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Effects of dietary exposure to aflatoxins on some plasma biochemical indices of common carp (*Cyprinus carpio*)

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Abstract

Aflatoxins are a group of secondary fungal metabolites that occur widely as natural contaminants of many feeds under high humidity and temperature, and are potentially dangerous to fish. Therefore, this study was designed to investigate the effects of aflatoxins on some plasma biochemical indices, as clinical biomarkers, in common carp, *Cyprinus carpio*. Fish were fed diets contaminated with 0 (control), 0.5, 0.7 and 1.4 mg aflatoxins per kg feed for 3 weeks. No significant changes (p>0.05) were observed in alanine aminotransferase (ALT) activity in plasma of fish. Alkaline phosphatase (ALP) activity, total protein and globulin levels in fish fed aflatoxins showed a significant (p<0.05) decrease; however, plasma aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) activities, glucose, cholesterol, triglyceride and creatinine levels were significantly higher (p< 0.05) than in the control group. The results showed that administration of 0.70 and 1.40 mg kg^{-1} of aflatoxins in fish significantly (p<0.05) increased albumin levels. The results of this study show that diets containing certain concentrations of aflatoxins (0.5, 0.7 and 1.4 mg kg^{-1} feed) caused serious toxic effects, including changes in plasma biochemical indices.

Keywords: Aflatoxins, Common carp, Biochemical indices, Aflatoxicosis

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Introduction

Mycotoxins are secondary toxic metabolites that are produced by the growing fungi in food products such as corns, peanuts, etc. (Ding *et al*., 2012). Therefore, using contaminated corn, wheat, peanuts and sorghum in commercial formulated diet or even storing feed in adverse conditions may provide the grounds for the occurrence of aflatoxicosis in the animals that consume such feeds (Bankole *et al*., 2010). Aflatoxin is produced by the fungi belonging to the genus *Aspergillus*, especially *A. flavus*, *A. parasiticus* and *A. nomius* (Ding *et al*., 2015). So far, 17 metabolites have been recognized as aflatoxins. Aflatoxin B1 (AFB1) is the major and most common form of aflatoxin which is usually found in contaminated cereal (Amiridumari *et al*., 2013). Aflatoxins rapidly enter the liver through the bloodstream and are then absorbed by hepatocytes (Guindon *et al*., 2007). Biologically, aflatoxins have a high potential in developing cancers, mutations, hepatotoxicity and teratogenicity (Amiridumari *et al*., 2013). When the accumulated aflatoxin B1 transfers from fish to humans, it may have carcinogenic, mutagenic and immunosuppressive effects (Huang *et al*., 2011). Therefore, recognizing clinical signs of aflatoxin poisoning in fish seems essential in providing the food safety of consumers and eliminating fish suspected of aflatoxicosis (Manning *et al*., 2005).

Reduced growth rate, behavioral abnormalities, immunosuppression, necrosis of liver cells, damage to the gonads, and aflatoxin accumulation in liver and edible tissues of fish are other consequences of feeding fish with aflatoxin-contaminated diets (Huang *et al*., 2014). Mycotoxins affect cells by producing free radicals and reactive oxygen species (ROS) (Marin and Taranu, 2012). By increasing the production of ROS, aflatoxins and especially aflatoxin B1 can damage cells of target organs such as the liver. Following this increase, there is a significant change in blood biochemical indices as well as an increase in lipid peroxidation metabolites in the liver (Alpsoy and Yalvac, 2011) and kidney and a decrease in the cellular total antioxidant in rats (Rastogi *et al*., 2001; El-Nekeety *et al*., 2011; Hathout *et al*., 2011), mice (Adedara *et al*., 2010; Kanbur *et al*., 2011; Eraslan *et al*., 2013), and birds (Sirajudeen *et al*., 2011). Biochemically, aflatoxins may affect organisms by influencing the energy budget and metabolism of carbohydrates, lipids, nucleic acids and proteins (Amiridumari *et al*., 2013).

Although aflatoxicosis was first reported in rainbow trout in 1960 (Raghavan *et al*., 2011), symptoms of aflatoxicosis have also been studied in other farmed fish species such as *Ictalurus punctatus* (Manning *et al*., 2005; Manning *et al*., 2011), *Oreochromis niloticus* (Tuan *et al*. 2002), *Cyprinus carpio* (He *et al*., 2010), *Labeo rohita* (Sahoo *et al*., 2003), *Fenneropenaeus indicus* (Ghaednia *et al*., 2013), and *Penaeus monodon* (Boonyaratpalin *et al*., 2001). The LD_{50} content of aflatoxin in cultured species is reported between 0.3

to 17.9 mg per kg body weight (Andleeb *et al*., 2015). Behavioral changes and clinical signs of aflatoxicosis are reported in gibel carp that were fed concentrations of aflatoxin in a range between 3.2 and 991.5 µg per kg feed for 12 weeks (Huang *et al*., 2011). It is reported that diets containing less than 1641 µg aflatoxin per kg feed had no effects on the mortality of tilapia during 20 weeks (Deng *et al*., 2010). However, mortality rates in sturgeon fed aflatoxins at 41.7 µg kg^{-1} feed were more than 50% (Raghavan *et al*., 2011). These findings indicate differences in the physiological responses and tolerance threshold of varied species to aflatoxin. Cold-water fish are more sensitive to aflatoxin compared to warm-water fish (Tuan *et al*., 2002). In most of these researches, hematological changes, histopathological damages, as well as growth indices have been studied. However, we have little information on aflatoxin effects on blood biochemical indices. Since the study of blood biochemical indices is a fairly quick and accurate method for diagnosis of damage to internal organs (Soleimany *et al*., 2016), the relevant findings in fish treated with oral aflatoxin could be useful in evaluating fish health.

Common carp (*C. carpio*) is an important farmed fish in Iran. Since formulated diets of common carp mainly consist of herbal raw materials, carp are more vulnerable to aflatoxin, compared to other farmed species in Iran. That is why we used common carp as our laboratory model to assess toxic effects of aflatoxin. This study aimed at investigating blood biochemical indices in common carp which was treated with an aflatoxin diet for 21 days by feeding fish with sub-lethal concentrations of aflatoxin.

Materials and methods

A. 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). All the test tubes were then placed in an incubator at 37 °C for seventeen days (Shotwell *et al*., 1996). The fungal spores were transferred from the inoculated test tubes on to 200 g dried bread which was soaked in 30 ml distilled water. The material was shifted to eight 500 mL sterilized conical flasks and put on an orbital shaker at 28 °C and 150 rpm for a period of one month. After 30 days, the aflatoxins were extracted from the culture medium with methanol, acetone (70:30 ratios) and diluted water and then used for aflatoxin analysis by HPLC method (Raghavan *et al*., 2011).

All the ingredients of the commercial feed were powdered, sieved, blended and extruded through a kitchen noodle maker with a 3 mm die, dried at 55 °C overnight and stored in a freezer. The experiment diet had the same composition as that of the control diet to which varying concentrations of 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 was allowed to evaporate. The ingredients were mixed with water, extruded and then dried. Aflatoxin concentration was calculated according to the following formula:

$$x = \frac{log\, b - log a}{n - 1}$$

(b): LD_{50} dose of aflatoxins for carp: 12.6 mg kg^{-1}; (a): minimum sub-lethal dose of aflatoxins for carp: 0.5 mg kg^{-1} (Sahoo *et al*., 2003); n: treatments

Common carp (*C. carpio*) samples were obtained from the culture ponds of a private farm, Ahvaz, Khuzestan Province, Iran. Fish were maintained in fiberglass tanks filled with fresh water under laboratory conditions. The water was changed daily to maintain water quality at an appropriate level. After a period of adaptation for two weeks, one hundred and eighty healthy fish with a mean weight of 30.7±4.5 g were transferred to fifteen experimental tanks and allowed to acclimatize to these tanks for a week. During this period, fishes were fed with 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 weeks, while a fourth group was fed on the diet containing extraction solution (methanol, acetone and diluted water) as a positive control and a fifth group was fed on a normal diet as the control group. Fish were deprived of food 24 hours before sampling. After 21 days, 12 fish were randomly captured from each group and then anesthetized with clove powder solution (200 mg L^{-1}). Next, fish blood was collected from the caudal vein of the fish, and stored in heparinized sterile glass vials at 4 °C. The blood was centrifuged for 10 min at 6000 g and at 4 °C. Plasma samples were immediately stored at -25 °C until biochemical analysis.

Biochemical indices of blood

Measuring the biochemical indices was done using the kits supplied by Pars Azmun Company and a UV/VIS spectrophotometer (model Biochrom Libra S22). Total plasma protein was measured by the Biuret reaction at 540 nm, albumin level was measured by the immediate Bromocresol Green reaction and at 630 nm, and the plasma globulin was measured based on the ratio of albumin versus total protein (Johnson *et al.*, 1999). Plasma glucose was measured by the glucose-oxidase method at 500 nm (Sacks, 1999), Plasma cholesterol levels were determined by the CHOD-PAP enzymatic method at 510 nm, triglyceride level was measured by GPO-PAP enzymatic method at 546 nm (Rifai *et al.*, 1999) and creatinine was measured by the JAFFE method and at 510 nm (Foster-Swanson *et al.*, 1994). The activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma was measured by NADPH consumption and its conversion to NAD^{+} at 340 nm,

lactate dehydrogenase (LDH) in plasma based on the conversion of pyruvate to lactate at 340 nm, alkaline phosphatase (ALP) based on converting nitro phenol phosphate into nitrophenol and phosphate at 405 nm (Moss and Henderson, 1999). All biochemical indices were measured according to the manufacturers' manuals.

Data analysis

Significant differences in the biochemical indices of specimens treated with the different concentrations of aflatoxin were assessed using one-way ANOVA. All the data were examined for normality (Kolmogorov-Smirnov test). The significant means were compared by Duncan's test and $p<0.05$ was considered statistically significant. Statistical analyses were performed using SPSS (IBM, Ver. 19) software. Data are presented as mean ± SE.

Results

The results of various blood biochemical indices are presented in figs. 1-11.

There was a significant difference ($p<0.05$) in the AST activity between the fish fed on contaminated diets with 0.70 and 1.40 mg kg^{-1} aflatoxins and uncontaminated diet. Further comparisons by Duncan's analysis revealed that AST activity in fish fed contaminated diets with 0.50 mg kg^{-1} aflatoxins was significantly lower than AST activity in other groups fed contaminated diets with higher doses of aflatoxins (Fig. 1).

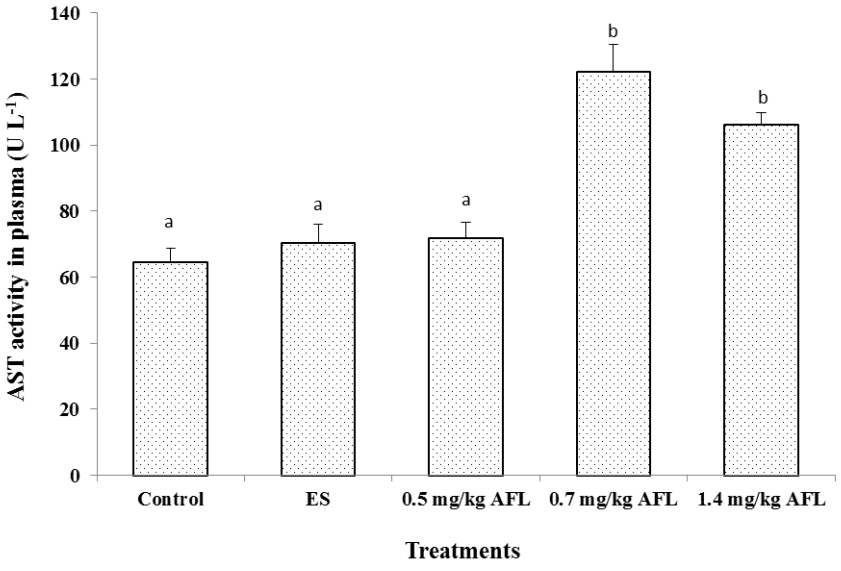

Figure 1: Aspartate aminotransferase (AST) activity in plasma of common carp fed on contaminated diets with different concentrations of aflatoxins. Significant differences between values when compared with control groups are shown with different letters ($p<0.05$), similar letters indicated no significant difference between experimental groups. Error bars represent mean+S.E.M; ES: Extract solution; AFL: Aflatoxins.

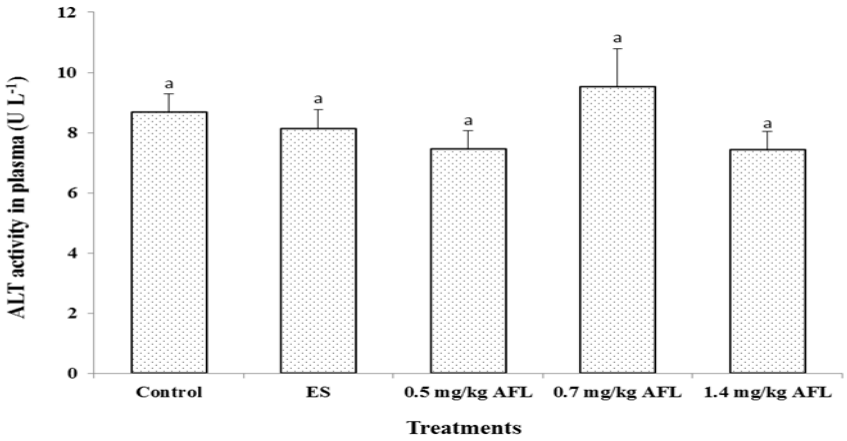

Figure 2: Alanine aminotransferase (ALT) activity in plasma of common carp fed on contaminated diets with different concentrations of aflatoxins. Significant differences between values when compared with control groups are shown with different letters (p<0.05), similar letters indicated no significant difference between experimental groups. Error bars represent mean±SEM; ES: Extract solution; AFL: Aflatoxins.

There were no significant changes (p>0.05) in ALT activity in the plasma of fish fed contaminated diets with aflatoxins when compared to control groups (Fig. 2).

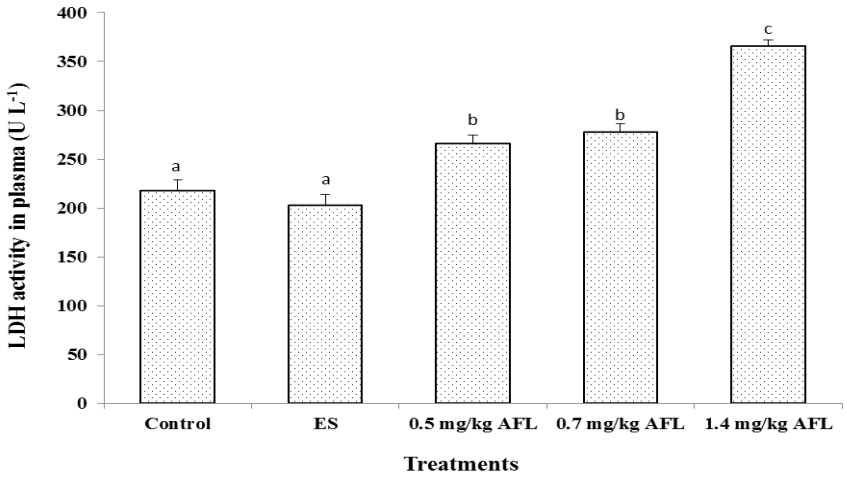

Figure 3: Lactate dehydrogenase (LDH) activity in plasma of common carp fed on contaminated diets with different concentrations of aflatoxins. Significant differences between values when compared with control groups are shown with different letters (p<0.05), similar letters indicated no significant difference between experimental groups. Error bars represent mean±SEM; ES: Extract solution; AFL: Aflatoxins.

Statistically there was a significant difference in the plasma and LDH activities of fish fed contaminated diets with aflatoxins when compared to control groups (Fig. 3).

One way ANOVA revealed that ALP activity was significantly decreased in plasma in fish fed diets containing aflatoxins (Fig. 4).

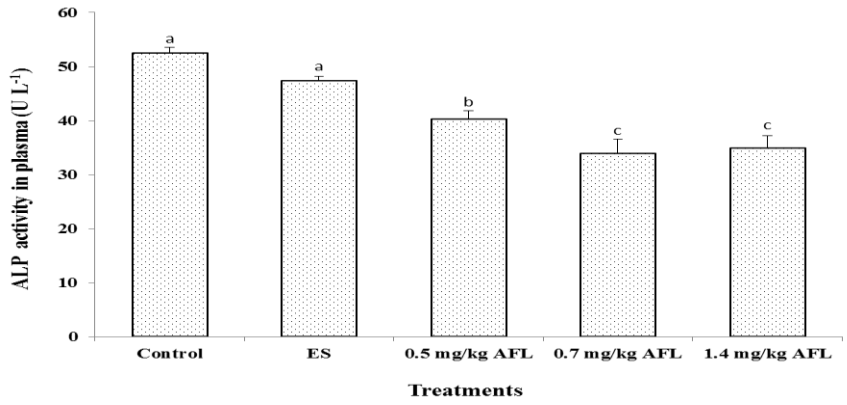

Figure 4: Alkaline phosphatase (ALP) activity in plasma of common carp fed on contaminated diets with different concentrations of aflatoxins. Significant differences between values when compared with control groups are shown with different letters (p<0.05), similar letters indicated no significant difference between experimental groups. Error bars represent mean±SEM; ES: Extract solution; AFL: Aflatoxins.

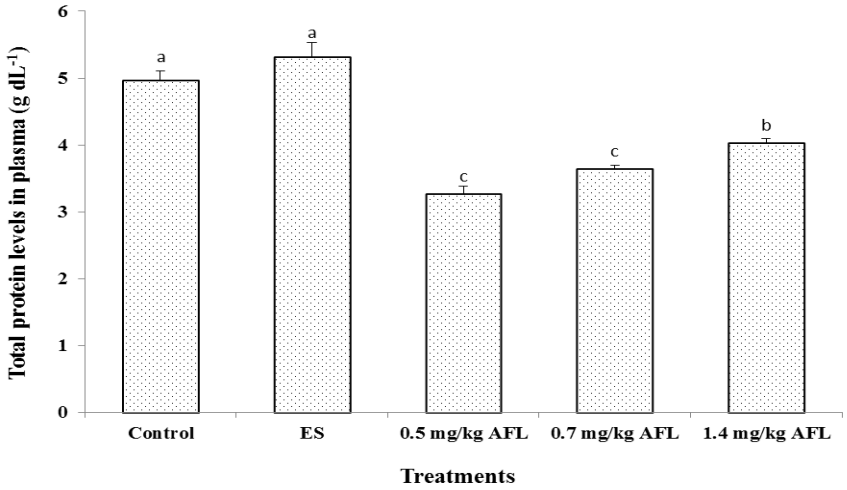

Figure 5: Total protein levels in plasma of common carp fed on contaminated diets with different concentrations of aflatoxins. Significant differences between values when compared with control groups are shown with different letters (p<0.05), similar letters indicated no significant difference between experimental groups. Error bars represent mean±SEM; ES: Extract solution; AFL: Aflatoxins.

A significant decrease was observed in plasma total protein was observed in fishes by oral feeding of contaminated diets with aflatoxins for 21 days (Fig. 5).

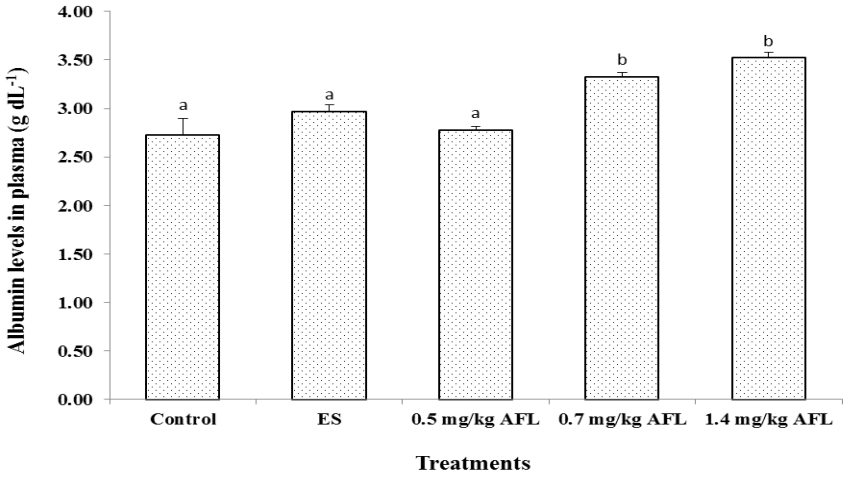

Figure 6: Albumin levels in plasma of common carp fed on contaminated diets with different concentrations of aflatoxins. Significant differences between values when compared with control groups are shown with different letters (p<0.05), similar letters indicated no significant difference between experimental groups. Error bars represent mean±SEM; ES: Extract solution; AFL: Aflatoxins.

Although there was a significant increase in plasma albumin levels in fish fed contaminated diets with 0.70 and 1.40 mg kg^{-1} aflatoxins, further comparisons by Duncan's analysis revealed that no significant difference was observed between fish fed 0.50 mg kg^{-1} aflatoxins and fish fed uncontaminated diets (Fig. 6).

A significant decrease was observed in plasma globulin levels of fish fed the diet containing different concentrations of aflatoxins (Fig. 7).

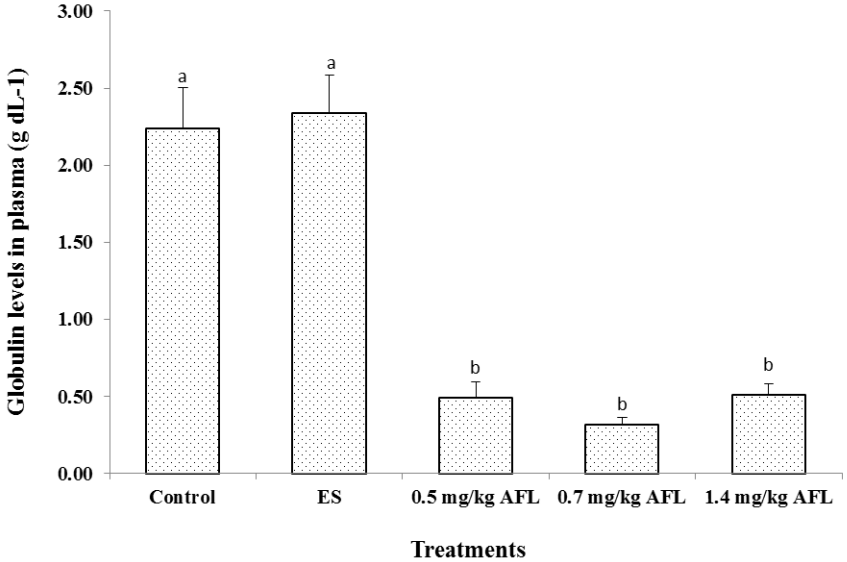

Figure 7: Globulin levels in plasma of common carp fed on contaminated diets with different concentrations of aflatoxins. Significant differences between values when compared with control groups are shown with different letters (p<0.05), similar letters indicated no significant difference between experimental groups. Error bars represent mean±SEM; ES: Extract solution; AFL: Aflatoxins.

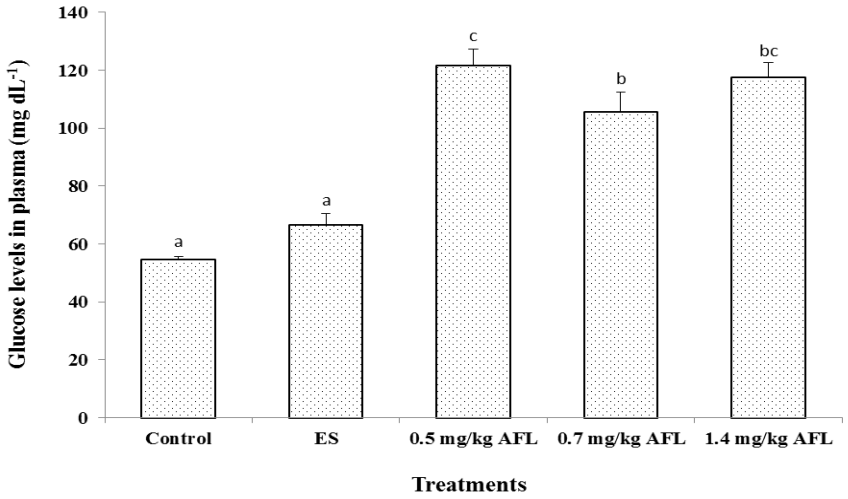

Figure 8: Glucose levels in plasma of common carp fed on contaminated diets with different concentrations of aflatoxins. Significant differences between values when compared with control groups were showed by alphabet letters (p<0.05), similar alphabet letters indicated no significant difference between experimental groups. Error bars represent the mean + S.E.M; ES: Extract solution; AFL: Aflatoxins.

A significant increase was observed in glucose levels plasma of fishes fed with different concentrations of aflatoxins (Fig. 8).

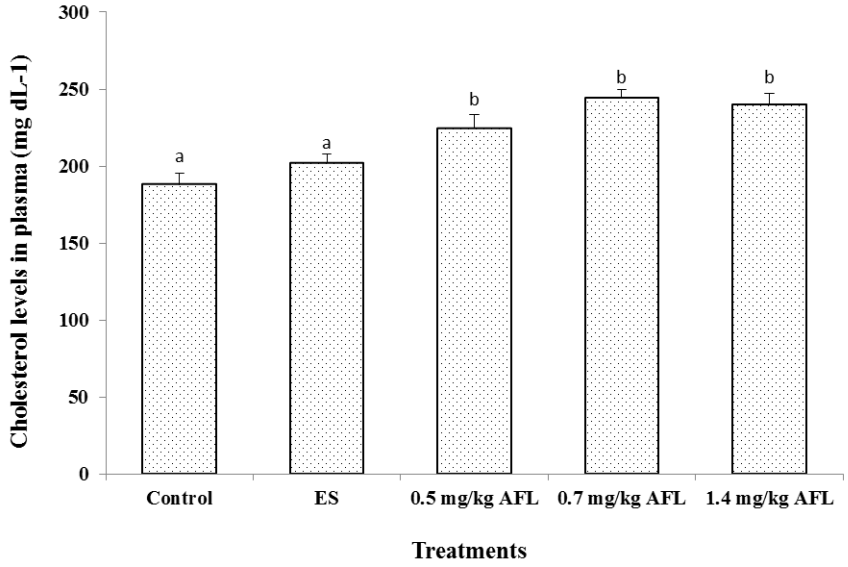

Figure 9: Cholesterol levels in plasma of common carp fed on contaminated diets with different concentrations of aflatoxins. Significant differences between values when compared with control groups are shown with different letters (p<0.05), similar letters indicated no significant difference between experimental groups. Error bars represent mean±SEM; ES: Extract solution; AFL: Aflatoxins.

The results presented in Fig. 9 indicated that fish treated with aflatoxins showed a significant increase in plasma cholesterol levels compared to the control group or those treated with extract solution alone.

There was a significant increase in triglyceride levels of plasma of fish fed contaminated diets with different concentrations of aflatoxins (Fig. 10).

A significant increase was found in creatinine levels of plasma of fishes fed contaminated diets with aflatoxins (Fig. 11).

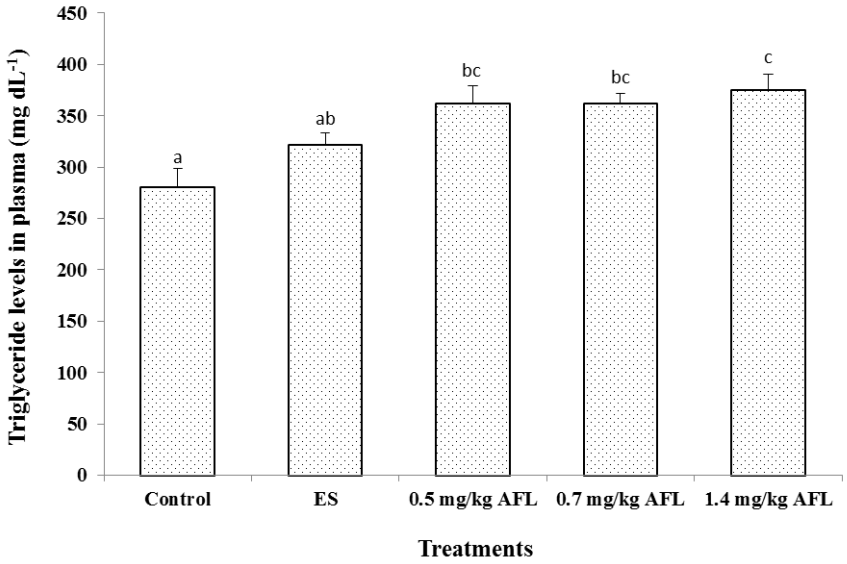

Figure 10: Triglyceride levels in plasma of common carp fed on contaminated diets with different concentrations of aflatoxins. Significant differences between values when compared with control groups are shown with different letters (p<0.05), similar letters indicated no significant difference between experimental groups. Error bars represent mean±SEM; ES: Extract solution; AFL: Aflatoxins.

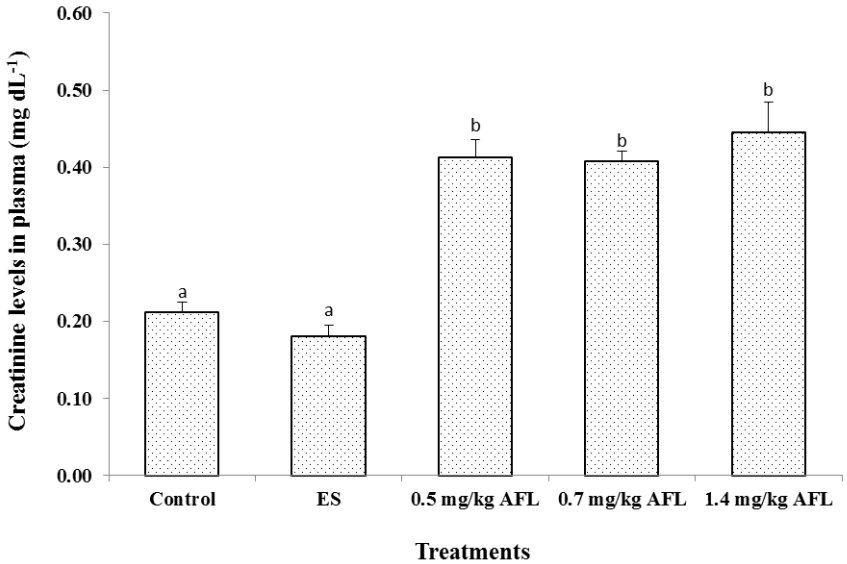

Figure 11: Creatinine levels in plasma of common carp fed on contaminated diets with different concentrations of aflatoxins. Significant differences between values when compared with control groups are shown with different letters (p<0.05), similar letters indicated no significant difference between experimental groups. Error bars represent mean±SEM; ES: Extract solution; AFL: Aflatoxins.

Discussion

In this study, no mortality was observed in the experimental groups. However, fish treated with different concentrations of aflatoxin showed clinical signs of aflatoxicosis, including internal bleeding, liver damage and pale gills at the end of the experiment. Similar results were reported by other researches after oral exposure to aflatoxin (Tuan *et al*., 2002; Deng *et al*., 2010; Huang *et al*., 2011; Raghavan *et al*., 2011).

In the positive control group, adding extract solution to the diet had no significant effect on plasma biochemical indices of fish which is because of acetone and alcohol evaporation during food pelleting.

In the present study, aspartate aminotransferase (AST) activity increased significantly in plasma of aflatoxin-treated fish which indicates damage to cell membranes, especially hepatocytes membrane and tissue necrosis. The elevated activity of plasma AST following an increase in concentrations of aflatoxins is indicative of changes in the hepatic tissues (Abdel-Wahhab *et al*., 2010). Elevations of AST activity is one of the clinical signs in cultured animals (Coulombe *et al*., 2005; Moghaddam-Jafari *et al*., 2014). An increase in AST in rats (Moghaddam-Jafari *et al*., 2014) and cultured quails (Tessari *et al*., 2010) treated with aflatoxin is also reported. He *et al*. (2010) found that aflatoxin B1 may destruct the cell membranes and increase the activity of AST, ALT and LDH in supernatant of hepatic cells of common carp.

In the present study, no significant change was observed in the activity of alanine aminotransferase (ALT) in fish which were fed contaminated diets with different concentrations of aflatoxin. This is in agreement with the results of a study done on gibel carp treated with different concentrations of aflatoxin (Huang *et al*., 2011).

Alkaline phosphatase (ALP) is one of the main liver enzymes involved in detoxification. Therefore, an increase in ALP in tissues may reflect an increase in aflatoxin up to the tolerance and detoxification threshold level of liver cells (Huang *et al*., 2011). However, a significant decrease in the activity of ALP following an elevation in aflatoxin accounts for liver cell necrosis, especially cells around the bile ducts, or is the result of damage to the intestinal epithelium cells, as well as disturbance in ALP biosynthesis in liver. An increase in the red blood cell hemolysis in these fish may also address a decrease in the activity of ALP in plasma (Farah *et al*., 2012).

Aflatoxins induce damage to parenchymal cells of the liver as indicated by the elevation of LDH activity in plasma of fish after 21 days. An increase in the activity of LDH in rats treated with aflatoxin is reported (Moghaddam-Jafari *et al*., 2014).

A significant decrease in plasma glucose in groups treated with aflatoxin may be due to an increase in the degradation rate of liver glycogen stores to glucose and the mobilization of energy sources against toxic effects of aflatoxin. Disturbance in the carbohydrates' metabolism following

aflatoxin toxicity leads to an increase in plasma glucose (Huang *et al*., 2011). A decrease in the activity of Glucose-6-phosphate dehydrogenase and glycogen stores in the liver is usually the primary reason for the increase in blood glucose in response to aflatoxin toxicity (Rastogi *et al*., 2001). Faphunda *et al*., (2008) found that an increase in blood glucose in rats treated with aflatoxin was due to disturbance in the endocrine system which is responsible for regulating plasma glucose.

In the current study, a significant decrease in plasma total protein in fish fed aflatoxin-contaminated diets may be the result of liver necrosis or disturbance in kidney function (Abdel-Wahhab *et al*., 2007). By inhibiting protein synthesis in liver, aflatoxin can reduce plasma proteins, especially globulin (Tessari *et al*., 2010). A decrease in protein synthesis in liver is caused by an increase in the proteolytic activity in the liver of fish treated with aflatoxin. In addition, degradation of tissue protein sources to free amino acids may be a good source of energy through tricarboxylic acid cycle (Murray *et al*., 2003). A decrease in plasma globulin can be attributed to protein synthesis and damage incurred to fish treated with aflatoxin. Moreover, increased albumin levels in plasma of fish treated with 0.7 and 1.4 mg aflatoxin are because of albumin function in distributing aflatoxin in the blood. Faraji *et al*., (2011) reported that aflatoxin in diets (1-3%) can be distributed in the blood by binding to albumin.

A significant increase in plasma cholesterol and triglyceride in fish fed with aflatoxin-contaminated diet is the result of lipoprotein biosynthesis, disturbance in the endocrine glands, disturbance in lipid metabolism (Huang *et al*., 2011), severe damage to the liver and kidney and an increase in the lipid peroxidation of cell membranes. Degradation of fat stores in tissues in order to provide energy to deal with aflatoxin toxicity and anorexia after damage to the nervous system justifies triglyceride and cholesterol levels in plasma of fish.

Creatinine is the final product of creatinine metabolism in the skeletal muscles and is excreted from the body through the kidney. The amount of plasma creatinine is proportional to the muscle mass. Since plasma creatinine is known as an index of kidney function, an increase in creatinine proportional to elevated levels of aflatoxin in the diet indicates damage to muscles and kidney or disturbance in kidney function in excreting creatinine.

In general, damage to target organs such as the liver or kidney may account for changes found in plasma biochemical indices in fish fed with aflatoxin-contaminated diets. Moreover, the findings of this study indicated that most changes in plasma biochemical indices were observed in fish treated with 0.7 and 1.4 mg aflatoxin per kg food.

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