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THE EFFECTS OF ACUTE AND CHRONIC AFLATOXICOSIS ON THE IMMUNE FUNCTIONS OF *OREOCHROMIS NILOTICUS* IN EGYPT

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SUMMARY

The present study was oriented toward assessing the effects of aflatoxin on *O. niloticus* with particular reference to clinical manifestations and suppression of macrophage phagocytic activity. Ten day LD_{50} of intraperitoneally administered crude aflatoxin was 0.5mg / kg bw. Long term effects of levels of chronic aflatoxicosis included mortality (63.5 %), growth suppression and reduced macrophage phagocytic activity. Changes in macrophage phagocytic activity were observed even in the absence of clinical manifestations, hence phagocytic assays may be useful as an early warning indicator of environmental toxicosis.

INTRODUCTION

Tilapia are an increasingly important cultured foodfish in Egypt, because they are readily adaptable to intensive culture conditions and provide a relatively economical source of high quality protein. However, like other animals maintained under intensive conditions, disease resistance may be compromised by various environmental pollutants (Matthews et al 1990). One such pollutant, aflatoxin, is widely distributed in the environment and represents one of the most important food pollutants to humans and other animals. Literature concerning the role of aflatoxicosis among the cultured fishes in Egypt is scanty. Although Hegazy (1984 and 1988) and Moharram et al (1988) conducted mycologic studies on fish meal and El - Banna et al (1992) and Srour (1992) studied the effect of different dietary levels of aflatoxin B_1 (AFB_1) on mortality and weight gain of Oreochromis niloticus, studies focusing on the effects of aflatoxin on immune functions of *O. niloticus* appear not to have been reported. Macrophages play an important role in both cellular and humoral immune defense mechanisms of fish (Wolke 1992), hence the present study was designed to assess the effects of acute and chronic aflatoxicosis on the phagocytic activity of *O.niloticus* using *E. coli* as a model system.

MATERIAL AND METHODS

Fish and their maintenance:

Two hundred and seventy eight apparently normal 50 - 60 g *O. niloticus* were maintained in continuously aerated chlorine free filtered tap water, PH 7.5 (±0.4), 26 (±1) °C. The fish were fed twice daily at a rate of 3% body weight (Jauncy and Ross, 1982) using a locally produced diet formulated according to El - Banna (1991).

Production and extraction of crude aflatoxin (AFB_1, AFB_2, AFG_1, AFG_2): Toxigenic *Aspergillus flavus* . NRRL 999 was cultured and the crude aflatoxin extracted according to Davis et al (1965). Aflatoxin analysis was conducted by thin layer chromatography (A. O. A. C., 1980).

Phagocytic assay:

The phagocytic assay was a modification of that described by Mathews et al (1990). Head kidneys were aseptically collected, rinsed in teleost buffered saline (TBS) and extruded through a sterile stainless steel mesh into cold minimum essential

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medium - S (MEM - S) followed by light centrifugation (<2000 xG) on Ficole ® histopaque (Sigma). Macrophages were collected and washed in 5 volumes TBS and resuspended in Hanks Balanced Salt Solution (HBSS). Cell identity, viability and concentration of the preparation were assessed microscopically in a hemocytometer after diluting the cell suspension 10^{-1} in 0.5 % (w / v) trypan blue. Culture tubes containing 2.5×10^5 viable macrophages were mixed with 2.5×10^7 formalin killed *E. coli* cells in a total volume of 1 ml and incubated for 30 min at 17°C. Ice cold TBS was added and the preparations centrifuged for 5 min at 1000 x G. The sedimented cells were collected with capillary tubes, smeared on microscopic slides, Giemsa stained and the phagocytic index microscopically estimated at 1000 x as the percentage of macrophages containing 2 or more bacteria i.e.:

% phagocytosis = (The total number of phagocytes with ingested bacteria) / (Total number of phagocytes) x 100.

Statistical analysis: The experimental data were statistically analyzed using Statview 512 software (1986).

Experimental design: Three studies were conducted, viz study 1 was designed to determine the ten day LD_{50} of the crude aflatoxin; study 2 was designed to assess short term aflatoxicosis and study 3 was designed to assess long term effects of aflatoxicosis. To determine the crude aflatoxin ten day LD_{50}, seventy fish (50 - 60 g) were divided into groups of ten fish per group. Fish in the first six groups were injected intraperitoneally with crude aflatoxin containing AFB_1, AFB_2, AFG_1,AFG_2 (0.6, 0.2, 0.6 and 0.2 mg aflatoxin / ml CHCI3 respectively). The injected doses per group were 0.2 , 0.4 , 0.6 , 0.8 , 1 and 4 mg crude aflatoxin / kg body weight respectively. The seventh group served as the control. Injected with the diluent, Fish were observed daily for clinical signs and the number of deaths which occurred over the ten days period was recorded and served as the basis for calculating the ten day LD_{50}.

Short term aflatoxicosis studies were conducted with 40 fish. The fish were divided into four groups, 10 fish per group. Fish of groups 1-3 received diets containing 0.25 , 0.5 and 1.0 mg / kg body weight crude aflatoxin daily. Group four fish served as the control group and received uncontaminated diet. The fish were observed over a 10 day period, during which time clinical manifestations and mortality were recorded. The phagocytic index of kidney macrophage was assessed from fish necropsied on the eleventh day. Chronic aflatoxicosis studies were conducted over a 22 week period. For this purpose 168 fish were subdivided into 14 groups, 12 fish per group. Fish of groups 1 - 7 were fed crude aflatoxin contaminated diet at a rate of 50µg aflatoxin / kg feed (AFB_1, 1.33 mg ; AFB2 0.56 mg ; AFG_1, 1.05 mg - per kg corn). Fish in other groups were fed uncontaminated diet throughout the experimental period. Fish were examined over the experimental period to ascertain mortality, clincal signs and body weight. Phagocytic activity was determined in moribund and

Table (1): The mortality percent in *O. niloticus* for detection of LD_{50} of crude aflatoxin

Gp. #	No. of fish / gp	Dose of crude aflatoxin / kg b.w.	Number of dead fish in 10 days										Total # of dead fish
			1	2	3	4	5	6	7	8	9	10	
1	10	0.2 mg	0	1	1	1	0	0	0	0	0	0	3
2	10	0.4 mg	0	2	1	1	0	0	0	0	0	0	4
3	10	0.6 mg	0	4	1	1	0	0	0	0	0	0	6
4	10	0.8 mg	1	3	2	2	0	0	0	0	0	0	8
5	10	1 mg	5	3	1	0	0	0	0	0	0	0	9
6	10	4 mg	5	5	0	0	0	0	0	0	0	0	10
control	10	0 mg	0	0	0	0	0	0	0	0	0	0	0

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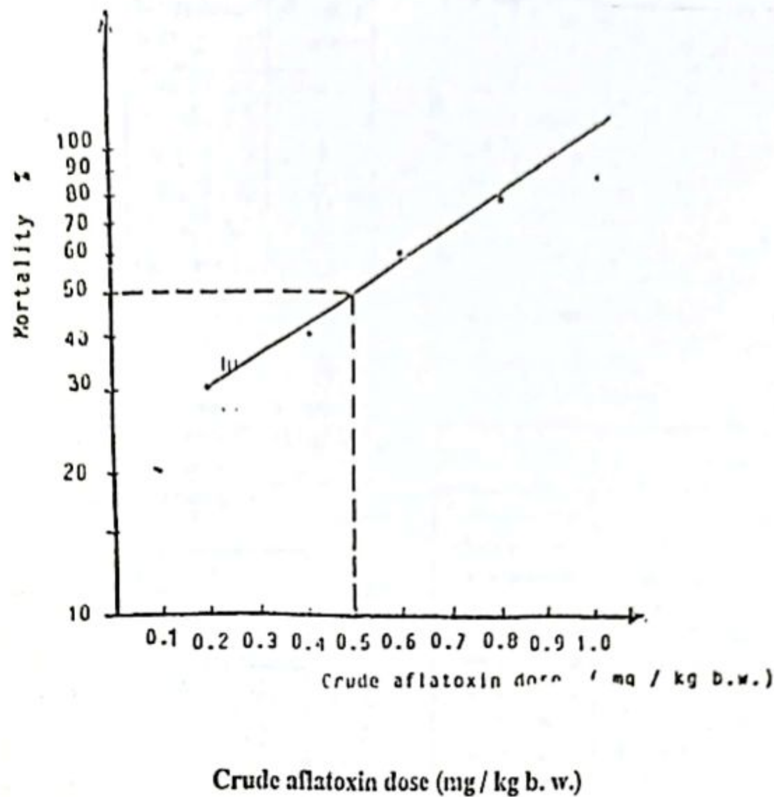
Fig. (1). Logarithmic representation of LD50 in *Oreochromis niloticus* I / P inoculated with crude aflatoxin.



Crude aflatoxin dose (mg / kg b. w.)

sacrificed fish on a monthly basis.

RESULTS

As demonstrated in the data of table 1 and illustrated in figure 1, the 10day LD50 was 0.5 mg / kg body weight. Clinical manifestations of injected fish prior to death included sluggish swimming activity, opercular distention, increased opercular rhythm and skin darkening. Neither mortality nor clinical manifestations were observed in the short term study; however, phagocytic activity of the kidney macrophages derived from orally aflatoxicated fish were significantly decreased when compared with the untoxicated controls (Table 2).

By the end of 22 weeks, mortality rate was 63.5 % in aflatoxicated *O. niloticus* at the indicated levels (Table 3). Clinical signs exhibited in fish exposed to aflatoxin included skin darkening, anemia and a significant ($p = < 0.01$) decrease in body weight (Table 4). The phagocytic activity of those fish was significantly reduced (Table 5).

DISCUSION

Much concern has been expressed over the adverse effects of various environmental pollutants upon aquatic ecosystems. Because the immune system plays a key role in host defense mechanisms, factors such as environmental pollutants which could affect the immune system, would likely influence productivity of important cultured fish species. Aflatoxins which are widespread in the environment and various grain products used in fish diets are some of the more potent toxins to some species of fish (Halver et al 1969). Although several studies on aflatoxicosis in some of the more important commercial species of fish in the United States exist (e.g. Bauer et al 1969; Halver et al 1969); Jantrarotai et al 1990), reports on the effects of aflatoxins on *O. niloticus*, a commercially important species in Egypt, are somewhat limited.

With a 10 day LD_{50} of 0.5 mg / kg bw, the present study suggests that *O. niloticus* is more susceptible to aflatoxcosis than rainbow trout (*Mt. slasta*)

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Table (2): Phagocytic activity of the kidney macrophages derived from orally aflatoxicated fish

Crude aflatoxin dose (mg / kg b. w.) per day	Phagocytic %
0.25	78 + 2.1*
0.5	43 + 0.6**
1.0	42 + 0.1**
control	80 + 0.1

* Significant difference between control & aflatoxicated fish ($P<0.05$)

** High significant difference between control & aflatoxicated fish ($P< 0.01$)

Table (3): Mortality % in *O. niloticus* after long expoure to oralaflatoxin

Time in weeks	Number of living fish	Cumulative # of dead fish	Cumulative mortality %
0	96	0	0
4	82	14	14.58
8	54	34	35.42
12	40	44	45.83
16	31	47	48.96
20	61	55	57.30
22	7	61	63.54

Table (4): *Growth rate of O. niloticus after long expousure to oral aflatoxins*

Time in weeks	Mean weights of Experimental Fish (g)	Mean weights of Control fish (g)	Growth reduction rate (g)	
0	47.57 + 0.24	47.43 + 0.39	0.39	0.00
2	51.80 + 0.82 *	54.45 + 0.54	0.54	2.65
4	52.85 + 0.88 *	57.03 + 0.63	0.63	4.18
6	58.25 + 1.22 *	62.85 + 0.70	0.70	4.60
8	60.70 + 1.06 *	67.05 + 0.80	0.80	6.35
10	58.87 + 0.99 *	66.05 + 0.86	0.86	7.18
12	60.50 + 1.83 *	69.05 + 0.86	0.86	8.55
14	59.50 + 1.49 *	71.45 + 0.79	0.79	11.95
16	60.24 + 1.55 *	74.15 + 0.72	0.72	13.91
18	61.09 + 2.61 *	74.18 + 0.72	0.72	13.09
20	58.57 + 1.05 *	75.38 + 0.02	2.02	16.81
22	53.40 + 4.97 *	72.68 + 0.52	0.52	19.18

Table (5): Phagocytic activity of the kidney macrophages derived from *O. niloticus* after long exposure to oral aflatoxins

Time in weeks	Phagocytic % Experimental group	Phagocytic % control group
1	18.70 + 0.40 *	60.30 + 0.30
2	27.90 + 2.90 *	67.70 + 2.20
3	40.70 + 0.60 *	78.30 + 3.70
4	23.70 + 4.40 *	72.60 + 1.30
5	29.90 + 0.60 *	77.00 + 3.70

* Significant difference between control & chronic aflatoxicated *O. niloticus ($P<0.01$)*

(0.81 mg / kg bw) (Bauer et al 1969), channel catfish *(Ictalurus punctatus)* (11.5 mg / kg bw) (Jantrorotai et al 1990) or coho salmon (1.0 mg / kg bw). The increased susceptibility of *O.niloticus* to aflatoxin correlates to the ability of the fish to reduce the toxin into the more toxic metabolite aflatoxicol by cytoplasmic NADPH - dependent enzyme (Bailey et al 1988). The enzyme may be more active or the toxin less effectively excreted in *O.niloticus* in comparison to the other species. Rainbow trout for example, do not possess the enzyme sulfotransferase but excrete AFB_1 as glucuronide conjugates (Melancon and Lench 1976; Lovelan et al 1984). Clinical signs associated with intraperitoneal administration of aflatoxin to O.niloticus was similar to those described in salmonids by Halver (1968) and ictalurids by Jantrarotai et al (1990).

Exposure of *O.niloticus* to 0.2, and 1.0 and 2.0, 1.0 and 2.0 LD_{50} aflatoxin resulted in clinical responses which were milder following oral administration than those associated with intraperitoneal exposure. While clinical manifestations were not readily apparent follwing short term exposure, phagocytic activity was drastically reduced (table 2). Clinical manifestations which included redced body weight, darker coloration and occasional hyperactivity were apparent in the long term (22 week) exposure studies. Reduced phagocytic activity of anterior kidney macrophage was also observed in the long term studies (table 5). These observations are similar to those of Halver et al (1966) and Lee et al (1978), who observed that sublethal levels of crude aflatoxin (320 ppb / kg bw) and of AFB_1 (450 ppb / kg bw) suppressed the growth of rainbow trout. Similar observations on growth reduction were observed in other fish

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i.e. channel catfish (Jantrarotai and Lovell 1990) and tilapia fingerlings (El Banna et al 1992); Srour 1992).

The observed mortality of young of the year adult O.niloticus in the present study (63.5 %) is somewhat higher than that observed in *O.niloticus* fingerlings (10 % - 16.7 %) reported by El - Banna et al (1992) or in red tilapia and nile tilapia fingerlings (5 % and 12.5 % respectively) reported by Srour (1992).

The present study was largely based upon the hypothesis that sublethal effects of certain environmental pollutants, particularly aflatoxin, may be associated with suppression of phagocytic activity. This hypothesis and the data of the present study are consistent with suggestions of otheres e.g. Mathews et al (1990) who suggest that chronic exposure to toxic pollutants could lead to decreased to viral, bacterial and oter parasitic diseases. Anderson and Siwicki (1994) suggest that reduced phagocytic activity in fishes may be indicative of the presence of contaminants, chronic infection, low dietary protein, vitamin deficiencies or other stressors, while enhanced phagocytic activity may be indicative of initial phases of bacterial infection.

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