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Histopathological outcomes designating the toxicological aspects of Fumonisin B1 on Cultured Nile Tilapia, *Oreochromis niloticus*

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Abstract
Fumonisins are mycotoxins produced by *Fusarium verticillioides* and *F. proliferatum*. They occur worldwide and are found predominantly in maize and in maize-based animal feeds. A total of 100 Nile tilapia, *Oreochromis niloticus* were grouped and fed ration contaminated with different levels of Fumonisin B1 and observed for one week to determine the median lethal dose (LD_{50}). Another experiment was operated to determine the clinicopathological picture of *O. niloticus* when experimentally fed with ration containing 1/10 and ½ of the LD_{50} of Fumonisin B1 and then observed for another six weeks. The clinical signs postmortem (PM) lesions and mortalities were recorded daily. Kidney, gills, brain, liver and spleen were sampled and examined for any histopathological alterations. It was found that the LD_{50} of Fumonisin B1 was 0.6 ppm. Additionally, there were no any pathognomonic clinical signs. The results of histopathological sections were in the form of hepatotoxic and nephrotoxic alterations, hyperplasia of gill filaments, ischemic neuronal injury and demyelination of the brain and depletion of the lymphoid elements and melanomacrophage centers of spleen.

Keywords: Fumonisin B1 - Histopathological alterations - *Oreochromis niloticus* - LD_{50}

Introduction
Mycotoxins are secondary fungal metabolites associated with severe toxic effects to vertebrates. They are produced by many phytopathogenic and food spoilage fungi including the *Aspergillus, Penicillium, Fusarium*, and *Alternaria* species. Food and feed contamination with mycotoxins is a worldwide problem. Beside their toxic effects on other organ systems, mycotoxins are neurotoxins that can produce a wide spectrum of behavioral and cognitive changes, ataxia, and convulsions.

Fumonisins are produced by *Fusarium verticillioides* (formerly = *F. moniliforme*), *F. proliferatum*, and other *Fusarium* species [1, 2, 3]**.** The most frequent and potent among them is fumonisin B1 (FB1). Fumonisin B1 which, is produced by the genus Fusarium, is the best known and most toxic of the Fusarium toxins [4]. FB1 frequently occurs with FB2 and they are mainly produced by *F. verticillioides* and *F. proliferatum*, which occur predominately in maize. The toxicological and pathological effects of fumonisins have been extensively studied in laboratory animals; the liver and kidney are the major target organs but species-, strain-, and sex-dependent differences in dose–response occur [5].

FB1 can also have adverse effects on the central nervous system in carp (*Cyprinus carpio*). FB1 is a hydrophilic molecule with low molecular weight (500 Da), which can pass through the blood brain barrier (BBB) of young individuals. The BBB is a structure that allows selective entry of oxygen and glucose into the brain and spinal cord while preventing the entry of a spectrum of large, potentially toxic molecules. Once FB1 reaches the brain, it produces edemas and cell degeneration [6]**.**

Further, fumonisins also have immunosuppressive effects in many species as it was found that FB1 reduces the amount of macrophages, inhibiting immunological function activity against pathogens and diminishing levels of antibodies. FB1 can therefore lead to increased vulnerability to infectious diseases [7]**.**

In all animal species analyzed, FB1 has been proven to be both hepatotoxic and nephrotoxic [8, 9]. Therefore, our study was focused mainly to evaluate the histopathological findings due to Toxicological aspects of FB1 in cultured *O. niloticus*.

2. Materials and Methods

2.1. Fish and experimental conditions:-

A total number of 100 apparently healthy *O. niloticus*, with average body weight of (40 ± 5 g /fish) were obtained from a private fish farm at Kafre-Elsheikh governorate and transported a live to the laboratory of the department of poultry and fish diseases, Faculty of veterinary medicine, Alexandria University in large plastic bags containing water enriched by oxygen (2/3). All fish were placed in aquaria and left acclimated for 2 weeks prior to the experiments. Experiments were conducted in prepared glass aquaria (90 x 50 x 35 Cm), supplied with chlorine free tap water [10]. The continuous aeration was maintained in each aquarium using an electric air pumping compressors. Settled fish wastes were cleaned daily by siphoned with three quarters of the aquarium's water, which was replaced by aerated water from the water storage tank. Water temperature was kept at 22± 1 °C and pH 8.5 during the experimental period. During this period, fish were hand-fed commercial feed, *ad libitum*, twice a day.

2. 2. Fumonisin B1

Fumonisins were biosynthesized in a liquid culture medium (yeast extract 20 g; sucrose 40 g; 1000 mL sterile water, pH 7.4) inoculated with a *F. verticillioides* isolate from a corn sample from faculty of agriculture, Alexandria university, Egypt.

One-millilitre suspension of conidia was inoculated into 1000 mL of the medium and incubated at 25 °C for 15 days. All chemicals used for the extraction of FB1 were purchased from Lab service (Egypt). The procedure of extraction was repeated to collect the necessary amount of FB1. A stock solution of FB1 was prepared in distilled water (5 mg mL-1) and kept refrigerated at 4 °C. This stock solution was further diluted with distilled water for uniform admixing to the experimental feed.

2. 3. Feed

Pellets were prepared weekly. Equal parts of crushed commercial pellets for *O. niloticus* (protein content 25%), wheat flour and an adequate amount of distilled water (with or without FB1) were mixed to obtain the dough. A household machine was modified to form pellets from the dough. After drying, the feed was stored in a refrigerator.

2.4. Experimental design

Experiment (I):- Determination of median lethal dose (LD_{50})

Seventy of the acclimated *O. niloticus* were subdivided into seven groups each of 10 fish and were fed to diets containing different levels of FB1 as followings; 0.20, 0.30, 0.50, 0.60, 0.80 and 1.00 mg / Kg feed respectively. The last group was fed a diet contain zero FB1 and used as a control group. The fish groups were closely observed for one week and mortalities were recorded daily.

Table 1: Design for determination of median lethal dose (LD50) of FB1

Group number	Dose of FB1 (mg / kg feed)	Number of fish
I	0.20	10
II	0.30	10
III	0.50	10
IV	0.60	10
V	0.80	10
VI	1.00	10
VII	Basal diet (Control group)	10

The LD_{50} was determined [24] using the following formula:

$$LD_{50} = \frac{\text{Mortalities above 50\% - 50}}{\text{Mortalities above 50\% - Mortalities below 50\%}}$$

Experiment (II)

Thirty of the acclimated fish were subdivided into 3 groups each of 10 fish and each group was reared in a separate aquarium. The fish of the first group were fed a diet containing 1/10 of the LD_{50} of FB1; the second group was fed a diet containing 1/2 of the LD_{50} of FB1, while the last one fed on basal diet. Fish groups were observed for six weeks, the clinical signs and mortalities were recorded daily and the dead fish were taken for any post mortem examination and histopathological changes.

2.5. Histopathological studies

Following complete necropsy of the freshly dead fish, specimens were collected from kidney, liver, brain, gills and spleen for histopathological examination. Thereafter, these specimens were rapidly fixed in 10% natural formalin buffered phosphate for at least 24 hours, after that the specimens washed by running tap water then dehydrated through ascending grads of ethanol, cleaning in by chloroform and embedded in paraffin wax at 60 C. Paraffin block were prepared and from which 5 microns thick sections were obtained by microtome. These sections were stained by Haematoxylin and eosin stain (H & E) **[11]**.

Results

3. 1. Results of the median lethal dose (LD_{50}):-

The LD_{50} of FB1 for tested fish was (0.60 mg / Kg feed) (Table 2).

Table 2: Design for determination of median lethal dose (LD_{50}) of FB1

Group No.	Dose of FB1 (mg / kg feed)	No. of fish	Total No. of dead fish	Mortality %
I	0.20	10	0	0
II	0.30	10	2	20
III	0.50	10	3	30
IV	0.60	10	5	50
V	0.80	10	6	60
VI	1.00	10	8	80
VII	Basal diet (Control group)	10	0	0

The dead fish have no any pathognomonic clinical signs.

3. 2. Histopathological findings of *O. niloticus*

3. 2. a. Liver damage

Normally, the liver of Nile tilapia was consisted of round polygonal hepatocytes which arranged in cord-like structures that are limited at one side by the hepatic capillaries or sinusoids. Central to each cord are thin bile canaliculi adjacent to the hepatocytes. Tilapia liver showed also presence of the pancreatic portion (hepatopancreas) which completely surrounding the afferent vein (Fig. 1).

Generally, fumonisin showed mainly hepatotoxic and nephrotoxic effects in a time dependent manner. The lesions were varied form degeneration to necrosis. Livers of fish subjected to FB1 toxicity for one week of exposure showed

mainly congestion of hepatic blood sinusoids, vacuolation of hepatocytes and abnormal fatty degeneration (Fig. 2).
On the 2nd week, liver showed marked abnormal vacuolation of lipid-type (Fig. 3). Similar lesions were noticed in 3rd week in addition perivascular hepatic degeneration (Fig. 4). Degenerative changes of pancreatic portion associated with loss of zymogen granules were markedly seen (Fig. 5). Also, perivascular necrosis was increased within the hepatic tissue at the 4th week (Fig. 6). After the 5th week of FB1 exposure, the liver showed interesting multi-focal eosinophilic degeneration consisted from degenerated and apoptotic hepatocytes (Fig. 7). After the 6th week of exposure of FB1, the liver showed diffuse hepatocytes degeneration with focal hepatocytes necrosis (Fig. 8).

3. 2. b. Kidney damage
Regarding to the kidney, the posterior kidney of fish in control group showed normal renal tubules and glomeruli (Fig. 9). Kidneys of fish exposed to fumonisin showed similar degeneration and necrosis lesions that shown in liver. On the 1st week, the kidney showed vacuolation of renal tubular epithelium (Fig. 10). Focal renal tubular degeneration in addition to interstitial hemorrhage was observed on the 2nd week (Fig. 11). After three weeks of fumonisin toxicity, there was renal tubular degeneration and interstitial infiltration of inflammatory cells (Fig. 12). On the 4th week, degeneration of renal tubular epithelium and with presence of many apoptotic bodies and diffuses interstitial leucocytic infiltration in the renal tissue (Fig. 13). The 5th week group showed, there was feature of interstitial nephritis with marked leucocytic infiltration in 5th week (Fig. 14). The 6th week group showed severe degree of tubular degeneration and interstitial inflammatory cells infiltration (Fig. 15).

3. 2. c. Spleen lesions
Spleen of fish in control groups showed normal red and white pulp (Fig. 16). Fish exposed for one week with FB1 were similar to the normal one (Fig. 17). Starting from the 2nd week, the spleen showed marked congestion of splenic sinusoids of red pulp with decrease in lymphoid elements and melanomacrophage centers (Fig. 18). The 3rd and 4th demonstrated lymphoid depletion (Fig. 19 & 20). Interestingly, the degree of lymphoid depletion was correlated with time of fumonisin exposure especially in the 5th and 6th week of administration which showed marked necrosis in lymphocytes (Fig. 21 & 22).

3. 2. d. Gill lesions
Gills of fish in control group showed normal gill filaments and lamellae with normal epithelial lining. After the 1st week of exposure to FB1, the gills showed a slight focal proliferation interlamellar cells and with occasional some epithelial lifting (Fig. 23). After two weeks of the FB1 exposure, the gills showed hyperplasia of interlamellar epithelium and lamellar epithelial cell lifting (Fig. 24). The 3rd week, the gills showed increased the interlamellar hyperplasia with focal fusion of the secondary filaments (Fig. 25). On the 4th week, there was extensive fusion of gill lamellae with hyperplasia of mucous cells (Fig. 26). The 5th and 6th week exposure to fumonisin, the gills showed severe loss of secondary gill lamellae with marked epithelial hyperplasia (Fig. 27 & 28) respectively.

3. 2. e. Brain lesions
Interestingly, the brain of *O. niloticus* after 6th week of FB1 exposure showed ischemic neuronal injury and demyelination (Fig. 29).

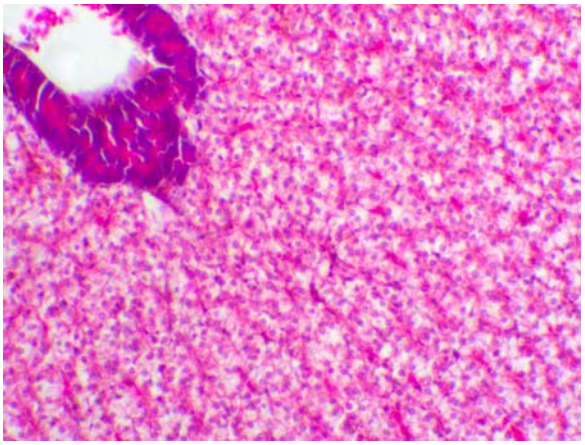

Fig 1: Liver of *O. niloticus* (Control group) showed normal hepatocytes and hepatopancreas, H & E (X 200).

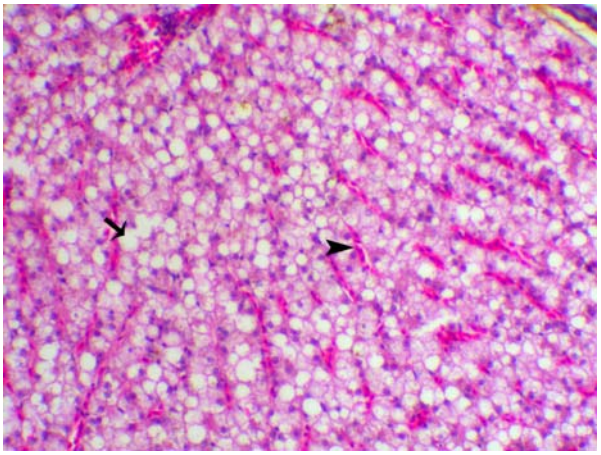

Fig 2: Liver of *O. niloticus* after one week of FB1 exposure showed congestion of hepatic sinusoids (arrowhead) and abnormal fatty degeneration (arrow), H & E (X 200).

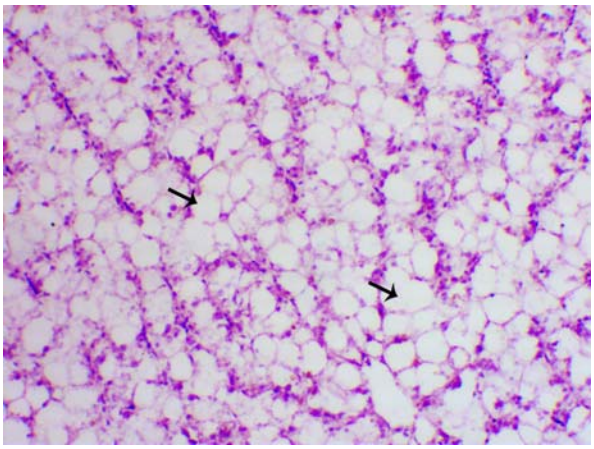

Fig 3: Liver of *O. niloticus* after 2nd week of FB1 exposure showed marked degree of hepatocytes vacuolation consistent with fatty change (arrow), H & E (X 200).

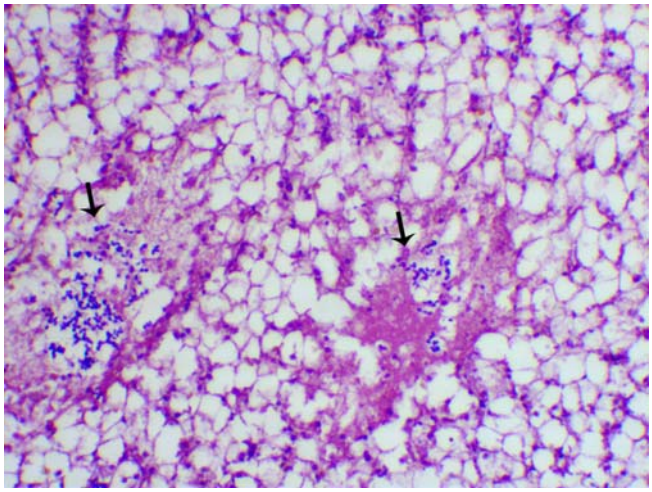

Fig 4: Liver of *O. niloticus* after 3rd week of FB1 exposure showed marked hepatocytes and centrolobular hepatic degeneration (arrow), H & E (X 200).

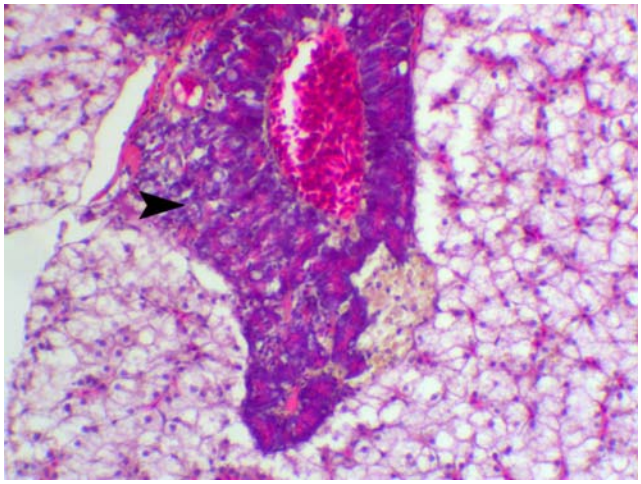

Fig 5: Liver of *O. niloticus* after 3rd week of FB1 exposure showed degeneration of pancreatic portion (arrow), H & E (X 200).

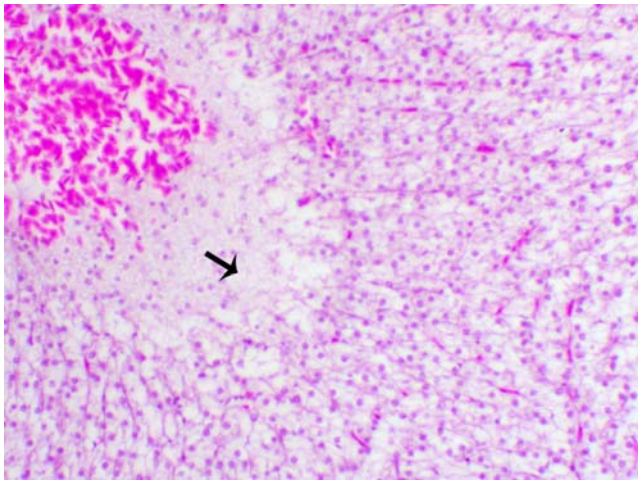

Fig 6: Liver of *O. niloticus* after 4th week of FB1 exposure showed marked degree of perivascular hepatic necrosis (arrow), H & E (X 200).

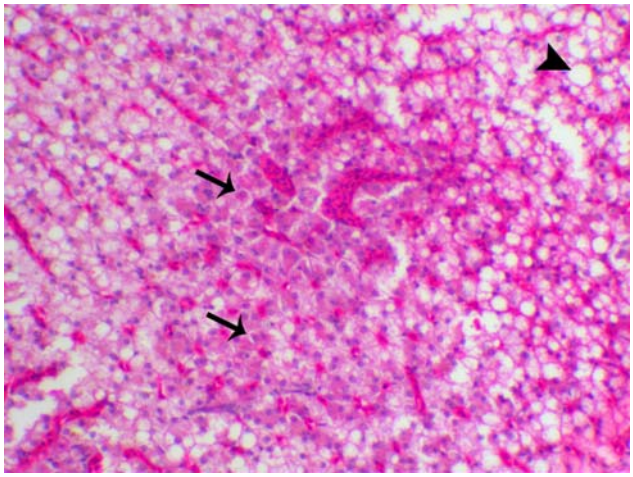

Fig 7: Liver of *O. niloticus* after 5th week of FB1 exposure showed vacuolation of hepatocytes (arrowhead) in addition to eosinophilic foci of hepatocytes degeneration with presence of many apoptotic bodies, (arrow), H & E (X 200).

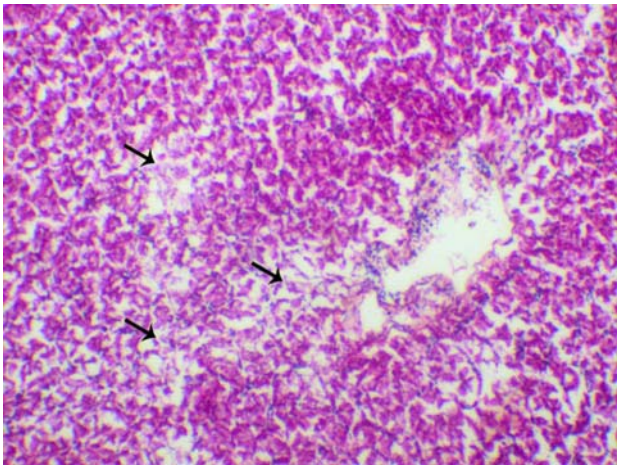

Fig 8: Liver of *O. niloticus* after 6th week of FB1 exposure showed vacuolation of hepatocytes (arrowhead) in addition to eosinophilic foci of hepatocytes degeneration with presence of many apoptotic bodies (arrows), H & E (X 200).

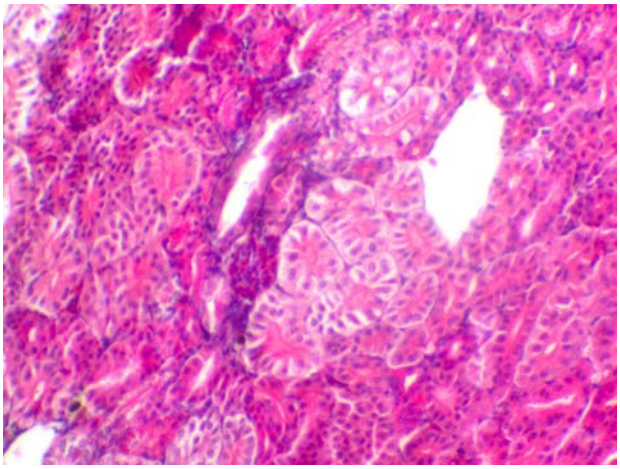

Fig 9: Kidney of *O. niloticus* (Control group) showed normal renal tubules, H & E (X 200).

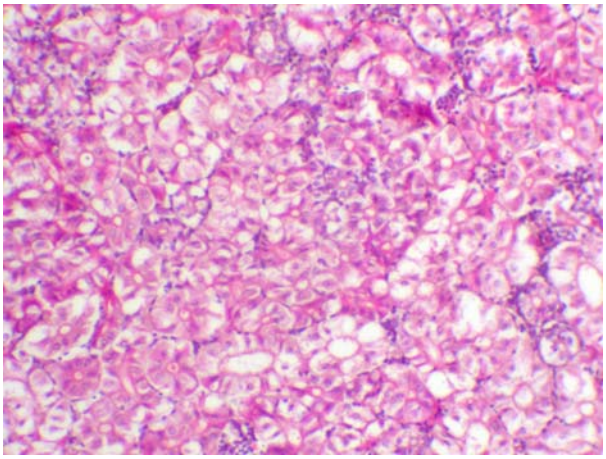

Fig 10: Kidney of *O. niloticus* after 1st week of FB1 exposure showed vacuolation of renal tubular epithelium, H & E (X 200).

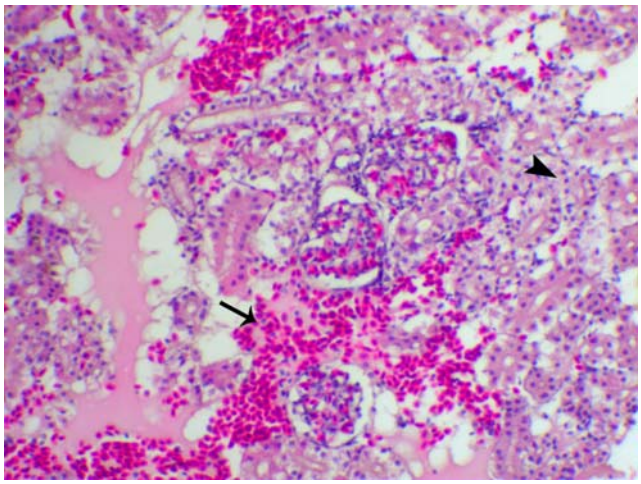

Fig 11: Kidney of *O. niloticus* after 2nd week of FB1 exposure showed interstitial hemorrhage (arrow) and focal tubular degeneration, H & E (X 200).

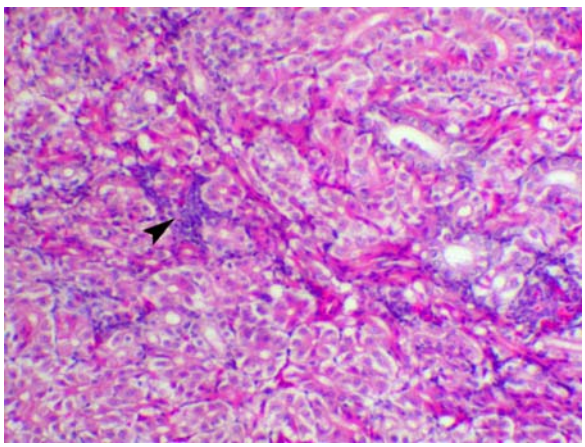

Fig 12: Kidney of *O. niloticus* after 3rd week of FB1 exposure showed focal degeneration of renal tubules with interstitial leucocytic infiltration (arrowhead), H & E (X 200).

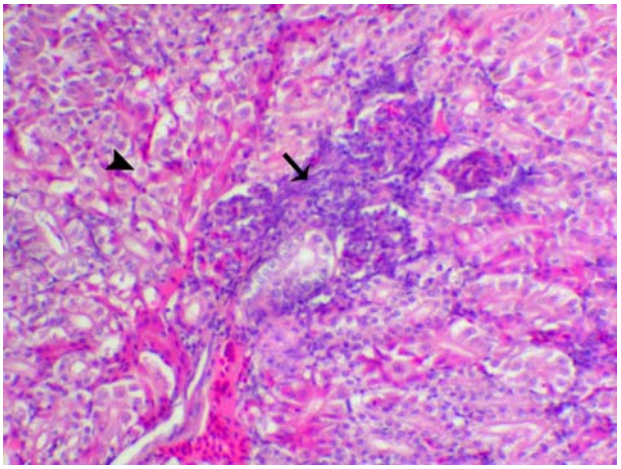

Fig 13: Kidney of *O. niloticus* after 4th week of FB1 exposure showed degeneration, apoptosis (arrowhead) of renal tubules and marked interstitial leucocytic infiltration (arrow), H & E (X 200).

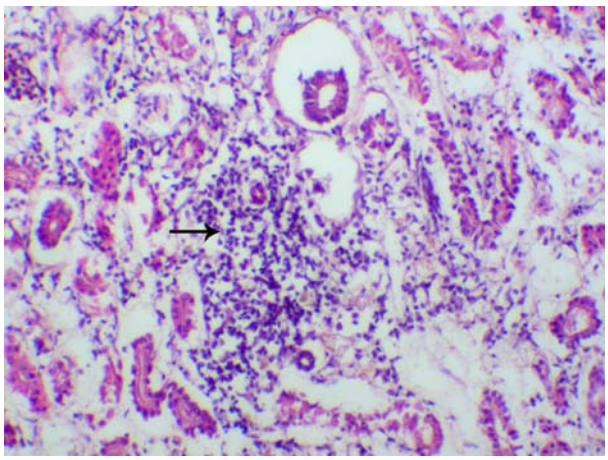

Fig 14: Kidney of *O. niloticus* after 5th week of FB1 exposure showed tubular degeneration and interstitial leucocytic infiltration (arrow), H & E (X200).

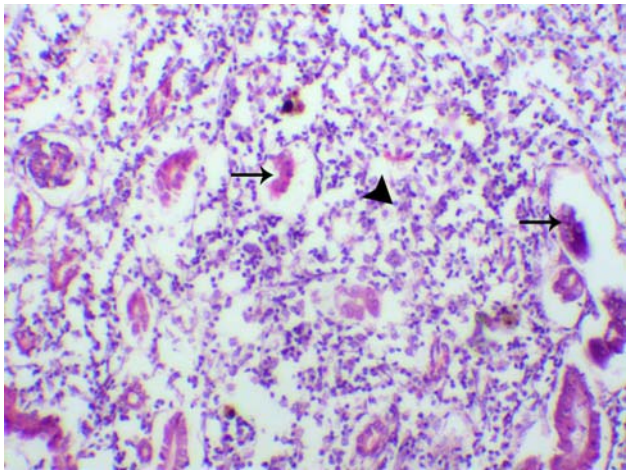

Fig 15: Kidney of *O. niloticus* after 6th week of FB1 exposure showed marked degree of tubular degeneration (arrow) and diffuse interstitial leucocytic infiltration as well, H & E (X 200).

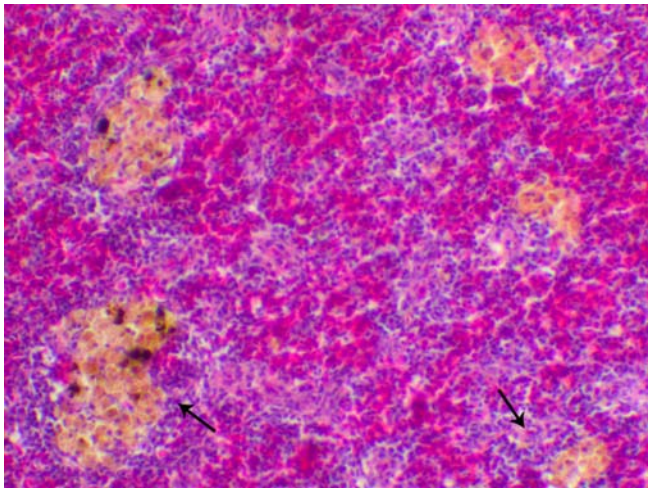

Fig 16: Spleen of *O. niloticus* (Control group) showed normal melanomacrophage centers, lymphoid elements (arrow) and blood sinusoids, H & E (X 200).

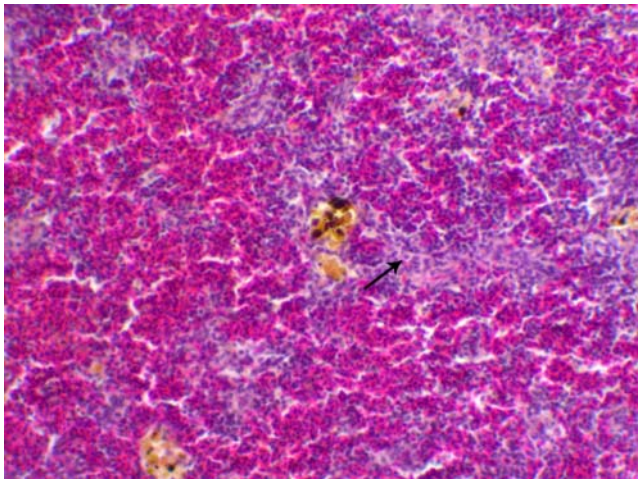

Fig 17: Spleen of *O. niloticus* after 1st week of FB1 exposure showed melanomacrophage centers and lymphoid elements (arrow) were within the normal limits, H & E (X 200).

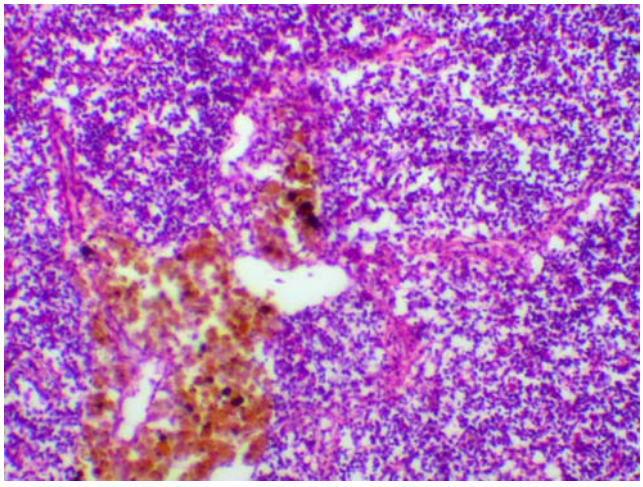

Fig 18: Spleen of *O. niloticus* after 2nd week of FB1 exposure showed mild degree of lymphoid depletion, H & E (X 200).

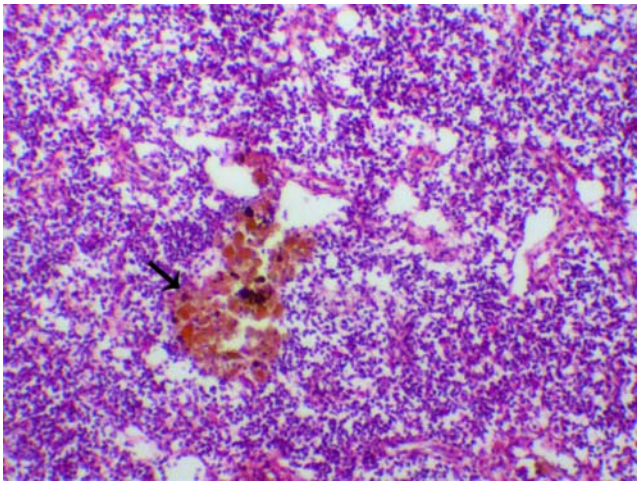

Fig 19: Spleen of *O. niloticus* after 3rd week of FB1 exposure showed mild to moderate degree of lymphoid depletion, H & E (X 200).

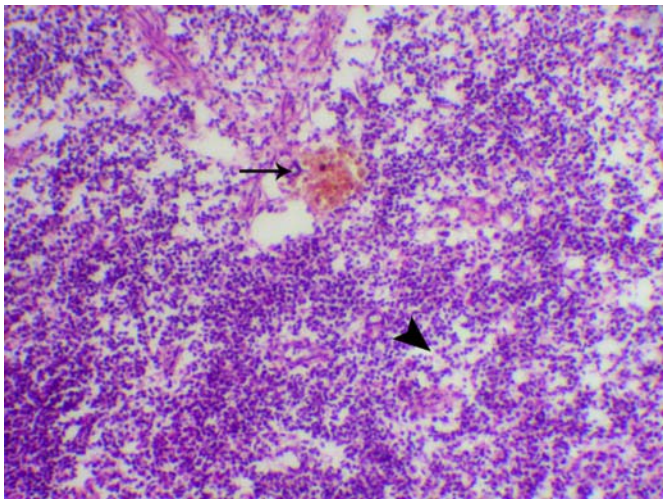

Fig 20: Spleen of *O. niloticus* after 4th week of FB1 exposure showed moderate degree of lymphoid depletion (arrowhead), H & E (X 200).

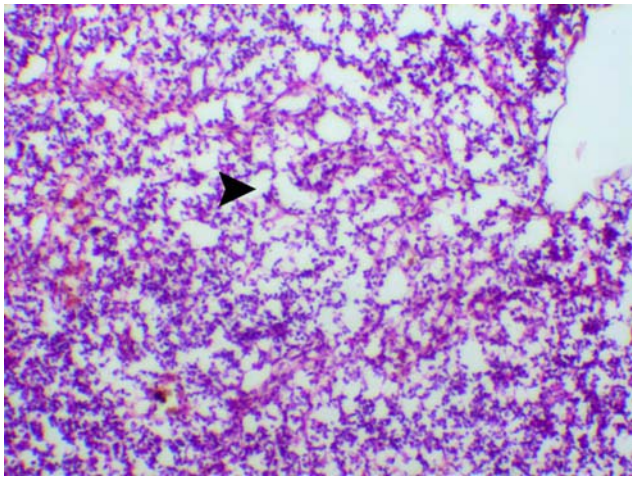

Fig 21: Spleen of *O. niloticus* after 5th week of FB1 exposure showed marked degree of lymphoid depletion (arrowhead), H & E (X 200).

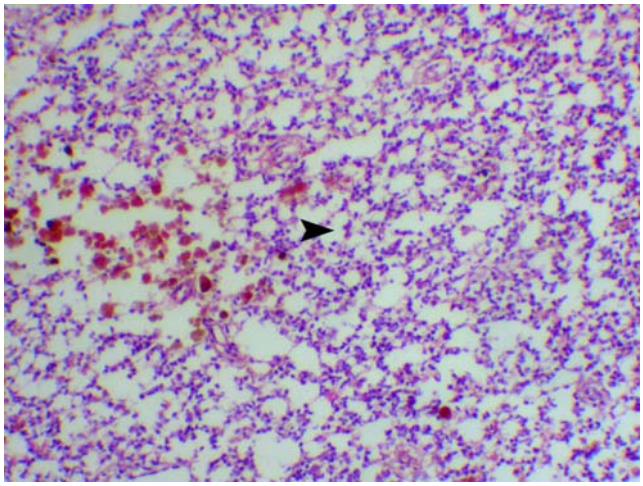

Fig 22: Spleen of *O. niloticus* after 6th week of FB1 exposure showed severe degree of lymphoid depletion (arrowhead), H & E (X200).

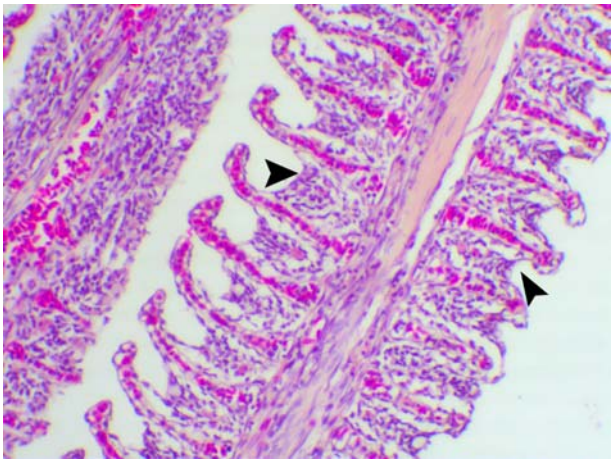

Fig 25: Gills of *O. niloticus* after 3rd week of FB1 exposure showed progressive increase of interlamellar hyperplasia (arrowhead), H & E (X200).

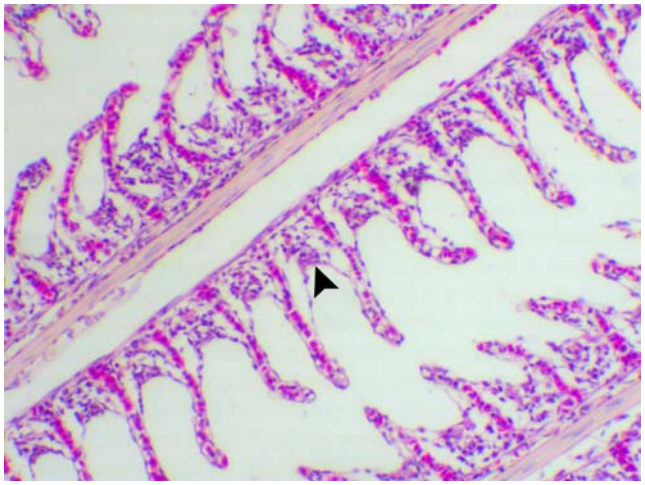

Fig 23: Gills of *O. niloticus* after 1st week of FB1 exposure showed mild interlamellar hyperplasia and epithelial lifting (arrowhead), H & E (X 200).

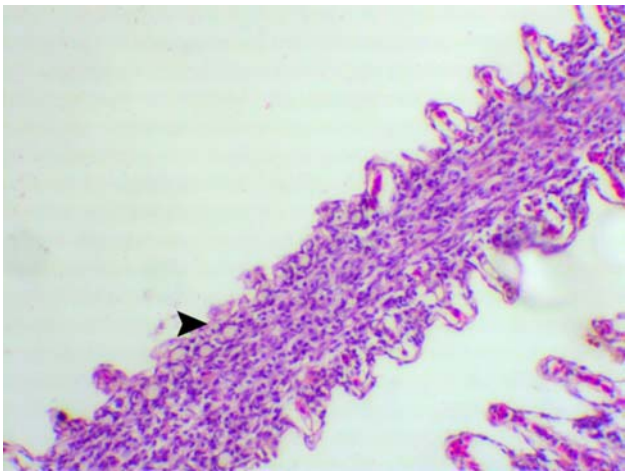

Fig 26: Gills of *O. niloticus* after 4th week of FB1 exposure showed loss of secondary lamellae with hyperplasia of mucous cells (arrowhead), H & E (X200).

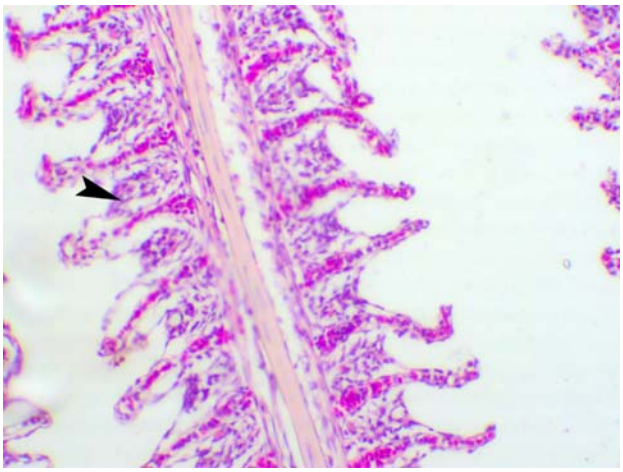

Fig 24: Gills of *O. niloticus* after 2nd week of FB1 exposure showed increase of interlamellar hyperplasia and epithelial lifting (arrowhead), H & E (X 200).

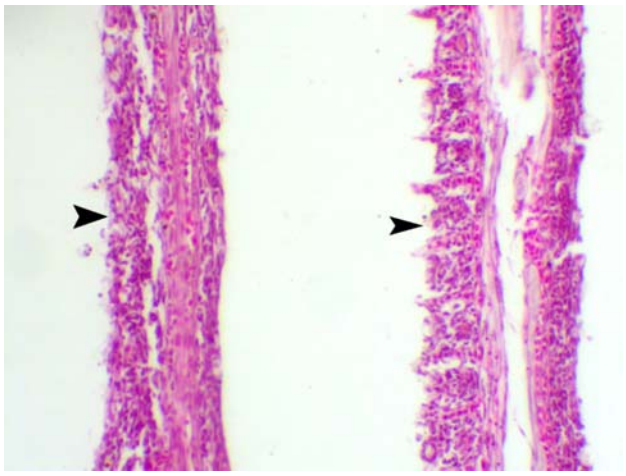

Fig 27: Gills of *O. niloticus* after 5th week of FB1 exposure showed severe degree of interlamellar epithelial hyperplasia with complete fusion of the secondary lamellae (arrowhead), H & E (X 200).

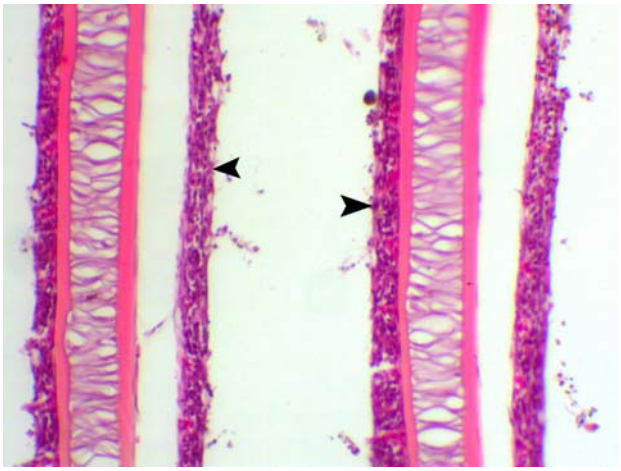

Fig 28: Gills of *O. niloticus* after 6th week of FB1 exposure showed severe complete loss of secondary lamellae (arrowhead), H & E (X 200).

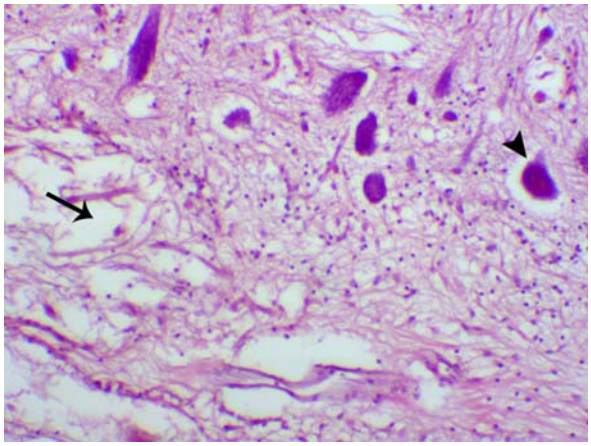

Fig 29: Brain of *O. niloticus* after 6th week of FB1 exposure showed marked ischemic neuronal injury (arrow head) and severe degree of nerve fiber demyelination (arrow), H & E (X 200).

Discussion

This experiment examined the effects of a mycotoxin, fumonisin B1, which affects the health and survival of many fish species in aquaculture [4]. FB1 is a toxin produced by mold of the genus *Fusarium* that is found primarily in maize based feeds.

Depending on animal species, FB1 can cause neurotoxicity, hepatotoxicity, nephrotoxicity, immunosuppression, developmental abnormalities, liver tumors, and other disorders [5].

The toxicological and pathological effects of Fumonisins have been extensively studied in laboratory animals. Comprehensive reviews of the subject are available [12, 13, 14, 15] and therefore only the main points are summarized here. The liver and kidney are the major target organs but species-, strain-, and sex-dependent differences in dose–response occur. For example, the no observed effect and lowest observed effect levels for kidney are lower than those for liver in Sprague–Dawley [5]. And Fischer 344 [16, 17, 18] rats and males are more sensitive than females in regard to nephrotoxicity. This is not the case however for the BD IX rat, in which the liver is the only major target organ [19]. Rabbits are very sensitive to the renal effects of FB1 [20]**.** While mice are less sensitive to nephrotoxicity than Sprague–Dawley or Fischer 344 rats [18]. In both liver and kidney, apoptosis alone or together with increased mitosis is an early microscopic indication of tissue injury [16 21]. Cytomegaly, anisocytosis, anisokaryosis, cytoplasmic vacuolation (hepatocellular) and necrosis become evident in both tissues with increasing dose. Biliary epithelial or oval cell proliferation, foci of cellular alteration, and fibrosis have also been observed in more advanced liver lesions. Kidney lesions are at first confined to the proximal tubules of the outer medulla (in some reports designated as the corticomedullary junction) [12, 14, 16, 21] and characterized by detachment and sloughing of the apoptotic epithelial cells (small, round, with pyknotic nuclei) into the lumen. This is accompanied by an increase in the number of mitoses in the tubular epithelium and, as the severity of injury and regeneration increase, the epithelium displays cytoplasmic basophilia and is more cuboidal. Foci of tubular hyperplasia may also be present. Severely damaged kidneys exhibit progressively more apoptosis and sloughing, tubular atrophy, focal tubular hyperplasia, interstitial fibrosis, inflammation and, in the most severe cases, overt tubular necrosis.

FB1 disrupts sphingolipid metabolism. Sphingolipids are essential components of cellular lipoprotein membranes and they are involved in specific functions such as cell regulation, recognition and signaling. Fumonisins inhibit the enzyme sphinganine Nacyltransferase (ceramide synthase) activity, which catalyzes sphingolipid production. Free sphingoid bases (sphinganine and sphingosine) are toxic and persist in kidney and liver because the bases persist longer than the toxin itself, inducing cell death and leading to the accumulation of sphinganine. Sphinganine concentration is positively correlated with kidney and liver damage [22, 23]

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