney and comparatively to controls, significantly decrease of AST activity ($P<0.05$) were observed in fish fed diet contaminated with different concentrations of aflatoxins (Figure 1). In respect to kidney, there was a significant increase in ALT activity in fish fed diet contaminated with 0.7 and 1.4 mg kg^{-1} of aflatoxins as compared with control groups (Figure 2). Although significant increase of LDH activity ($P<0.05$) were found in kidney of fish fed diet contaminated with 0.5 mg kg^{-1} of aflatoxins, LDH activity in kidney of fish fed diet contaminated with 0.7 and 1.4 mg kg^{-1} of aflatoxins were significantly lower than its activity in control groups (Figure 3).

Significant decreases in ALP activity in kidney ($P<0.05$) were occurred after 21 d for the treatment fish with aflatoxins, comparatively to controls. CAT activity in kidney increased significantly ($P<0.05$) in fish fed diet contaminated with different concentrations of aflatoxins after 21 d (Figure 5). A significant

increase ($P<0.05$) were observed in MDA levels in kidney of fish fed diet contaminated with different concentrations of aflatoxins for 21 d (Figure 6). There was a significant decrease ($P<0.05$) of total antioxidant levels in kidney of fish fed diet contaminated with aflatoxins as compared with control groups (Figure 7).

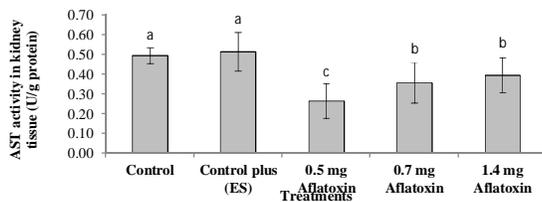


Figure 1. Aspartate aminotransferase (AST) activity in kidney of common carp fed on contaminated diets with different concentrations of aflatoxins. Significant differences between values when compared with control groups were showed by alphabet letters ($P<0.05$), similar alphabet letters indicated no significant difference between experimental groups. Error bars represent the mean \pm S.D.; ES: Extract solution.

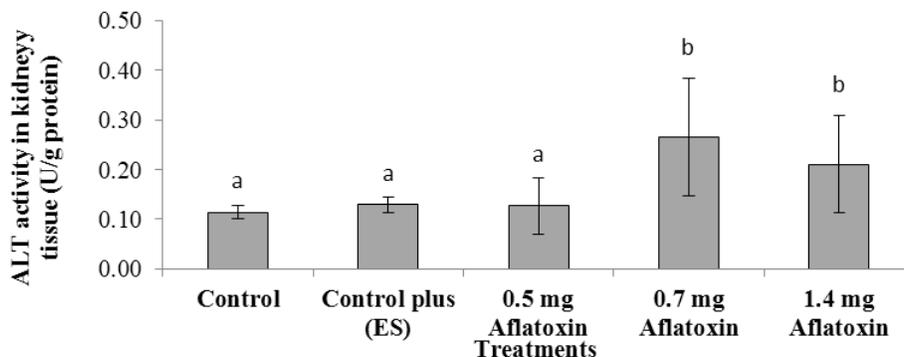


Figure 2. Alanine aminotransferase (ALT) activity in kidney of common carp fed on contaminated diets with different concentrations of aflatoxins. Significant differences between values when compared with control groups were showed by alphabet letters ($P<0.05$), similar alphabet letters indicated no significant difference between experimental groups. Error bars represent the mean \pm S.D.; ES: Extract solution.

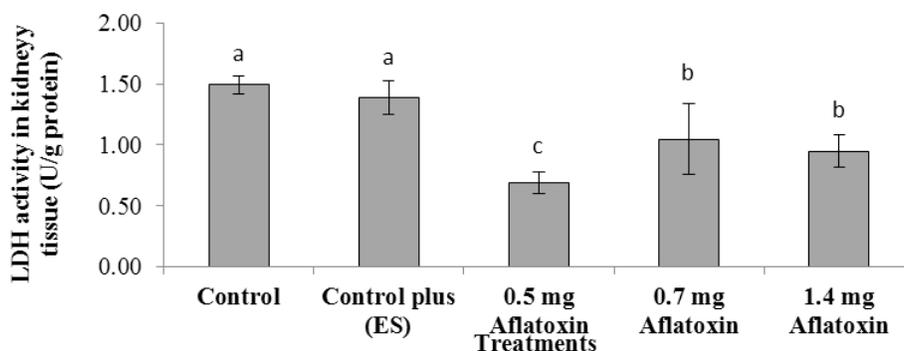


Figure 3. Lactate dehydrogenase (LDH) activity in kidney of common carp fed on contaminated diets with different concentrations of aflatoxins. Significant differences between values when compared with control groups were showed by alphabet letters ($P<0.05$), similar alphabet letters indicated no significant difference between experimental groups. Error bars represent the mean \pm S.D.; ES: Extract solution.

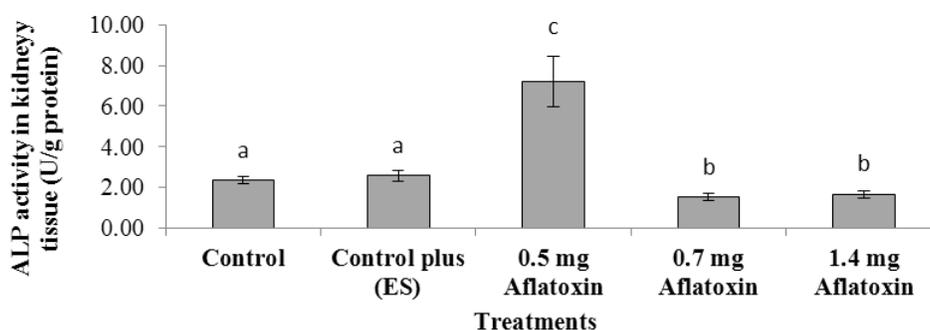


Figure 4. Alkaline phosphatase (ALP) activity in kidney of common carp fed on contaminated diets with different concentrations of aflatoxins. Significant differences between values when compared with control groups were showed by alphabet letters ($P<0.05$), similar alphabet letters indicated no significant difference between experimental groups. Error bars represent the mean \pm S.D.; ES: Extract solution.

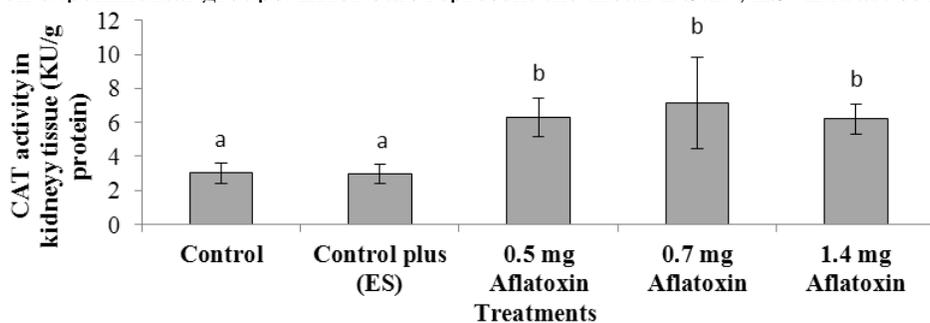


Figure 5. Catalase (CAT) activity in kidney of common carp fed on contaminated diets with different concentrations of aflatoxins. Significant differences between values when compared with control groups were showed by alphabet letters ($P<0.05$), similar alphabet letters indicated no significant difference between experimental groups. Error bars represent the mean \pm S.D.; ES: Extract solution.

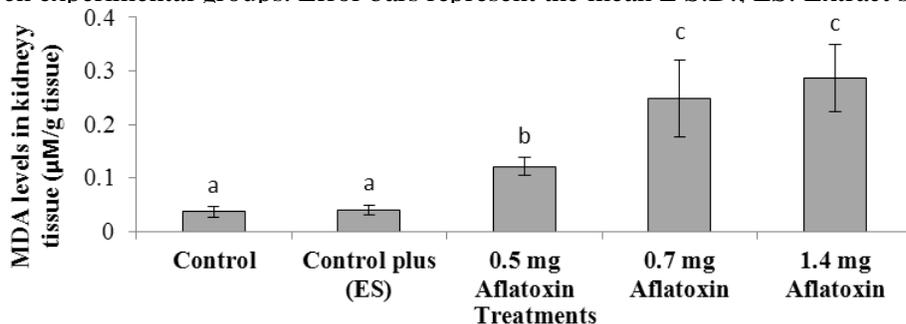


Figure 6. Malondialdehyde (MDA) levels in kidney of common carp fed on contaminated diets with different concentrations of aflatoxins. Significant differences between values when compared with control groups were showed by alphabet letters ($P<0.05$), similar alphabet letters indicated no significant difference between experimental groups. Error bars represent the mean \pm S.D.; ES: Extract solution.

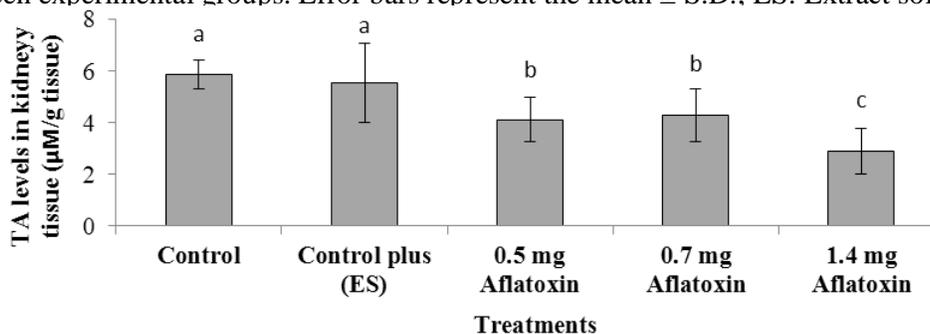


Figure 7. Total antioxidant (TA) levels in kidney of common carp fed on contaminated diets with different concentrations of aflatoxins. Significant differences between values when compared with control groups were showed by alphabet letters ($P<0.05$), similar alphabet letters indicated no significant difference between experimental groups. Error bars represent the mean \pm S.D.; ES: Extract solution.

DISCUSSION

Mycotoxins often cause cytotoxicity through several mechanisms, including the production of free radicals [10]. Poisoning with aflatoxins may provide the grounds for the production of peroxide radicals (O_2^-) in cells [10]. Since peroxide radicals (O_2^-) in cells may convert to other ROS compounds, the peroxidation rate of micro-molecules increases [23].

Consequently, the increase in malondialdehyde (MDA), as the final metabolite of lipid peroxidation in kidney cells of fish treated with aflatoxin 0.7 and 1.4 mg kg⁻¹ feed for 21 d can be attributed to an increase in ROS compounds and lipid peroxidation rate. The cytotoxic nature of aflatoxins may underlie increased MDA [24-26]. Increased lipid peroxidation (LOP) is reported in kidney of mice treated with aflatoxin B1 [27]. By damaging the digestive system, aflatoxins negatively affect the absorption of nutrients, especially vitamins and minerals. Therefore, a decrease in absorption and level of certain vitamins and minerals might weaken the defense mechanism of the cellular antioxidant system [28]. The reduced capacity of cellular antioxidant defense system and reduced level of vitamins such as vitamin A and E, which have an important role in inhibition, and prevention of lipid peroxidation by aflatoxin may lead to an increase in MDA [28]. The MDA produced during lipid peroxidation may form covalent bonds with other components of the cell membrane and therefore affect the physiological function of cell membranes [27].

The cellular antioxidant defense system, including antioxidant enzymes and non-enzymatic antioxidants, may help in eliminating free radicals; however, a significant decrease ($P<0.05$) in total anti-oxidant (TA) level of kidney cells of fish can accelerate the process of oxidative stress, which can be due to aflatoxin. Changes in the activity of antioxidant enzymes and a decrease in non-enzymatic antioxidants caused by aflatoxins [28] might be the main factor in reducing the cellular total antioxidant level. A decrease in the cellular antioxidant capacity is reported in blood cells treated with aflatoxin B1 [26]. A decrease in enzymatic and non-enzymatic antioxidant levels was reported in kidney of rats treated with aflatoxin B1 [29].

Catalase (CAT) is one of the main antioxidant enzymes that is responsible for

degradation of the hydrogen peroxide formed in cells under normal circumstances. Therefore, a significant ($P<0.05$) increase in the activity of CAT in kidney cells of fish treated with different concentrations of aflatoxin may be a physiological response to an increase in hydrogen peroxide in cells. Increased CAT activity was observed in kidney of mice treated with aflatoxin B1 [27].

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) have a key role in the final stages of protein breakdown in order to produce ATP [30]. Furthermore, increased activity of these enzymes in kidney cells of fish may be effective in using amino acids in oxidation process of glycogenesis in cells to provide energy for coping with the toxic effects of aflatoxin. Amino acids participate in detoxification by conjugating with toxic metabolites [31]. Thus, the increased activity of AST and ALT could be an appropriate mechanism to increase free amino acids to accelerate detoxification process. An increase in AST and ALT is reported in various tissues of rats [32, 33] and Nile tilapia, *Oreochromis niloticus*, [3] treated with aflatoxin B1.

The effect of aflatoxin-contaminated diets on LDH activity was dose dependent. A significant increase ($P<0.05$) in LDH activity in renal cells of fish treated with aflatoxin 0.5 mg kg⁻¹ feed in 21 d may signify a disturbance in oxidative phosphorylation in mitochondria, alteration in glycolysis and NADPH re-oxidation under anaerobic conditions. There was a significant increase in LDH activity in various tissues of rats [33] treated with aflatoxin B1. A significant ($P<0.5$) decrease in LDH in renal cells of fish fed with aflatoxin 0.7 and 1.4 mg kg⁻¹ feed could be the result of a disturbance in LDH biosynthesis in cells. Alkaline phosphatase (ALP) is one of the cell membrane enzymes that have a major role in hydrolysis of phosphate groups in biological molecules such as nucleotides and proteins. A significant ($P<0.05$) decrease in ALP activity in kidney cells of fish treated with different concentrations of aflatoxin may be due to damage to cell membranes and alteration in the biological nature of ALP. Decreased ALP level in intestinal mucosa of rats treated with aflatoxin is also reported [34].

CONCLUSION

Feeding fish with diets contaminated with different concentrations of aflatoxin (0.5, 0.7 and 1.4 mg kg⁻¹ feed) can lead to oxidative stress. An

increase in lipid peroxidation, a decrease in the cellular total antioxidant capacity and changes in other markers of oxidative stress show that aflatoxin cytotoxicity could be the result of an increase in ROS compounds.

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