

*Indian J. Fish.*, **50**(4) : 421-426, Oct.-Dec., 2003

## **Changes in serum proteins and immune response in *oreochromis mossambicus* induced by aflatoxin B<sub>1</sub>**

SHERLY ZACHARIA, P. C. THOMAS AND M. P. PAULTON

*Central Marine Fisheries Research Institute, Cochin - 682 014, India*

### **ABSTRACT**

Studies were conducted to evaluate the molecular changes induced by aflatoxin, the most frequently encountered dietary carcinogen in *Oreochromis mossambicus*. Three sub-lethal test doses of aflatoxin B<sub>1</sub> incorporated in feed (1.5, 5.0 and 15.0mg/kg feed) were given to the fishes for a period of 30 days and the changes in serum protein profile were resolved through SDS PAGE at regular intervals. Similarly, the changes in immune responses were also studied. The study revealed significant changes in both protein profile and immune response in the experimental fishes compared to the control, indicating that the toxin exerts its effect even at sublethal levels.

### **Introduction**

Aflatoxicosis is an important and frequently encountered problem seriously affecting fish production in tropical countries. It is mostly produced by the fungal strains of *Aspergillus flavus*. Aflatoxins are the only naturally occurring dietary carcinogens currently recognized by the International Agency for Cancer Research (IARC, 1993) as carcinogenic to humans. It functions as an electrophilic reactant, which after biotransformation induces carcinogenic effects. The toxin is best known for their hepatotoxic, nephrotoxic, carcinogenic, mutagenic and immunosuppressive properties in animals.

Initial changes due to aflatoxins are expected to be manifested in the transporting medium, *viz.*, blood. This aspect has received ample attention

especially in fish immunological studies. A prolonged time for blood clotting was reported by Woerr *et al.* (1987) indicating severe coagulopathy. The defect was mainly due to the failure of hepatic synthesis of factors V, VI, VII and fibrinogen (Baker and Green, 1987). Anticoagulant activity has been attributed to aflatoxin mainly due to its chemical relation to caumarin, which can bind to serum albumin. Severe anaemia together with the changes in haematocrit, haemoglobin concentration and erythrocyte and leukocyte counts were observed as prominent signs of acute aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) toxicity in channel catfish (Jantrarotori *et al.*, 1990). Determination of the serum proteins in fish contributes to an appraisal of the health of fish. The activities of serum constituents were found to be different in many farm animals on aflatoxicosis.

The total protein levels were found to be decreased in aflatoxin-fed animals, but the protein expressions were found to be elevated. Expressions of serum cholesterol, albumin and globulin were decreased in AFB<sub>1</sub> fed cockerels, while the serum globulins were elevated (Jakab, 1994). Reductions in the total serum protein and serum calcium and phosphorus levels were noted in AFB<sub>1</sub> treated animals (Dafalla *et al.*, 1987; Singh *et al.*, 1987; Jakab, 1994).

The more advanced teleosts are reported to possess an immune system approaching the complexity and efficiency of higher vertebrates. Interest on the effect of xenobiotics, particularly on the immune system, has increased in the recent past making it a sensitive biological parameter to monitor the immunotoxicity of chemicals. Mycotoxins have been reported to suppress the immune function and reduce resistance to infection on chronic exposure to low levels of toxin in a number of species (Jakab, 1994). Although a number of studies have been made on the effects of xenobiotics on the immune response of fish, studies involving aflatoxin in fish are scanty.

Most of the available information on aflatoxicosis in teleost is based on the studies conducted on coldwater fishes, especially salmonids and cyprinids. With such information on tropical fishes lacking, an attempt was made in the present study to determine the changes in serum protein profile and antibody response in tilapia, *Oreochromis mossambicus*, fed a diet fortified with AFB<sub>1</sub>. Of the several blood components in fish, determination of changes in the serum protein profile, serum protein concentration and immune response can provide to be a reliable appraisal of the condition of fish.

## Material and methods

Tilapia (50 ± 10 g) collected from brackishwater ponds were used for the study. They were acclimatized to freshwater conditions in the laboratory and transferred to experimental units of circular polypropylene tanks of 40 l capacity. The animals were fed initially for a week with a semi-purified diet containing 35% protein as suggested by Jauncey and Ross (1982) for freshwater tilapia during the period of acclimatisation.

### Experimental diet

Three experimental diets, having the same composition as that of the control (35% protein) was prepared by incorporating different concentrations of AFB<sub>1</sub> from a stock solution (100 µg AFB<sub>1</sub>/ml of chloroform) prior to final mixing of the diets. Since studies had indicated that warmwater fishes are generally less sensitive to AFB<sub>1</sub> than coldwater fishes (Jantrarotai *et al.*, 1990) higher doses of AFB<sub>1</sub> were included in the present study. Diets with 1.5, 5.0 and 15.0 mg AFB<sub>1</sub>/kg feed were prepared by adding adequate aliquots of the stock solution before blending and chloroform was allowed to evaporate. The ingredients were then mixed with water, extruded and dried.

### Experimental design

Three experimental groups each consisting of nine animals were exposed to three dietary levels of AFB<sub>1</sub> - 1.5, 5.0, 15.0mg/kg diet (test groups I, II and III), while the fourth group, fed on semi-purified diet devoid of AFB<sub>1</sub> formed the control. The fishes, starved for two days prior to feeding the experimental diet, were fed at the rate of 2% body weight per day to ensure that they consumed the feeds offered. Each group was routinely examined for physical and behavioural abnormalities. Thirty days observation

was used in this study.

### ***Serum protein profile***

Blood samples were obtained by cardiac puncture on the 7<sup>th</sup>, 14<sup>th</sup>, 21<sup>st</sup> and 30<sup>th</sup> days of the treatment. Samples were allowed to clot at room temperature. Serum was separated immediately and stored at -20°C until they were analyzed electrophoretically. The changes in the serum protein profile were analysed by Sodium Dodecyl Sulphate Polyacrylamide Gel (11.5%), Electrophoresis (SDS PAGE) (Table 1) using 1.5 M Tris-HCl buffer (pH 8.8) along with a stacking gel of 6% at a constant voltage of 140 V (Laemmli, 1970). The molecular weights of the subunits were determined using molecular weight standards run along with the samples. The volumes of samples were adjusted to a concentration of 120 µg proteins per well.

On completion of electrophoresis, the gels were stained for proteins with 0.1% Coomassie brilliant blue R250 according to conventional methods and destained in a solution of 50% methanol containing 10% acetic acid. Relative mobilities ( $R_f$ ) of the various protein fractions estimated and the molecular weights were

determined in comparison with markers.

### ***Evaluation of immune response***

In order to study the changes brought about by AFB<sub>1</sub> in the immune response of tilapia, haemagglutination test was conducted (Ambrosius and Schaker, 1964). Rabbit erythrocytes suspended in normal saline were used as antigen in the study.

***Immunisation of the fishes:*** Treated and control fishes were immunised by intra-muscular injection of the antigen (rabbit RBC suspension containing 10<sup>4</sup>-10<sup>6</sup> cells / kl) emulsified with complete Freund's adjuvant. Booster doses were given on the 10<sup>th</sup> day after primary injection without the adjuvant.

***Antibody detection:*** The antiserum from the blood of immunized fishes were separated on the 30<sup>th</sup> day of exposure. The samples were analyzed for antibody production at different serial dilutions by haemagglutination test.

## **Results**

### ***Changes in serum profile***

Electrophoretic analyses of the serum protein profile of the test groups

TABLE 1. *Composition of 11.5% Gel*

<i>Separating Gel</i>	
Acrylamide and bisacrylamide	- 11.50 ml
Separating gel buffer	- 6.00 ml
10% SDS	- 300.00 µl
TEMED	- 30.00 µl
Ammonium persulphate	- 100.00 µl
Double distilled water	- 12.07 ml
<i>Stacking Gel</i>	
Acrylamide and bisacrylamide	- 2.00 ml
Separating gel buffer	- 2.50 ml
10% SDS	- 100.00 µl
TEMED	- 10.00 µl
Ammonium persulphate	- 40.00 µl
Double distilled water	- 5.40 ml

showed some variations from that of the control. Of the 35 proteins resolved by SDS PAGE in the control, only a few showed marked changes in their expression on exposure to AFB<sub>1</sub> on the 7<sup>th</sup> day in the experimental groups. The general expression of all the serum proteins of molecular weights above 98 kDa was weak in all the test groups compared to the control. The low molecular weight proteins exhibiting variation in the test groups were 81, 72, 70, 54, 24, 23 and 16.5 kDa (Fig. 1).

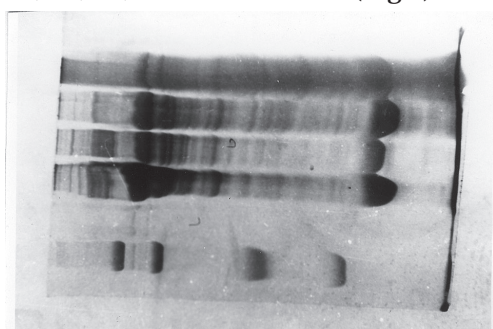


Fig 1. SDS PAGE analysis of serum proteins on 7<sup>th</sup> day

- Lane 1 - Molecular weight marker
- Lane 2 - Control
- Lane 3 - 1.5 mg AFB<sub>1</sub>
- Lane 4 - 5.0 mg AFB<sub>1</sub>
- Lane 5 - 15.0 mg AFB<sub>1</sub>

The moderate expression of the protein bands in the test groups I and II was maintained till the 21<sup>st</sup> day, while in test group III the protein expression became very strong by the 14<sup>th</sup> day and this level was maintained till the 21<sup>st</sup> day. On the 30<sup>th</sup> day of exposure, the expression of all the fractions became too weak to be detected in all the test groups.

### **Immune response**

The sera collected from both control and treated fishes which were immunized against rabbit erythrocytes were tested for antibodies by haemagglutination test using different serial dilutions of serum and rabbit RBC. The antibody titre of the

control group was 1:512, whereas in the fishes exposed to 1.5, 5.0 and 15.0 mg of AFB<sub>1</sub>/kg diet, the maximum titre at which agglutination took place was 1:64, 1:32 and 1:16, respectively (Fig. 2).

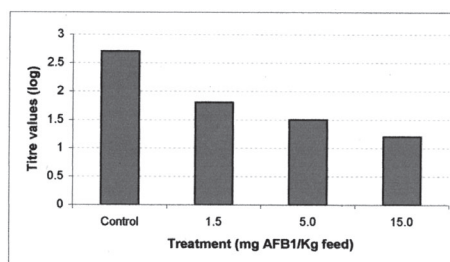


Fig. 2. Immune response of tilapia exposed to different concentrations of aflatoxin B<sub>1</sub> on the 30<sup>th</sup> day

### **Discussion**

Blood together with lymph is a homeostatically controlled internal environment and changes induced by carcinogens are expected to be initially manifested in this transporting medium. In the present study, the treated fishes exhibited dose-dependent changes in the normal serum protein profile on exposure to AFB<sub>1</sub> indicating their sensitivity to AFB<sub>1</sub>. The serum protein profiles of the test animals were similar to those of the controls except for the enhancement of expression. The proteins had an initial increase in their expression on the 7<sup>th</sup> day, which got further intensified by the 14<sup>th</sup> day and continued till the 21<sup>st</sup> day and thereafter declining by the 30<sup>th</sup> day of observation. Scarpelli (1969) reported elevated levels of serum proteins in electrophoretic analysis in rainbow trout with hepatocarcinoma where the composition of the serum was qualitatively similar to that of normal animals. In some cases, a three-fold increase over normal serum value was noted, which reflected in an increase of all the serum protein components. Snieszko (1961) made the first

observation that trout bearing liver tumours showed marked elevations of their serum proteins.

The changes in serum protein expression in the treated fishes may be due to DNA damage, which in turn, leads to changes in the expression of its products, *viz.*, proteins. Experimental results have demonstrated the covalent binding of aflatoxin to DNA, resulting in inhibitions of DNA replication and also inhibition of RNA synthesis thus, resulting in altered expression of the gene. Thus, the initial enhancement in the protein expression in the treated groups in comparison to the control may be due to the activation of the genes coding for these proteins under the influence of AFB<sub>1</sub>, while the depression in protein expression by the 30<sup>th</sup> day may probably be due to the genetic alteration progressing to the point of collapse of the mechanism of transcription of DNA and the subsequent translation into proteins.

Studies on the immunological aspects of AFB<sub>1</sub> have done much to exploit the special susceptibility of the very young and newborn members in contrast to the adults and aged members of the same species. In the present study, adult fishes exposed to aflatoxin when immunised with rabbit erythrocytes through intramuscular routes showed a decrease in agglutination titre as compared to control, indicating the immunosuppressive action of AFB<sub>1</sub>. The immunosuppressive effect of AFB<sub>1</sub> is corroborated by the serum protein changes also. Aflatoxin toxicity was reported to impair the systemic innate and acquired defence by suppressing peritoneal macrophage phagocytosis and splenic antibody production resulting in increased susceptibility to infection by the domesticated animals (Jakab, 1994). Similarly, references to AFB<sub>1</sub> initiated

reduction in blood parameters (haematocrit, haemoglobulins, RBC and WBC) and reduction in leukocytes and macrophages in splenic corpuscles were reported in channel catfish (Jantrarotai *et al.*, 1990).

The molecular weight of tetramers, H chain and L chain of the immunoglobulin IgM4 reported in teleost are 610-720 kDa and 24-26 kDa respectively (Roberts, 1978). The serum protein bands exhibiting variations in the present study, *viz.*, 81, 72 and 70 kDa are regarded as  $\alpha$ 1,  $\alpha$ 2 and  $\gamma$  protein fractions of the H chain and 24, 25 and 16.5 kDa are their corresponding L chains. The reduced expression of the serum protein fraction as well as the general reduction in the total serum proteins in the treated fishes suggest a decline in immunoglobulins leading to dose-dependent reduction in the antibody titre on exposure to AFB<sub>1</sub>.

The present study demonstrate that tilapia exposed to AFB<sub>1</sub> exhibit variation in the protein expression suggesting an alteration in the genes which encode them. Thus, it could be used as a rapid biological test for detecting carcinogenic potential of chemical compound. This approach has the advantage that fish from the same treatment group could be maintained to determine the long-term effects. However, the exact mechanism underlying the increase in expression remains to be established. The present study confirms the extreme sensitivity of tilapia to sublethal levels of dietary AFB<sub>1</sub>.

### Acknowledgements

We are thankful to the Director, Central Marine Fisheries Research Institute, Kochi, for providing facilities. One of the authors wishes to express thanks to the Indian Council of

Agricultural Research, New Delhi, for the award of a fellowship.

## References

- Ambrosius, H. and W. Schaker 1964. Contribution to the immunobiology of poikilothermic vertebrates. I. Immunological investigations on the common carp, *Cyprinus carpio*. *Zool. J. Physiol.*, **71**: 74-88.
- Baker, D. C. and R. A. Green 1987. Coagulation defects of aflatoxin intoxicated rabbits. *Vet. Pathol.*, **24** (1): 62- 70.
- Dafalla R., A. Yagi and A. Adam 1987. Experimental aflatoxicosis in hybrid type chicks, sequential changes in growth and serum constituents and histopathological changes. *Vet. Hu. Toxicol.*, **29**(3): 222-226.
- IARC, 1993. *Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Aflatoxins, Vol.56* International Agency for Cancer Research, Lyon, p. 245 - 396.
- Jakab, G. T. 1994. Respiratory aflatoxicosis: suppression of pulmonary and systemic host defences in rats and mice. *Toxicol. Appl. Pharmacol.*, **125** (2): 198- 205.
- Jantrarotai, W., R.T. Lovell and J.M. Grizzle 1990. Acute toxicity of AFB<sub>1</sub> to channel catfish. *J. Aquat. Anim. Health*, **2**: 237-247.
- Jauncey, K. And B. Ross 1982. *A Guide to Tilapia Feeds and Feeding*. Scotland Institute of Aquaculture, University of Stirling, 111 pp.
- Laemmli, U.K. 1970. Cleavage of structural protein during the assembling of the head of bacteriophage T4. *Nature*, **227**: 680-685.
- Roberts, R. J. 1978. *Fish Pathology*. Bailliere Tindall, London.
- Scarpelli, D. J. 1969. Comparative aspects of neoplasia in fish and other laboratory animals. In: *Fish in Research*, O. W. Neuhaus and J. E. Halver (Eds.). Academic Press, New York, p 45- 49.
- Singh, J., R. P. Tiwari, G. Singh, S. Singh and D. V. Vadhera 1987. Biochemical and immunological effects of aflatoxins in rabbits. *Toxicol.*, **35** (2-3): 225- 230.
- Snieszko, S. F. 1961. Hepatoma and visceral granuloma in trouts. *N. Y. Fish and Game J.*, **8**(2): 145-147
- Woerr, J. A., W. E. Huff and P. B. Hamilton 1987. Increased sensitivity to staphylococcal beta-haemolysin of erythrocytes from chicken during aflatoxicosis. *Poult. Sci.*, **66**(12): 1929-1933.