Effects of aflatoxin B_1 on growth performance, health indices, phagocytic activity and histopathological alteration in *Fenneropenaeus indicus*

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Abstract

Mycotoxins contamination of feedstuff for aquatic animals is common in regions with humid tropical conditions. In this study Indian white shrimp, *Fenneropenaeus indicus*, (11.79 ± 1.76 g) were fed with diets containing 0, 20, 50, 100, 200, 400, 800 and 1600 ppb levels of aflatoxin B_1 ($AFLB_1$) for 8 weeks. Final weight, aflatoxin B_1 residue (2-week intervals), Total Hemocyte Count (THC), Total Plasma Protein (TPP), Phagocytic Activity (PA), Survival rate (4-week intervals) were determined. Histopathological alterations in hepatopancreas, midgut and muscle tissues were studied at the end of 4 and 8 weeks. Shrimps fed with the 1600, 800 and 400 ppb concentrations of $AFLB_1$ exhibited slow growth, and more reddish discoloration disseminated over the body at 4^{th} week. Growth parameters, survival rate and health indices (THC, TPP) of *F. indicus*, are affected by the different doses of $AFLB_1$ in diets. At the end of 8^{th} week, doses of $AFLB_1$ in the diets showed negative correlation to final weight, survival rate, THC and TPP (r = - 0.312, -0.603, -0.237 and - 0.649 at $P<0.001$, respectively). Moreover, significant histopathological alterations in the hepatopancreas, midgut and muscle tissues of exposed shrimps to different levels of $AFLB_1$ were observed and these alterations are obviously indicated by changes in the health indexes (THC and TPP).

Keywords: Aflatoxin B1, *Fenneropenaeus indicus*, growth performance, health indices, histopathological alteration

Introduction

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Most of the problems currently facing the shrimp culture activities are related to the widespread occurrence of disease, e.g., bacterial and viral infections or parasitic invasions. These diseases can lead to drastic scathe to the industry; thus the researchers have focused much of their attention to study such threats. Nonetheless, there are other potential threats caused by other agents, such as the environment or the feed which can also affect seriously the success of the shrimp culture industry (Bintvihok et al., 2003). These agents have been some way neglected by the shrimp industry. Intercontinental trade in agricultural commodities has imparted significantly to the discussion about potential hazards involved and has particularly increased the consciousness of mycotoxins (Binder et al., 2007).

Mycotoxins are produced by the growth of molds (fungal spoilage) on a wide range of foodstuffs which can occur at many stages during food production during plant growth, harvesting, storage and processing (Anklam et al., 2002). Due to the omnipresent nature of fungi, it has been estimated that approximately twenty percent of whole food products (chiefly of plant origin) are contaminated with substantial toxin. About 300 different mycotoxins have been reported that are produced by approximately 200 dissimilar fungal species. All the same, there are only twenty mycotoxins that are often found in food and feedstuffs at concentrations likely to pose a health hazard for people and animals consumption so-called “primary exposure” (Anklam et al., 2002). However, all fungal growths don't lead to mycotoxin producing. Thereupon detection of fungi does not show inevitably the presence of mycotoxins (D'Mello and Macdonald, 1997).

Consumption of a food containing mycotoxins can induce acute or long-term chronic effects resulting in teratogenic, carcinogenic, oestrogenic and immunosuppressive effects. Consumption of mycotoxin contaminated feed lead to: reduced feed intake, feed refusal, diminished body weight gain, increased feed conversion, disease prevalence (due to reduce immunity defense functions) and decrease in reproductive capacities (Malins and Ostrander, 1994; Meerdink, 2002; Fink-Gremmels and Malekinejad, 2007). Feed commodities with high deal of starch and lipids may be susceptible to fungal contaminations if not properly dehydrated. For example, peanuts and peanut by-products, tree nuts, copra, maize, cottonseed meal and sunflower meal are especially susceptible to fungal colonization, most often by *Aspergillus flavus*, but occasionally by *Fusarium* spp. and *Altemaria* spp. *Aspergillus* spp. which can produce several classes of aflatoxins. $AFLB_1$ is known as an actual toxin and hepatocarcinogen (Jantrarotai et al., 1990; Achim, 1997; D'Mello and Macdonald, 1997). $AFLB_1$ is a potent toxin to a number of cell types, plants, invertebrates and vertebrates (Coulombe and Roger, 1993).

Fungal contamination of feedstuff for aquatic animals is common in regions with humid tropical condition, such seaports. Some factors, such as improper quality of feedstuff ingredients and incompatible storage of feed can increase probability of fungal contamination (Bintvihok et al., 2003). Many aquatic vertebrates are known to suffer toxic

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effects of dietary $AFLB_1$. For example, dietary administration of $AFLB_1$ at 1 ppb has shown hepatocarcinogenic effect in rainbow trout. It's believed, warm water species are often less sensitive to $AFLB_1$ than cold water species (Lovell, 1998). In marine shrimp, $AFLB_1$ can lead to decrease in growth, low apparent digestibility, physiological disorders and histolopathological changes, mainly in the hepatopancreas tissue (Lightner et al., 1982; Sindermann and Lightner, 1988). Hepatopancreas is the major digestive organ in decapod crustaceans. This organ has some vital biological functions, such as synthesis and secretion of digestive enzymes (Frandson et al., 2009). The effects of dietary $AFLB_1$ on aquatic invertebrates, particularly Penaeid shrimps, have been some deal defined. Study of juvenile Pacific white shrimp, *Litopenaeus vannamei* (Lightner, 1988), indicated that shrimp feed containing 100 ppb $AFLB_1$, (from AF-contaminated peanut meal) resulted in a moderate incidence of aflatoxicosis within a 171-day grow out period. Some researchers described mortalities caused by aflatoxicosis among juvenile *L. vannamei* after they were fed diets containing 50-300 ppm $AFLB_1$, for 28 days (Wiseman, 1980; Wiseman et al., 1983). Bautista et al. (1994) have reported histopathological changes in the hepatopancreas tissue of shrimp chronically exposed to $AFLB_1$. The degree of histopathological changes correlated with the concentration of $AFLB_1$. In the current study, the effects of different concentrations of $AFLB_1$ in diets on health indexes, haemocyts phagocytic activity and histopathology of shrimps were deliberated.

Materials and methods

Juvenile Indian white shrimps, *F. indicus*, of 11.79±1.76g average weight were stocked into six glass aquaria (30 shrimps each). Shrimps were obtained from Tiab region in Hormozgan province and transferred to a research station in Iran Shrimp Research Center (ISRC) in Bushehr province in the south of Iran. Shrimps were placed in fiberglass containers ($4m^3$) to acclimatize (26.06 ± 2.80°C, 30.09 ± 2.84ppt, DO 7.71 ±0. 74mg/l and pH 8.23± 0.32) for two weeks. During the acclimation period, shrimps were fed three times daily with a pelleted shrimp feed (provided from Havourash Company, Bushehr).

One batch of commercial diets (Havoorash feed Company, Bushehr, Iran), consisting preliminary of soybean meal, wheat, fish meal and shrimp meal was analyzed prior to use and were found to be free from $AFLB_1$. Analytical reagent grade $AFLB_1$ (Sigma Chemical Co. No. A6636 or Calbiochem No.121741) was purchased. The crystalline $AFTB_1$ was first dissolved in ethanol to prepare solutions of $AFLB_1$ and then according to Boonyaratpalin et al. (2001) eight homologous diets contain desire levels of $AFLB_1$ were prepared as follows: treatment 1 (control) contained 0 ppb (µg/ml) $AFLB_1$, treatments 2 to 8 contained 20, 50, 100, 200, 400, 800 and 1600ppb $AFLB_1$, respectively. For all treatments, diets were processed by a meat grinder with about one third moisture and then pelleted. This process was followed by 6 h of drying at 65°C until the moisture content came down to 10%. The dried pellets were stored in plastic bags in a Laboratory refrigerator until used. The shrimps were fed these aflatoxin-contaminated feeds

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twice daily for 8 weeks. Moribund or dead shrimps were removed and recorded. Moribund and recently dead animals were preserved in Davidson fixative for histopathological examination.

Haemocyte lyses or degranulate *in vitro* quickly. Therefore, 10% formalin in 0.45 M NaCl was used for the fixation of the collected haemolymph. Haemolymph (0.1ml) was collected from the abdominal surface and the base of a walking leg by using a syringe containing 0.1ml fixative, transferred to Eppendorf tube and mixed gently. After 10 min at 4°C, 10 µl of fixed haemolymph was placed on Haemocytometer (improved Newbauer bright line) and counts were done under microscope (Nikon Photolab, Japan) with magnification of 40× for 5/25 squares to calculate THC per ml hemolymph (0.5 × count × 10^5 × dilution factor).

Concentrations of plasma protein in each sample were determined by the Lowry method (Lowry et al., 1951).

For evaluation of phagocytic activity, the glassware and solutions were pyrogen-free to avoid enzymatic interruption and all chemicals were of analytical reagent grade. Phagocytic activity was determined by the method of Jiang et al. (2004). Briefly, 25ml of collected haemolymph was placed on a dichromate-cleaned glass slide and incubated for 30 min at room temperature. Subsequently, 25µL of a *Staphylococcus aureus* suspension (1×10^8 cells/ml) was added to each sample and the preparation was incubated for an additional 30 min. Then, each slide was washed with anticoagulant, fixed with 4% glutaraldehyde in the solution of anticoagulant for 1 min, rinsed in distilled water for 1 min, post fixed with 95% ethanol for 1 min, and air-dried. The slides were then stained with toluidine blue for 5 min and decolorized in running tap water. Numbers of ingested *S. aureus* and numbers of haemocytes that had ingested *S. aureus* were counted from any 200 haemocytes observed using a light microscope at a magnification of ×100 (Nikon, Photolab, Japan) (Jiang et al., 2004). Percentage of phagocytosis was calculated as below:

Percentage of phagocytosis= (number of cells ingesting bacteria/number of cells observed) ×100

After 4 and 8 weeks of the feeding trial, tissue samples from the hepatopancreas, midgut and muscle of live shrimps were obtained and examined using an optical microscope. Briefly, three cephalothoracic regions and muscles (first segment) from each treatment were fixed in Davidson's fixative overnight at the start point, on the fourth and the eighth week of the experimental rearing. Fixation was accomplished by first injecting 0.3 ml of fixative (depending on the size of the animal being preserved; using a 1 ml syringe) directly into the hepatopancreas. Then the cuticle for the length of the animal was opened along the lateral midline using dissecting scissors, and the animal was immersed into fixative. After 24 to 72 h in Davidson's fixative, preserved shrimp were transferred to 50% ethyl alcohol for storage. Tissues were prepared for light microscopy using routine paraffin techniques, and were stained with hematoxylin and eosin. Tissues were processed for paraffin embedding in a 110KP automatic tissue processor (KP-110) and sections of 5-6 µm thickness were cut with a Leica Jung RM 2045 semi-automatic

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rotary microtome. Longitudinal and transverse sections of the tissues were taken. Histological terms used to describe the tissues and organs in this study were according to Lightner (1996).

All data were subjected to analysis of variance (Two-way (univariate) ANOVA) to determine differences in means (P<0.05).

Results

The actual $AFLB_1$ content in final diets after feed preparation was determined from 5g samples of each. Actual feed levels were 20.44, 42.50, 85.10, 212.50, 425.50, 793.20 and 1380.00 mg/kg in the final prepared diets (Table 1).

Discolorations on the ventral part of the body were evident during the second week of treatments in shrimps fed diets containing more than 200 ppb $AFLB_1$. The body and appendages changed from off white to yellowish (in the first week) and eventually had a reddish discoloration (after 2 weeks). The color of fecal matter changed from brown to brownish red. These changes were not seen in shrimps fed with dies containing less than 800 ppb $AFLB_1$, even in the last weeks of treatment. Hepatopancreas of shrimps treated by diet containing 800 ppb and 1600 ppb $AFLB_1$ change to red and later to off white and were reduced to half of its size than the control group. Moribund shrimps showed abnormal movements and soft shell, specially the shrimps that were fed with diets contain more than 800 ppb $AFLB_1$.

Normal distribution was certified by SPSS 18 software. Four weeks after treatment, shrimps fed with the 1600, 800 and 400 ppb concentrations of $AFLB_1$ exhibited slow growth, and more reddish discoloration disseminated over the body and the tail, which was not observed in the other treatments. They also recorded the lowest final weight, daily weight gain, specific growth rate and survival rate, which was significantly different from other concentrations treatments (Table 2).

Table 1: Aflatoxin B_1 ($AFLB_1$) content of experimental diets

$AFLB_1$ content (ppb)	Diets							
Amount incorporated diet	0	20	50	100	200	400	800	1600
Amount after analysis	0.00	20.44	42.50	85.10	212.50	425.50	793.20	1380.00

Table 2: Final weight, weight gain and survival

Parameters*	$AFLB_1$ concentration (ppb)							
	0	20	50	100	200	400	800	1600
final weight (g)	13.50±1.22^{b}	13.75±0.95^{b}	1397±0.82^{b}	13.63±1.02^{b}	13.53±0.92^{ab}	13.06±1.23^{ab}	13.07±1.05^{ab}	12.42±1.36^{a}
Specific Growth Rate (%)	0.10±0.062^{cd}	0.06±0.009^{cd}	0.14±0.058^{d}	0.02±0.009^{cd}	0.01±0.009^{c}	0.11±0.025^{b}	0.11±0.039^{b}	0.30±0.068^{a}
Survival (%)	98.89±0.57^{d}	95.56±1.15^{cd}	100.00^{d}	97.78±1.15^{cd}	94.44±0.57^{cd}	88.89±0.57^{c}	68.89±1.52^{b}	58.89±3.05^{a}

* **Values are (mean ± SD) for each row. Means with the same superscript are not significantly different (P < 0.05).**

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Eight weeks after commencing the feeding trial, shrimps fed with more than 200 ppb of $AFLB_1$ showed slow growth, and more reddish discoloration disseminated over the body and the tail, which was not observed in the other treatments. They also exhibited the lowest final weight, daily weight gain, specific growth rate and survival rate, which was significantly different from treatments given the diets with less than 200 ppb of $AFLB_1$. At the end of week 8, the concentration of $AFLB_1$ in the diets was negatively correlated with the final weight at the 0.001 level (2-tailed) (r = -0.339**). At the same time, dose of $AFLB_1$ in the diets and duration of feeding were showed negatively correlated with the survival rate at the 0.001 level (2-tailed) (r = -0.603** and -0.571**).

Table 3: Final weight, weight gain and survival rate in *F. indicus* fed with different concentrations of aflatoxin B1 ($AFLB_1$) for 8 weeks

Parameters*	$AFLB_1$ concentration (ppb)							
	0	20	50	100	200	400	800	1600
Final weight (g)	14.15±1.19^{c}	14.22±1.01^{c}	14.12±1.09^{c}	13.80±1.03^{c}	13.42±1.15^{bc}	12.86±1.17^{b}	12.49±1.27^{ab}	11.78±1.14^{a}
Specific Growth Rate (%)	0.03±0.002^{d}	0.05±0.005^{d}	0.01±0.025^{d}	0.02±0.005^{cd}	-0.01±0.001^{c}	-0.02±0.019^{b}	-0.08±0.005^{b}	-0.09±0.033^{a}
Survival (%)	98.89±0.57^{e}	93.33±0.00^{de}	91.11±0.57^{de}	94.44±1.15^{de}	83.33±1.00^{cd}	76.67±1.73^{c}	54.44±3.21^{b}	38.89±2.88^{a}

* **Values are (mean ± SD) for each row. Means with the same superscript are not significantly different ($P < 0.05$).**

Shrimps fed diets containing 0 (control) to 100ppb of $AFLB_1$, gave significantly higher growth compared with those given increased levels of $AFLB_1$. Daily weight gain started to slow down at day 28 for shrimps fed diets with more than 400 ppb $AFLB_1$ but Shrimp fed a diet with 1600 $AFLB_1$, started to lose weight in the first week and the daily weight gain and specific growth rate were negative, from the first week.

In the control group, the survival rate after 8 weeks was 98.89 ± 0.57 %, while that of the shrimps that received 400, 800 and 1600 ppb of $AFLB_1$ were affected after 4 weeks. The survival rate in higher concentrations of $AFLB_1$ (800 and 1600 ppb), fed group as decreased to 68.89 ± 1.52 % and 58.89 ± 3.05 %, respectively. After 8 weeks the survival rate in two last groups reduced to 54.44 ± 3.21 % and 38.89 ± 2.88 % (Table 3).

There was a noticeable increase in THC in the juvenile *F. indicus* given feed with high levels of $AFLB_1$ (more than 200 ppb) after 2 weeks. The mean THC in the control group was 76.15 ± 8.62, 74.48 ± 5.36 and 75.81 ± 10.95 ($\times 10^5$ cells/ml) at the end of 2, 4 and 8 weeks, respectively. Afterward, THC exhibited a decrease after 4 weeks in 400 and 1600 ppb treatments while at the end of 6th week, there was a gradual decrease in the number of circulating haemocytes in 200 and 800 ppb treatments (Table 4).

Concentrations of $AFLB_1$ in the feed and duration of feeding were positively correlated with THC at the 0.05 level (2-tailed) in the second week (r = 0.334* and 0.301*) while at the end of 8th week, concentrations of $AFLB_1$ in the dates and duration of feeding showed

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negatively correlated with THC at the 0.001 level (2-tailed) (r = -0.237** and -0.523**).

Table 4: Total Haemocyte Count in *F. indicus* fed with different concentrations of aflatoxin B_1 ($AFLB_1$) for 8 weeks.

Total Haemocyte Count ($\times 10^5$ cells/ml)*	$AFLB_1$ concentration (ppb)							
	0	20	50	100	200	400	800	1600
2nd week	76.15±8.62[a]	70.40±8.96[a]	70.40±18.07[a]	58.70±5.50[a]	82.20±25.22[a]	98.76±11.96[a]	97.39±3.87[a]	93.28±17.55[a]
4th week	74.48±5.36[ab]	71.32±5.61[ab]	53.41±3.73[ab]	49.84±3.85[a]	80.06±16.09[b]	47.14±11.80[ab]	69.92±3.87[b]	51.04±22.50[ab]
8th week	75.81±10.95[a]	60.74±24.94[ab]	55.19±25.51[ab]	51.86±32.00[b]	39.77±18.97[ab]	40.08±17.31[b]	30.61±15.82[ab]	31.56±10.16[b]

* **Values are (mean ± SD) for each row. Means with the same superscript are not significantly different ($P < 0.05$).**

The Total plasma protein means in control group at the end of 4 and 8 weeks was 74.48±5.36 and 75.81±10.95 mg/ml, respectively. The TPP showed drastic decrease in the groups that were fed with diet containing 800 and 1600 ppb of AFLB1. The TPP values at the second week showed a drastic decrease to 36.21 ± 6.25 mg/ml and 17.49 ± 3.44 mg/ml, respectively. The TPP values were reduced to 15.16 ± 5.33 mg/ml at the end of the eighth week in the group fed with the highest concentration of $AFLB_1$ (Table 5)

The correlation between dose of $AFLB_1$ in the diets and TPP at the 0.001 level (2-tailed) at 4 and 8 weeks demonstrated that there was a negative correlation (r = - 0.542** and r = - 0.649**).

Table 5: Total Plasma Protein in *F. indicus* fed with different concentrations of aflatoxin B_1 ($AFLB_1$) for 8 weeks

Total Plasma Protein (mg/ml)*	$AFLB_1$ concentration (ppb)							
	0	20	50	100	200	400	800	1600
2nd week	77.10±13.36[ab]	85.06±21.01[b]	82.24±19.80[b]	76.08±15.19[b]	84.01±13.04[b]	71.12±25.02[ab]	36.21±6.25[ab]	17.49±3.44[a]
4th week	80.99±9.73[c]	79.43±17.86[c]	83.43±12.26[c]	79.86±23.35[c]	70.89±17.86[c]	48.15±5.85[bc]	34.99±6.25[ab]	20.55±12.71[a]
8th week	78.52±5.80[bc]	81.14±12.19[c]	82.10±12.17[c]	79.36±8.57[c]	71.54±26.05[bc]	56.47±16.70[b]	30.37±11.5[a]	15.16±5.33[a]

* **Values are (mean ± SD) for each row. Means with the same superscript are not significantly different ($P < 0.05$).**

Phagocytic activities were found to increase in the 20,50,100,200,400 ppb groups when analyzed at the end of the 2nd week, while the decreased phagocytic activities were observed after 8 weeks in the groups fed with diets containing 800 ppb and 1600 ppb $AFLB_1$ (Table 6).

Two-way ANOVA test showed no significant difference in experiment period.

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Table 6: Phagocytic activities in *F. indicus* fed with diets contain different levels of aflatoxin B_1 ($AFLB_1$) for 4 and 8 weeks

Phagocytic activity (%)*	$AFLB_1$ concentration (ppb)							
	0	20	50	100	200	400	800	1600
2nd week	35.86±8.44	41.41±17.73	43.76±4.54	42.14±9.28	40.32±6.56	39.09±8.04	35.01±16.29	34.43±12.55
4th week	37.16±18.41	37.22±17.73	35.98±7.18	36.47±9.28	37.04±11.63	32.48±13.10	30.98±13.58	30.97±12.78
8th week	36.70±14.97	35.22±12.93	36.28±7.79	36.73±9.25	36.67±8.61	32.08±8.49	30.75±12.40	28.57±8.66

* **Values are (mean ± SD) for each row. Means with the same superscript are not significantly different ($P < 0.05$).**

The $AFLB_1$ residue levels detected in the shrimp carcass in the different treatments are shown in the Table 5. $AFLB_1$ residues were not detected in the control group. After receiving diets containing $AFLB_1$ for 8 weeks, the highest concentrations of $AFLB_1$ residue were found in the muscles of shrimps which received diets containing 50 to 400 ppb $AFLB_1$, 4 weeks after commencing the trial. These levels of $AFLB_1$ residues decreased after 8 weeks. The residues found in the head were smaller than those found in muscle and was stable throughout the treatment period (Table 7). The correlation between duration of feeding and residue at the 0.001 level (2-tailed) at 4 weeks, verified that there was a high positive correlation (r = 0.836**). The correlation between concentration of $AFLB_1$ in the feed and duration of feeding with residue of $AFLB_1$ in the shrimp carcass at the end of week 8 were significant at the 0.001 level (r = 0.300** and 0.512**). Paired samples t-Test showed a significant difference between $AFLB_1$ residue in head and muscle (df = 47, t = -3.753 and P <0.05).

Table 7: $AFLB_1$ residues in the carcass of *F. indicus* after dietary treatments with diets contain different doses of aflatoxin B_1 ($AFLB_1$) at the end of weeks 4 and 8

$AFLB_1$ in diet (ppb)	$AFLB_1$ residue (ppb)			
	Head		Muscle	
	4 weeks	8 weeks	4 weeks	8 weeks
0	0.00^{a*}	0.00^{a}	0.00^{a}	0.00^{a}
20	1.00^{a}	0.85^{ab}	2.25^{a}	1.30^{b}
50	2.55^{b}	0.75^{b}	12.75^{c}	0.85^{de}
100	3.80^{bc}	1.75^{c}	13.65^{c}	1.55^{e}
200	6.55^{d}	4.90^{e}	12.15^{c}	0.30^{de}
400	8.80^{e}	6.40^{f}	11.60^{c}	0.35^{d}
800	3.15^{bc}	5.85^{d}	7.95^{b}	0.65^{c}
1600	4.35^{c}	7.10^{e}	7.50^{b}	0.15^{c}

* **Means with the same superscript in each column are not significantly different ($P < 0.05$).**

The normal hepatopancreas tissue has a tubular pattern. The hepatopancreas as a midgut gland is the essential organ of digestion in crustacean. It is made of blind ending tubules consisting of four cell types. The E cells (embryo cells or Stem cells) at the summit of the tubules mature into R cells (absorption and storage of nutrients), F cells (production of digestive enzymes) and B cells (presumed to be secretory in function) (Bell and Lightner, 1988). The differences in the size and shape of the epithelial cells lead to the star appearance of the tubule lumen (Figure 1A). Hepatopancreas has been observed to be very sensitive to an altering in the normal (Dall, 1990) diet and water borne pollutants and R-cells are the first

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severely affected cell type in the tubular epithelia of the hepatopancreas thus it is used as a monitor organ to control the effects of various toxicants.

The Star shape appearance of tubule lumen was observed in 20, 50 and 100 ppb treatments but at 200 ppb, a reduction in the lipid content in the hepatopancreas tubule because of R-cell atrophy was observed. No inflammatory reaction was observed in the hepatopancreas of shrimps at less 200 ppb treatments. In the histological sections of the hepatopancreas of shrimp fed a diet with 200 ppb $AFLB_1$, infiltration of haemocytes around the tubules and in the interstitial sinuses, was observed.

The initial sign of inflammation was haemocytic infiltration in the interstitial spaces in between the tubules. More advanced lesions were in the form of and necrosis, either in the tubule itself or the sinuses around it. These lesions were most common in the proximal region of the hepatopancreas. Changes in B cell structure from cylindrical to cubic shape, loss of brush-border appearance, extinction of secretory tubule aperture and reduction in E cells count were observed in shrimps given more than 400 ppb $AFLB_1$ for 4 weeks (Figure 1B). In shrimps received 400 ppb and more $AFLB_1$ in the diet for 8 weeks, degenerative changes, necrosis of the hepatopancreatic tissues were observed. Furthermore, individuals with such symptoms had poorer growth performance.

The effects of $AFLB_1$ on histopathological alteration are directly correlated with the concentrations of $AFLB_1$ and the time period of the feeding according to the statistical analysis. This experiment obviously shows that when more than 400 ppb concentrations of $AFLB_1$ were introduced for 8 weeks, it led to the histopathological alteration in the hepatopancreas tissue of all the samples examined. When lower concentrations of $AFLB_1$ were fed, there were fewer shrimps showing histological abnormality.

At the end of week 4, the shrimps given the feed contain 800 and 1600 ppb $AFLB_1$ developed noticeable alterations in hepatopancreas tissue. R-cells became atrophied and severe necrosis and shrinkage of the tubules could be observed. Infiltration of haemocytes and fibroblastic cells produced a fibrosis-like exterior look. The cytoplasmic content of degenerated cells and and pyknotic nuclei were observed in the lumen (Figure 1D). Such changes were detected in nearly half of the samples examined. After 8 weeks, the shrimps fed with 800 and 1600 ppb $AFLB_1$, had showed severe histopathological alterations, similar to those observed at week 4. Spread necrosis and complete destruction of B cell were observed. Presentation of fibroblastic cells in the tubule structure inhibit of its complete demolition (Figure 1C). In 1600 ppb treatment connective tissue was completely isolated from hepatopancreas tissue and in midgut tissue, separation between mucousal and sub mucousal layers as well as necrosis and structural destruction were clearly seen (Figure 2A). In muscular tissue, in shrimps have been fed with diet contain more than 800 ppb $AFLB_1$, separation among muscular fibers were obviously observed (Figure 2B).

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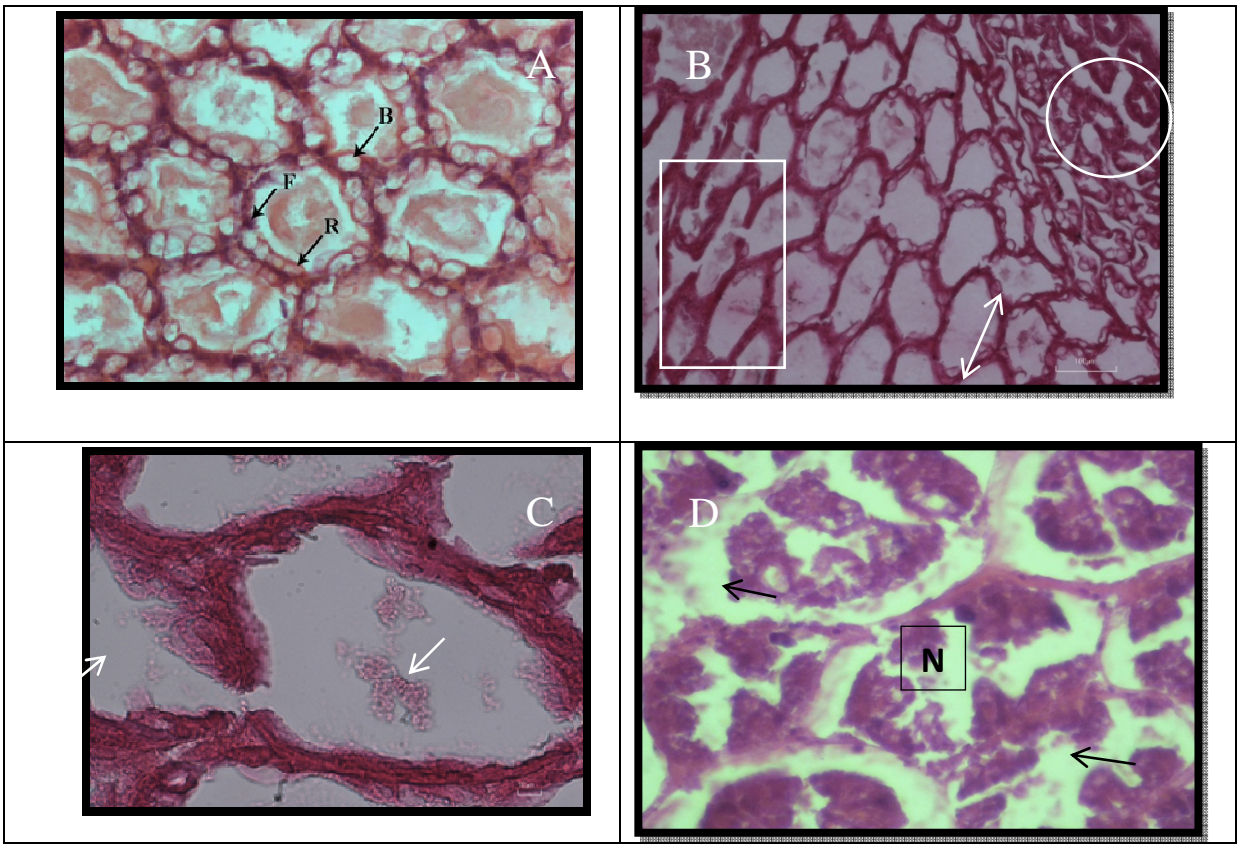


Figure 1: Transverse section (TS) of the hepatopancreas, lumen and muscle of the *F. indicus* feed with diets containing aflatoxin B_1 ($AFLB_1$) for 8 weeks. The structure of hepatopancreas in shrimps fed with diet containing 50 ppb $AFLB_1$ and Black arrows indicate three types of cells, × 200 (A). Changes in B cell structure from cylindrical to cubic shape, extinction of secretory tubule aperture (white arrow), Early degenerative changes (rectangle) and reduction in E cells count (circle) in shrimps given more than 400 ppb $AFLB_1$ for 4 weeks, × 100 (B). Spread necrosis and complete destruction of B cells and subversion of necrotic tissue into the tubule lumen (white arrow), × 400 (C). Cytoplasmic content of degenerated cells and pyknotic nuclei (N letter) in the lumen, × 400 (D). H&E, Haemotoxylin and Eosin.

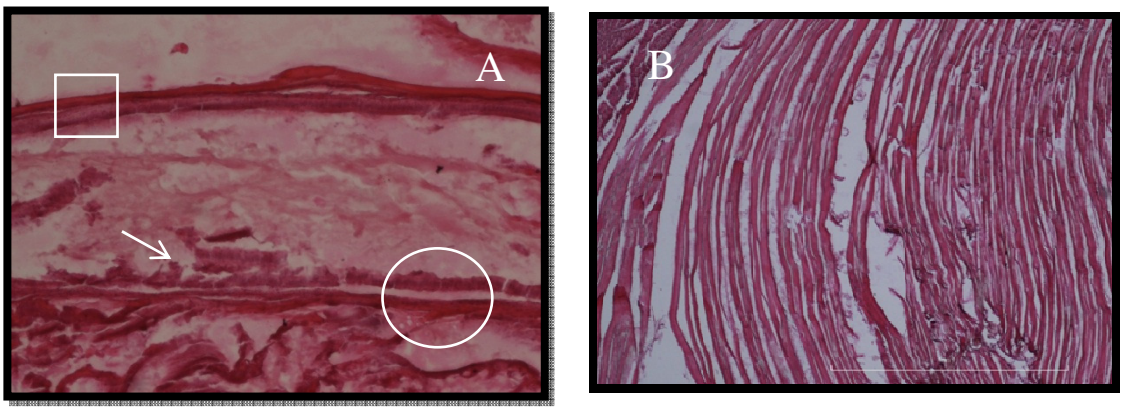


Figure 2: Separation between mucousal and submucousal layers (circle) versus normal structure (rectangle), necrosis and structural destruction of midgut tissue (arrow) in shrimp received more than 1600 ppb aflatoxin B_1 for 8 weeks (A). Abnormality in muscle fibres (B). × 40, H&E, Haemotoxylin and Eosin.

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Discussion

Aflatoxins contamination is an inconvenience come across by food and feed producers and raw material suppliers especially in the humid tropical countries. $AFLB_1$ has known as the most toxic mycotoxin, and nearly all the information available on the bioactivity of aflatoxins in animals has been attentive on $AFLB_1$ and its metabolites. The effects of $AFLB_1$ on *F. indicus* were studied considering the importance of the species in shrimp aquaculture and the inadequate information in the shrimp industry.

The THC was found to increase suddenly at the end of 2nd week in shrimps fed with diet containing 400, 800 and 1600 ppb while reduction was observed at 4 and 8 weeks. The high THC in shrimps fed higher levels of $AFLB_1$ during the first 2 weeks, may be due to response of the immune system to eliminate of $AFLB_1$ from shrimp haemolymph. Decrease in THC at the end of 4 and 8 week, could be related to the weakening of the defense mechanism. It was must consider, the circulating haemocyte frequency is a health or stress indicator but this parameter was non-specific and show fluctuation according environmental, and physico-chemical stresses (Soderhall and Cerenius, 1992). Environmental and nutritional stresses can modulate the mitotic activity of haemopoietic tissues and lead to the reduction of the turnover of hemocytes in decapoda (Johnson, 1980).

The TPP showed a drastic decrease in 400, 800 and 1600 ppb treatments at the end of 2nd week but after 4 weeks, there was a gradual decrease in the TPP. Albumin and globulin are two major serum proteins. The total plasma and serum protein are involved mainly in nutrition, water distribution, acid-base balance as well as immunity and metabolic needs (Lehninger et al., 1993). Decrease in TPP was correlated with damage severity of hepatopancreas organ. On the other hand, $AFLB_1$ disrupts endoplasmic reticulum and reduces the RNA biosynthesis, attachment of poly ribosomes to endoplasmic reticulum, and disruption in ribosomes functions also, thus severely affecting protein biosynthesis. In addition $AFLB_1$ interacts with the complex structure of nuclear chromatins, by forming DNA-Aflatoxin complex (Scarpelli and Trump, 1964). The reduction in protein biosynthesis and nuclear damage may be the reasons for the decrease in the total protein content in the present investigation. The TPP reduction trend was shown homology with similar studies (Boonyaratpalin et al., 2000, 2001; Gopinath and Paul Raj, 2009).

The reduction of TPP leads to a reduction in growth and survival rate in affected shrimps. It seems that, shrimp physiology prefers to the use of their tissue protein to redress reduction in protein biosynthesis. Thus, alterations in muscle texture, in 800 and 1600 ppb treatments may be indicative of impaired nutrient absorption, impotence and weight loss.

Phagocytic activity is used to show the percentage of hemocytes containing endocytosed yeast or bacteria (Soderhall and Cerenius, 1992). The decrease of PA can be a consequence of alterations in THC in exposed shrimps. Phagocytosis is contributed mainly by hyalinocytes and also in the lower level by semigranulocytes (Brehélin and Arcier, 1986). Gopinath and Paul Raj (2009) reported the

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increase of phagocysis percentage in shrimps exposed to $AFLB_1$ at the end of 4 weeks in a dose dependent manner, while decreasing phagocytic ratio was observed after 8 weeks.

Aflatoxin B_1 residues in carcasses of exposed shrimps were separately detected at the end of 4 and 8 weeks. According to cooking pattern in Iran and some country, only muscular edible part of the shrimp's body is used. The results from the current study showed that the $AFLB_1$ residue was highest in the muscle of shrimps in 50, 100, 200 and 400 ppb treatments after 4 weeks of feeding. However, $AFLB_1$ residue in muscle gradually decreased at the end of 8 weeks to a level which was lower than that detected in the head of carcasses (0.30-1.55 ppb). This finding was agreed with Boonyaratpalin et al. (2001) and Bintvihok et al. (2003). In spite of some studies, the mechanisms of toxic substance elimination from the shrimp body is not obviously proved (Linares and Ochoa 2009). According to Boonyaratpalin et al. (2001), increasing level of alkaline phosphatase, show direct relation with level of $AFLB_1$ in diets.

The histopathological studies of the hepatopancreas, midgut and muscles of the control shrimps conformed to the structure described by Bell and Lightner (1988). The general disorder in hepatopancreas tissue was similar to histopathological effects of $AFLB_1$ exposure described by Lightner et al. (1982). Atrophy of hepatopancreatic tubules, was the first histopatological sign of aflatoxicosis observed in *the F. indicus*. Atrophy of hepatopancreas was followed by the destruction of E, R and B cells, cellular inflammation, infiltration of haemocytes, necrosis and infiltration of fibbroblastic cells between the tubules of the hepatopancreas. The effect of $AFLB_1$ on the hepatopancreas appears to be directly correlated with its concentration and the duration of feeding. Similar changes were reported in Penaeid shrimps fed $AFLB_1$ (Bautista et al., 1994) and $AFLB_1$ levels above 100 ppb caused inflammation, necrosis, infiltration of hemocytes and severe degeneration of tubules. The degree and severity of the histopathological alterations observed in the hepatopancreas, midgut and muscles were undoubtedly related to the decreased final weight and survival rate in the 800 and 1600 ppb $AFLB_1$ exposed shrimps.

The results of the current study show that the growth parameters, survival rate and health indices (THC, TPP) of Indian white shrimp, *F. indicus*, are affected by the different doses of $AFLB_1$ in diets. $AFLB_1$ in the diet showed a highly negative correlation to final weight, survival rate. Moreover, significant histopathological alterations in the hepatopancreas tissue of shrimps exposed to different levels of $AFLB_1$ were observed. These alterations are evidently indicated by changes in the THC and TPP. From the practical point of view, these indicate that the feeding of shrimps with diets containing less than 200 $AFLB_1$, will not significantly affect the growth of culture shrimps after 8 weeks.

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تأثیر آفلاتوکسین B_1 روی رشد، سلامتی، فعالیت فاگوسیتوز و تغییرات آسیبی در *Fenneropenaeus indicus*

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دریافت: مرداد ۱۳۹۱ پذیرش: بهمن ۱۳۹۱

چکیده

آلود شدن جیره غذایی مورد استفاده در تغذیه آبزیان به مایکوتوکسین‌ها در مناطق گرم و مرطوب امری بدیهی می باشد. در این مطالعه میگوهای سفید هندی (*Fenneropenaeus indicus*) (۱/۷۶≠۱۱/۷۹گرم) به مدت ۸ هفته با جیره‌های غذایی حاوی مقادیر صفر،۲۰،۵۰،۱۰۰،۲۰۰،۴۰۰،۸۰۰ و ۱۶۰۰ ppb آفلاتوکسین B_1 ($AFLB_1$) تغذیه شدند. وزن نهایی و میزان باقی مانده بافتی هر دوهفته یک بار و تعداد هموسیت کل (THC)، پروتئین پلاسما کل (TPP)، فعالیت فاگوسیتی (PA) و درصد بازماندگی هر چهار هفته یک‌بار اندازه‌گیری گردید. در پایان هفته‌های چهارم و هشتم تیماربندی، تغییران بافت‌شناسی در بافت هپاتوپانکراس، روده میانی و عضله مورد بررسی قرار گرفت. در پایان هفته چهارم، در میگوهای تغذیه شده با جیره‌های حاوی ppb ۴۰۰، ppb۸۰۰ و ppb ۱۶۰۰ $AFLB_1$ میزان رشد کمتر و قرمزشدگی بیشتری در سطح بدن مشاهده شد. پارامترهای رشد، درصد بازماندگی و شاخص‌های سلامتی (THC و TPP) در میگوهای *F.indicus* تحت تاثیر تغذیه با جیره‌های حاوی مقادیر مختلف $AFLB_1$ قرار گرفت. در پایان هفته هشتم همبستگی منفی بین دوز $AFLB_1$ موجود در جیره با وزن نهایی، درصد بازماندگی، THC و TPP مشاهده شد (بترتیب r برابر با ۰/۳۱۲- ، ۰/۶۰۳- ، ۰/۲۳۷- و ۰/۶۴۹- با $P<0.001$). افزون براین، تغییرات بافت‌شناسی قابل مشاهده‌ای در بافت هپاتونکراس، روده میانی و عضله میگوهای تغذیه شده با جیره‌های حاوی مقادیر مختلفی از $AFLB_1$ مشاهده شد و تغییرات بافت‌شناسی با نوسانات شاخص‌های سلامتی هم‌خوانی نشان داد.

کلمات کلیدی: آفلاتوکسینB_1، *Fenneropenaeus indicus* ، رشد، سلامتی و تغییرات آسیبی

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