

Effects of Toxin Binder Biotox on Growth Performance Survival, Enzymatic Activity, Hematological and Biochemical Parameters of Fingerlings Rainbow Trout (*Oncorhynchus mykiss*) Fed Diets Contaminated with Aflatoxin

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Abstract

The aim of the study was to determine the effect of Biotox on growth performance, survival, enzymatic activity, hematological and biochemical parameters of fingerlings rainbow trout fed with aflatoxin. For this purpose, 900 fish were stocked in four treatments at the pools and fed 4 times a day by supplementing the basal diet with 0.1% Biotoxin¹ mg/kg aflatoxin (T1), 0.2% Biotoxin +1 mg/kg aflatoxin (T2), 1 mg/kg aflatoxin (T3) and basal diet as control group (T4) for 60 days. There were significant differences ($P < 0.05$) in survival in fish fed different diets for 60 days. Survival was higher in the T2. there were no significant difference ($P > 0.05$) was observed in growth performance, activity Alkaline Phosphatase (ALP) and hematological parameters, of fish in different treatments. Also, no significant difference ($P > 0.05$) in serum globulin, albumin and total protein was observed between treatments and control group. The results of the present study showed a significant ($P < 0.05$) increase in Glucose and triglyceride in T3, whereas serum cholesterol was higher in T3 and control group. Addition of 1-2 g Biotox/ kg feed did not affected growth performance and hematological parameters, but increased survival of fingerling fed with the diets of aflatoxin B1-exposed.

Keywords: Biotox; Growth performance; Enzymatic activity; Rainbow trout; Aflatoxin

Introduction

Aflatoxins are produced by *Aspergillus* fungi, which can infect many potential feedstuffs as corn, peanuts, rice, fish meal, shrimp and meat meals [1]. The aflatoxin B1 (AFB1) is very toxic and exists in a wide range of mycotoxins. Its high toxicity to both animals and humans makes AFB1 the most dangerous mycotoxin [2]. Liver is the target organ of aflatoxin B1, long-term intake of diets containing AFB1 can cause a reduction of weight gain, growth stunting and immunosuppression in animals, while increasing hepatocellular carcinoma incidence [3]. It seems that effects of aflatoxins on fish depend on the fish species and age, as fish fry are more susceptible than adults and species like Rainbow trout, (*Oncorhynchus mykiss*) and European seabass, (*Dicentrarchus labrax*) seem to be more susceptible than channel catfish, (*Ictalurus punctatus*) [4-6]. These species specific differences can be attributed to differences in the metabolism of AFB1 in the liver and affinity of AFB1-derived metabolites to hepatic macromolecules [7]. The negative effects of aflatoxin in different species such as Rainbow trout, Baltic salmon (*Salmo salar*), Nil tilapia (*Oreochromis niloticus*), coho salmon (*Oncorhynchus kisutch*), and Red Drum (*Sciaenops ocellatus*) [4,8-11] and shrimp (*Litopenaeus vannamei*) have been demonstrated. There are a number of approaches that can be taken to protect of animal from toxic effect of aflatoxins and these involve prevention of fungal growth, and strategies to reduce or eliminate mycotoxins from contaminated feeds, such as physical, chemical, nutritional, and biological techniques [12]. In the feed industry, the most common method to control AFB1 is the application of nonnutritive adsorptive materials so as to reduce the concentration of fungal toxins in the gastrointestinal tract of animals [13]. The aim of these additives is to inhibit the uptake of mycotoxins by an animal *in vivo*. The use of mycotoxin binding agents is occasionally recommended to farmers in order to protect animals against the harmful effects of mycotoxins occurring in contaminated feeds. The efficacy of the adsorption appears to depend on the chemical structure

of both the adsorbent and the mycotoxin [14,15]. Most studies related to the alleviation of mycotoxicosis by the use of adsorbents are focused on aluminosilicates, mainly zeolites, and aluminosilicate-containing clays, all consisting of aluminates, silicates and some interchangeable ions, mainly alkali metal and alkaline earth metal ions [14,16,17]. Mineral clay products such as bentonites, zeolites, and aluminosilicates are the most common feed additives which are effective in binding/adsorbing aflatoxins [15]. In the other hand, yeast or yeast cell walls show a potential as mycotoxin binders Besides its excellent nutritional value [14]. Biotox is one of the mycotoxin adsorbing agent and consists of various silicates combined with extracts of the cell walls of a yeast (*Saccharomyces cerevisiae*) that we have used in our study.

The number of studies addressing the effects of the addition toxin binders to fish feed is very limited. More research is needed to better understand the potential effects of these compounds in diet. Therefore, in the present study attempted was conducted to examine the influence of aflatoxin on growth performance, survival, enzymatic activity, hematological and biochemical parameters of fingerling rainbow trout (*Oncorhynchus mykiss*) and the antitoxic effect of Biotox.

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Ingredient (%)	Basal diet	Proximate composition (% wet weight)	Basal diet
Fishmeal	50	Crud protein	44.00
Soybean meal	20	Lipid	13.00
Wheat flour	12	Moisture	11.00
Fish oil	10	Ash	13.00
Vitamin premix ^a	2	-	-
Mineral premix ^b	1.5	-	-
Filler	4.5	-	-

^aContains (mg/kg food): E (30), K (3), niacin (40), thiamine (2), riboflavin (7), pyridoxine (3), folicin (1.5), pantothenic acid (18), biotin (0.7) and cyanocobalamin (0.18).
^bContains (mg/kg food): Mg (100), Zn (60), Fe (40), Cu (5), Co (0.1), I (1) and Antioxidant (100).

Table 1: The ingredient and proximate composition of basal diet.

Materials and Methods

Diet experimental design

Commercial trout diet as basal diet was purchased from Chineh Company, Tehran, Iran. Biochemical analysis of basal diet and treatment diets showed in Table 1. Biotoxin and AFB1 (C17H12O6, MW- 312.27, 98% pure) were purchased from Aria trading company Dallman (exclusive representative Biochem, Germany). four experimental diets were prepared by supplementing the basal diet with 0.1% Biotoxin +1 mg AFB1/kg (T1), 0.2% Biotoxin +1 mg AFB1/kg (T2), 1 mg AFB1/kg (T3) and basal diet as control group (T4).

The aflatoxin B1 was dissolved in chloroform and then added to Celufl, a non-nutritive bulking agent. The chloroform was evaporated in the dark under a fume hood. All dry ingredients (exception of the AFB1) was blend in a V-mixer, for 20 min. The dry ingredients were then mixed with the AFB1 in a Hobart mixer. The oil component and water were further added to the dry ingredients and mixed for 1h. Pellets are maintained in the refrigerator at 4°C until use.

Fish and experimental condition

Water quality parameters such as temperature and dissolved oxygen were measured daily by Thermometer and oxygen meter (Model WTW320I), respectively. They were kept under optimal range.

Nine hundred rainbow trout fingerlings (average weight $10 \pm 0/05$ g) were purchased from Private detective propagation and rearing of salmon (Lorestan, Iran). In this study, 9 Concrete pools (0.8 m × 4 m × 2 m) were designed. three feeding treatments (T1, T2 and T3) and one pool as control were conducted.

Three replicates were maintained for each treatment. Each pool was connected to a flow-through water system. After an initial 14 days acclimation period, fingerlings were randomly distributed in pools (100 fingerlings per pool).

Fingerlings were fed based on their body weight and temperature 4 times (06:00, 12:00, 18:00 and 24:00) a day at 3% of body weight for a period of 60 days.

Growth performance and survival rate

Growth performance, weight and length of Fish (20 fish per pool) was monitored once every 15 days following a 12-h starvation until the end of experiment. Growth performance and survival rate of the fingerlings rainbow trout were calculated using the following Formula:

$$SGR \% d^{-1} (\text{Specific growth rate}) = (Ln W_t - Ln W_0) \times 100 / t$$

$$GBM (\text{Gain of body mass}) = W_t - W_0; \text{Survival rate} = 100 \times (N_t / N_0)$$

Where: Wt and W0: final and initial body weight (g); t: time of rearing (days); Nt and N0: final and initial fish number.

Blood sampling

After a 60-day rearing period, five fish from each treatment (three from each tank) were quickly anesthetized using Clove powder (200 mg/l) then body surface of fingerlings rainbow trout were cleaned with blotting paper to avoid any contamination. Blood was collected using a hypodermic syringe from the caudal blood vessels [18]. The blood samples were transferred to heparinized tubes for hematological parameters and non-heparinized tubes to obtain serum for biochemical parameters. The blood was centrifuged at 3,000 rpm for 10 min at 4°C based on Pradhan et al. [19] and Nelson and Somogyi [20]. The collected plasma and serum were stored at -70°C for further analyses.

Hematological parameters

Red blood cells (RBCs) and white blood cells (WBCs) were counted under a light microscope using a Neubauer hemocytometer with Hayem's solution as the diluting fluid for RBC and Truck's solution for WBC [19,21]. The hemoglobin (Hb) concentration was analysed following the cyanmethemoglobin method [22]. Hematocrit (Hct) was determined by centrifuging blood in heparinized microhematocrit capillary tubes at 3,500 rpm for 10 min [23]. The relative numbers of the types (lymphocytes, neutrophils and monocytes) in the stained peripheral blood were recorded [24], and the percentage of each blood type was calculated as the average blood cell type/sum of averages of all types of blood cells × 100 [25].

Serum biochemical factors

Biochemical indices, including glucose, total protein, albumin, triglyceride and cholesterol in serum were analyzed using commercial kit (Pars Azmun Co. Ltd., Tehran, Iran) and measured by a spectrophotometer (Model WAP-S2000-UV/VIS, Cambridge-UK) following the manufacturer's instructions [20,26]. Globulin levels are calculated by subtracting the amount of albumin from total protein [27].

Enzymatic assays

Alkaline Phosphatase (ALP) was assayed using commercial kit (Pars Azmun Co. Ltd., Tehran, Iran) and measured by an autoanalyzer. ALP was measured an autoanalyzer according to Bessey et al. [28].

Data analysis

All data are shown as mean ± SD (standard deviations). Normality of data was tested by Shapiro-Wilk's test. Data were subjected to one-way ANOVA and significant difference between the treatments was determined by Duncan's test. The values of P<0.05 were considered significantly different. All analyses were performed using statistical software SPSS (version, 17).

Results

The growth performance and survival of fingerlings rainbow trout

Variables	GBM(g)	SGR (%)	Survival (%)
T1	77.20 ± 8.96 ^a	2.21 ± 0.15 ^a	98.33 ± 1.15 ^{ab}
T2	77.78 ± 0.85 ^a	2.28 ± 0.37 ^a	100 ± 0.00 ^a
T3	65.64 ± 11.47 ^a	2.14 ± 0.23 ^a	97.00 ± 0.00 ^b
Control	74.14 ± 11.06 ^a	2.16 ± 0.23 ^a	98.00 ± 1.73 ^{ab}

Values with different superscript letters denote significantly different means within each column (P<0.05).

Table 2: Effects of Biotox on Growth performance and Survival of *O. mykiss* fed with the diets of 1 mg AFB1/kg-exposed at the end of feeding trial.

Variables	RBC (° 10 ⁶ /mm ³)	WBC (° 10 ³ /mm ³)	Hct (%)	Hb (g/dL)
T1	0.82 ± 0.05 ^a	6.63 ± 2.14 ^a	60.67 ± 2.31 ^a	3.67 ± 0.57 ^a
T2	0.83 ± 0.18 ^a	5.37 ± 0.20 ^a	61.33 ± 7.57 ^a	3.62 ± 0.16 ^a
T3	0.70 ± 0.07 ^a	7.73 ± 0.90 ^a	50.33 ± 2.08 ^a	3.27 ± 0.40 ^a
Control	0.78 ± 0.03 ^a	8.39 ± 2.71 ^a	50.33 ± 15.37 ^a	3.94 ± 0.14 ^a

Values with different superscript letters denote significantly different means within each column (P<0.05).

Table 3: Effects of Biotox on hematological parameters of *O. mykiss* fed with the diets of 1 mg AFB1/kg-exposed at the end of feeding trial.

Variables	Monocytes	Neutrophils	Lymphocytes
T1	0.67 ± 1.15 ^a	4.00 ± 6.92 ^a	95.33 ± 8.08 ^a
T2	0.00 ± 0.00 ^a	6.67 ± 7.02 ^a	93.33 ± 7.02 ^a
T3	0.00 ± 0.00 ^a	4.67 ± 6.43 ^a	94.00 ± 8.71 ^a
Control	1.33 ± 2.31 ^a	2.67 ± 2.31 ^a	96.00 ± 4.00 ^a

Values with different superscript letters denote significantly different means within each column (P<0.05).

Table 4: Effects of Biotox on the percentage of the lymphocytes, neutrophils and monocytes in *O. mykiss* fed with the diets of 1 mg AFB1/kg-exposed at the end of feeding trial.

Variables	Glucose (mg/dl)	Triglyceride (mg/dl)	Cholesterol (mg/dl)	TP (mg/dl)	Albumin (mg/dl)	Globulin (mg/dl)
T1	143.00 ± 2.64 ^{ab}	305.67 ± 4.04 ^b	253.33 ± 8.32 ^{ab}	5.85 ± 0.08 ^a	2.01 ± 0.39 ^a	3.84 ± 0.34 ^a
T2	134.67 ± 3.05 ^b	295.33 ± 5.51 ^c	240.00 ± 13.23 ^b	5.92 ± 0.04 ^a	2.09 ± 0.38 ^a	3.83 ± 0.41 ^a
T3	146.33 ± 3.78 ^a	318.67 ± 7.77 ^a	271.67 ± 10.40 ^a	5.84 ± 0.02 ^a	1.96 ± 0.31 ^a	3.87 ± 0.33 ^a
Control	145.00 ± 9.53 ^{ab}	312.67 ± 2.52 ^{ab}	268.33 ± 7.64 ^a	5.82 ± 0.09 ^a	1.60 ± 0.10 ^a	4.22 ± 0.16 ^a

Values with different superscript letters denote significantly different means within each column (P<0.05).

Table 5: Effects of Biotox on biochemical blood parameters of *O. mykiss* fed with the diets of 1 mg AFB1/kg-exposed at the end of feeding trial.

Variables	ALP (U/L)
T1	577.89 ± 93.26 ^a
T2	623.28 ± 42.59 ^a
T3	726.65 ± 139.83 ^a
Control	594.67 ± 26.25 ^a

Values with different superscript letters denote significantly different means within each column (P<0.05).

Table 6: Effects of Biotox on ALP activity of *O. mykiss* fed with the diets of 1 mg AFB1/kg-exposed at the end of feeding trial.

fed treatment diets is presented in Table 2. No significant difference (P> 0.05) in growth performance was observed in fish fed different diets for 60 days. There were significant differences (P< 0.05) in survival in fish fed different diets for 60 days. survival in the T2 was significantly higher than the T3 (P<0.05). but there was no significant difference (P> 0.05) between T1 and control group, between T2 and T1, control group and between T3 and T1 (Table 2).

Effects of treatment diet on hematological parameters of fingerlings rainbow trout are presented in Table 3. No significant difference (P>0.05) in hematological parameters was observed in treatments and control group for 60 days (Table 3). The percentage of the lymphocytes, neutrophils and monocytes showed no significant changes between treatments and control group (Table 4).

Effects of varying doses of dietary Biotoxin and aflatoxin on biochemical blood parameters are shown in Table 5. No significant difference (P> 0.05) in serum Globulin, Albumin and total protein was observed in treatments and control group. Fish fed with T3 and control

group showed significantly (P< 0.05) higher Cholesterol contents. The serum Glucose and Triglyceride level were significantly (P< 0.05) increased in T3 (Table 5).

Effects of different levels of dietary Biotoxin and AFB1 on enzyme activity of fingerlings rainbow trout are presented in Table 6. There was no significant (P > 0.05) effect change in ALP between treatments and control group (Table 6).

Discussion

There are differences in vulnerability to mycotoxins among salmonid species. For example, rainbow trout is considered very susceptible to AFB1 [4,8]. The first documented incidences of aflatoxicosis affecting fish health occurred in the 1960s in trout hatcheries. Domesticated rainbow trout that were fed a pelleted feed prepared with cottonseed meal contaminated with aflatoxins, developed liver tumors and as many as 85% of the fish died in these hatcheries. Although cottonseed meal is no longer used as a major ingredient in feed formulations, poor storage of other feed ingredients and nutritionally complete feeds can lead to contamination with aflatoxins [29,30]. Santacroce et al. [8] explained that dosages of 0.5-1 AFB1 mg/kg feed resulted in 50% mortality. Other study showed that levels of 0.02 AFB1 mg /kg feed produced 96% incidence of liver tumors in rainbow trout. However, the same treatment had no effect on coho salmon [4]. In the present study survival was low in T3 that indicate the negative effect of AFB1 on survival. Although survival was negatively affected by AFB1, adding 2g Biotox/kg diet of rainbow trout fingerlings significantly increased survival of the fish. Our results indicated that Biotox especially at higher

dosage was effective to help improve survival. Previous study showed that the addition of adsorbents to feed can cause reducing aflatoxicosis in animal [31,32]. Also, NovaSil (a calcium montmorillonite clay) supplementation in the diets of AFB1-exposed fish resulted in a protective effect [33]. Other studies have reported that a 2% inclusion level of bentonite (a common clay containing montmorillonite) in trout feed reduced toxic AFB1 effects [1].

This study showed no significant change on GBM and SGR. While there was a slight increase in growth performance in T1 and T3, there was not significant. It is possible that the levels of AFB1 and/or Biotox used in this study were not capable of significant effect on growth performance. Sepahdari et al. [34] stated that the negative effect of AFB1 on fish growth seems to be correlated to the amount of toxin and duration of exposure. The toxicity of aflatoxin also varies according to many nutritional factors, and recovery from protein malnutrition is delayed by exposure to aflatoxin [35]. Similarly Santacroce et al. [8] stated that the feeds containing 0.5-1 AFB1 mg/kg feed did not affect the growth, feed intake or produced liver damage on Baltic salmon. Another study reported that there were no differences in the evaluated growth performance and feed intake of juvenile salmon exposed to AFB1 doses 0, 1, 5, 10 or 20 mg/kg feed [9]. This result is in agreement with our result that aflatoxin at a 1 mg/kg feed dose cannot change growth performance in fingerlings rainbow trout. Cagauan et al. [10] reported that when Nile tilapia were fed diets containing 1.8 mg AFB1/kg feed for 75 days growth rates reduced. Another study stated that Nile tilapia fed diets that contained 2.5, 10, or 100 mg AFB1/kg feed for 8 weeks had reduced weight gain [5]. Species-specific differences in vulnerability because of variations in toxin metabolism [7] and dose of exposure to AFB1 contaminated diet could explain the results.

Hematological parameters are important factors that could be considered during the fish diet quality, fish conditions and toxic effect of substances assessment [36]. These factors are considered valuable indices to assess fish health [37,38]. Our result showed no change on hematological parameters (RBC, WBC, PCV, Hb and MCV) as well as the percentage of Lymphocytes, Neutrophils and Monocytes. Mahfouz and Sherif [39] demonstrated that hematological profiles and monocytes, eosinophils, and lymphocytes were influenced by the dose and duration of exposure to AFB1 contaminated diet. As RBC, WBC, PCV, Hb and MCV levels were significantly decreased in the high AFB1 exposed group. Also, Tuan et al. [5] stated that Nile tilapia fed diets that contained 2.5 mg, 10 mg, or 100 mg AFB1/kg feed for 8 weeks had reduced red blood cell counts.

ALP is a membrane bound enzyme and its alteration is likely to affect the membrane permeability and produce derangement in the transport of metabolites [40]. An increase of these enzyme activities in the extracellular fluid or serum is a sensitive indicator of even minor cellular damage [41] in which cellular enzymes are released from the cells into the blood serum, which in turn indicates stress-based tissue impairment. Mahfouz and Sherif [39] showed that the activity of serum AST, ALT and ALP liver enzymes significantly increased in tilapia exposed to 100 ppb AFB1. Also, Mahfouz and Sherif [39] verified that AST, ALT and ALP were significantly increased in the serum of rats administered AFB1 (250 µg/kg/day for 2 weeks) indicating the presence of damaged and dysfunctional liver cells. Our result showed that dosages of 1 mg AFB1/kg feed had no effect on ALP of fingerlings rainbow trout. The difference between our result and the other studies is maybe due to the dose and duration of exposure to AFB1 contaminated diet.

One of the most principal target organs for aflatoxicosis is hepatopancreas [8]. After the invasion of aflatoxin into the

hepatopancreas, aflatoxin metabolites react negatively with different cell proteins, which lead to inhibition of carbohydrate, lipid metabolism and protein synthesis, finally inducing hepatopancreas necrosis, cell death or tumour. In relation with the disorder in hepatopancreas function, there is also derangement of the blood-clotting mechanism, jaundice and a decrease in total serum proteins and cholesterol synthesized by the hepatopancreas [10]. In this study, fish fed in all treatment showed no effect on total serum protein, Albumin, and Globulin. In agreement, Zeng et al. [42,43] dietary AFB1 had no effect on serum total serum protein and Albumin in white shrimp.

The addition of toxin binder or adsorbents to feed is considered the most promising and economic approach of reducing aflatoxicosis in animals [31,32]. The beneficial effects of using these additives in feed have been shown in a few studies. NovaSil (a calcium montmorillonite clay) supplementation in the diets of AFB1-exposed on Red Drum resulted in a protective effect, which was evident by the significant improvement in many of the tested parameters [33]. Ellis et al. [1] have reported that a 2% inclusion level of bentonite (a common clay containing montmorillonite) in trout feed reduced toxic AFB1 effects. Hassan et al. [44] in their studies suggest that a 0.5% inclusion level was sufficient to protect tilapia from 1.5 ppm AFB1. In another study, Bentonites have been added into fish feed at concentrations up to 10% with no alteration in whole-body proximate composition [45]. In the current study, addition of 1 and 2 g Biotox/kg feed did not ameliorate the effects of AFB1 on growth performance and hematological parameters, but increased survival of fingerling rainbow trout. Our result suggests that dietary Biotox may need to be used at levels higher than 1-2 g/kg feed to achieve protective effects against aflatoxin.

Conclusion

In conclusion, observed difference in different studies indicates differences in fish species, sources response to aflatoxin. Also, the dose and duration of exposure to AFB1 contaminated diet and the dose of Biotox are the other reason for these differences. Addition of 1-2 g Biotox/kg feed did not affect growth performance and hematological parameters, but increased survival of fingerling rainbow trout fed with the diets of 1 mg AFB1/kg-exposed.

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