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A multiparameter investigation into adverse effects of aflatoxin on *Oreochromis niloticus* health status

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Abstract Aflatoxin is a common contaminant of foods, particularly in the staple diets of many developing countries. To evaluate adverse effects of aflatoxin B1 (AFB1) toxicity on health status in the Nile tilapia *Oreochromis niloticus*, fish were fed diet contaminated with either 20 or 100 ppb AFB1 for 6 or 12 weeks. Growth indices, survival rate and hepatosomatic index (HSI) were assessed. Blood samples were collected for hematological profiles (e.g. RBCs and WBC count, Hb content). Liver enzyme activity; aspartate aminotransferase (AST), alanine aminotransferase (ALT) as well as alkaline phosphatase (ALP), were evaluated and toxin residues in the liver and musculature were detected. Liver histopathological investigations were carried out, whereas antioxidant glutathione peroxidase (GPx) and glutathione S-transferase (GST) gene expression were determined in this tissue by semi-quantitative RT-PCR. Furthermore, to test the fish immune status, challenge against *Aeromonas hydrophila* was conducted. Results indicated that 100 ppb AFB1 negatively impacted *O. niloticus* weight gain, feed efficiency, hematological profiles, HSI as well as liver histopathology, while increase in AST, ALT, ALP liver enzymes activity was evidenced. Further, the expression of liver *GPx* and *GST* down-regulated and AFB1 residues were always detected in the liver and only in the musculature in fish fed 100 ppb AFB1 for 12 weeks. The ability of fish to withstand *A. hydrophila* infection was remarkably lowered. Overall, the results herein demonstrate the toxic effects of AFB1 in *O. niloticus*. The observed alterations in fish status, especially in the liver coincide well with the expected oxidative stress resulting from the AFB1 toxicity.

Introduction

Aquaculture has sustained a global growth that continues to grow, and is expected to increasingly fill the shortfall in aquatic food products resulting from static or declining capture fisheries and population increase well into the year 2025 (De Silva, 2001). By 2030, aquaculture will provide close to two thirds of global food fish consumption as catches from wild capture fisheries level off and demand from an emerging global middle class substantially increases (The World Bank, 2014). Tilapia, once mooted as the "aquatic chicken" of the 1980s and the "poor man's fish" (Smith and Pullin, 1984),

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has established a highly pronounced position in aquaculture worldwide. It has been considered as the most important aquaculture species of the 21st century (Fitzsimmons, 2013). In Egypt, the Nile tilapia (*Oreochromis niloticus*) is one of the most commonly raised farmed fish. It represents a reliable model for mycotoxicosis, since this fish is extremely vulnerable to toxic insult from various chemicals and poisons (Kenawy et al., 2009).

Aflatoxins are toxic by-products of mold growth on certain agricultural commodities, which represent a health concern for populations that cannot properly store these commodities to limit mold growth or who have limited access to a wide variety of other foods (Roebuck, 2004). Factors that increase the production of aflatoxins in feeds include environmental temperatures above 27 °C (80 °F), humidity levels greater than 62%, and moisture levels in the feed above 14%. Contamination with aflatoxin is a common problem of aquaculture worldwide, causing disease with high mortality and a gradual decline of reared fish stock quality, especially in developing countries, as incorporation of plant-based ingredients into fish feed increases the risk of exposure (Santacroce et al., 2008; Selim et al., 2013; Zychowski et al., 2013). Among four major types of aflatoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* (Kurtzman et al., 1987; Kosalec and Pepeljnjak, 2005); B1, B2, G1, and G2, aflatoxin B1 (AFB1) is the most toxic to humans as well as animals including nonhuman primates, birds, fish and rodents (Yu, 2012). It exerts mutagenic, carcinogenic, teratogenic and cytotoxic actions (Bbosa et al., 2013).

Aflatoxin toxicity may ensue through the generation of intracellular reactive oxygen species (ROS) like superoxide anion, hydroxyl radical and hydrogen peroxide during the metabolic processing of AFB1 by cytochrome P450. High concentrations of ROSs lead to oxidative stress which can cause cellular damage (Halliwell and Gutteridge, 1999). This stress can be counteracted by enzymatic and non-enzymatic antioxidant systems. Among enzymatic systems, glutathione S-transferase (GST) and glutathione peroxidase (GPx) constitute essential components of cellular detoxification systems that defend cells against ROSs (Ren et al., 2009), hence play a major protective mechanism against oxidative stress (Almar et al., 1998). Both *GST* and *GPx* mRNA expressions have been used as biomarkers of exposure to environmental pollution/toxicity, as the mRNA levels represent a snapshot of the cell activity at a given time; and in many instances, single gene mRNA expressions can be useful biomarkers of stress in organisms (Fisher et al., 2006; Espinoza et al., 2012; Rios et al., 2014; Zheng et al., 2014). Furthermore, AFB1 chronic exposure may result in malnutrition, poor growth, suppressed immune response, physiological disorders as well as histological changes (Allameh et al., 2005; Lewis et al., 2005; Selim et al., 2013; Zychowski et al., 2013).

Because AFB1 damaging effects are largely species and dose-specific (Jiang et al., 2005; Zychowski et al., 2013), the current study was undertaken to investigate the effects of aflatoxin contamination in feeds on growth indices, blood components, immune function, liver histopathology as well as gene expression of *GST* and *GPx* oxidative stress biomarkers in *O. niloticus*. Furthermore, the AFB1 residues in the fish liver and muscle were also examined.

Materials and methods

AFB1 production and determination

AFB1 was produced through pellet fermentation using *A. parasiticus* NRRL 2999 according to the method described by Abdelhamid and Mahmoud (1996). The quantitative determination of AFB1 in ration and in fish tissues (liver and musculature) was performed by quantitative thin layer chromatography (TLC) according to the method of Eppley (1968).

Experimental fish and procedure

A total number of 98 apparently healthy *O. niloticus* were obtained from private fish farms at El-Riad, Kafr El-Sheikh Governorate; weighing 35 ± 0.50 g. Fish were acclimated to laboratory conditions in fiberglass tanks containing dechlorinated tap water (24 ± 2 °C and pH 7.2–8.2) for 15 days. Each tank was continuously supplied with compressed air from an electric compressor. Dechlorinated tap water was used to change one third of the water in each aquarium every day to avoid metabolite accumulations (static system). Feeding was done once daily using a basal diet (30% protein ration) at 3% of the fish body weight. Following acclimation, fish were randomly distributed into glass aquarium (50 × 40 × 40 cm) containing about 60 L of dechlorinated water and conditions were maintained as above. The fish were randomly divided into two groups, 49 fish each. As AFB1 cannot be eliminated completely from animal feed or human food supply (Abdelhamid et al., 1999; Roebuck, 2004), group 1 was fed basal diet contaminated with 20 ppb (μg/kg) AFB1 that mimics the safe level of toxin exposure (Lovell, 1992, 2001). This level, served as a control, agrees with that allowed to be used for feeding dairy and immature animals, including fish, by the national feed legislation in the USA (Lovell, 1992, 2001) and with the assigned permissible level by FAO (2004); which ranged from 0 to 50 ppb with an average of 20 ppb. Meanwhile, group 2 was fed the basal diet contaminated with a higher AFB1 concentration (100 ppb), which corresponds to the lowest common contamination level existing in animal feed in developing countries (Unnevehr and Grace, 2013). Each group was subsequently subdivided into 2 subgroups, the first (comprising 18 fish) lasts for 6 weeks and the second (with 31 fish) lasts for 12 weeks of exposure.

Challenge infection test

Investigation concerning the challenge infection test was performed to study the cumulative impact of AFB1. By the end of the AFB1 exposure duration (12 weeks), 10 fish from each group were injected intraperitoneally (IP) with the pathogenic *Aeromonas hydrophila* (0.3 ml of 10^8 CFU/ml) according to Schaperclaus et al. (1992). The bacterial strain was kindly obtained from the Fish Diseases Department, Animal Health Research Institute, Kafr El-Sheikh Branch. Further, pure saline solution (0.65%) was injected in a similar fashion, in 3 fish from each of the AFB1 groups, for negative control injection (Boijink et al., 2001). The injected fish were kept under observation for 14 days to record the mortality rate as follows:

Mortality rate % = no. of death in specific period/ total population during that period × 100.

Growth indices

Growth calculations and survival rate were estimated following either the 1st 6 weeks or the 2nd 6 weeks (12 weeks collectively) of exposure to AFB1. All calculations were performed for each fish individually. Growth indices were assessed in terms of total weight gain (TWG), average daily gain (ADG), relative growth rate (RGR), feed conversion ratio (FCR) and survival rate (SR) according to the following:

TWG = final body weight (g) – initial body weight (g) (Castell and Tiews, 1980).
ADG = final body weight (g) – initial body weight (g)/number of days in the feeding experimental period (Castell and Tiews, 1980).
RGR % = 100 × (final weight – initial weight)/initial weight.
FCR = dry feed fed (g)/[final body mass (g) – initial body mass (g)] (Tacon, 1987).
SR % = Final fish number/initial fish number × 100.

Hematological and biochemical analyses

Investigations regarding hematological and biochemical parameters were performed to study the cumulative impact of AFB1. After animals were anesthetized with benzocaine (3%), blood was drawn by caudal puncture, after 6 and 12 weeks, into heparinized tubes for plasma analyses. Blood specimens were assayed for: number of red blood cells (RBCs) and white blood cells (WBCs) by hemocytometer (Neubauer improved, Precicolor HBG, Germany) (Stoskoph, 1993); hematocrit or packed cell volume (PCV) by the micro-hematocrit technique; and hemoglobin level (Hb) by the cyanomethemoglobin method (Drabkin, 1964). For WBC differential count, blood films were prepared according to the method described by Lucky (1977). The following RBC indices were calculated: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) according to Dacie and Lewis (1975) as follows:

MCV = (PCV/RBCs) × 10 as m/mm^3.
MCH = (Hb content g/100 ml/RBCs) × 10 as m/mm^3.
MCHC = (Hb content g/100 ml/PCV) × 100 as%.

Other blood samples for serum separation were collected without the addition of anticoagulants and then centrifuged at 3500 rpm for 10 min. The activity of liver enzyme aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined colorimetrically according to Reitman and Frankel (1957), while alkaline phosphatase (ALP) was measured according to Rec (1972) using kits reagents supplied by Diamond Diagnostic Co. (Holliston, USA).

Liver examinations

On the completion of the experimental period, the fish were weighed individually, then sacrificed between 8:00 and 10:00 h to avoid any possible rhythmic variations in the antioxidant level (Ravinayagam et al., 2012). The fish were dissected according to the method described by Amlacher (1970), the abdominal wall was then removed and internal organs were investigated. For hepatosomatic index (HSI) determination, the liver was collected, weighed and the index was calculated according to Htun-Han (1978) as liver weight (g) × 100 × total body weight $(g)^{-1}$. For histopathological examination, the liver was collected and fixed in 10% buffered formalin. After alcohol dehydration and xylol clearing, specimens were embedded in paraffin, cut on 4 μm sections and stained with hematoxylin–eosin for a microscopic examination according to Bancroft et al. (1999). For molecular analysis, liver portions (100 μg) were immediately removed, fast frozen under liquid nitrogen and stored at −80 °C for later RNA extraction.

Molecular analysis of GPx and GST

RNA isolation and purification

Total RNA was extracted from 100 μg of liver tissue by the standard TRIzol extraction method (Invitrogen, Paisley, UK) and recovered in 100 μl of diethyl pyrocarbonate (DEPC)-treated water. Contaminating DNA was removed using 2 units of deoxyribonuclease (DNase I, RNase free, New England Biolabs, UK) for 30 min at 37 °C. RNA concentrations were measured spectrophotometrically at 260 and 280 nm (average ratio 1.89 ± 0.1). The purified RNA samples were preserved at −80 °C until used.

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA (1 μg) was reverse transcribed into cDNA in a total volume of 20 μl using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Carlsbad, CA, USA). The resulting cDNA was stored at −20 °C for later use or directly used as a template for semi-quantitative PCR.

Oligonucleotides PCR primers for *GST* were developed based on the published primers sequences (Cheng et al., 2012) that were designed using the sequence from GenBank EU107284, while primers for *GPx* were designed using the Primer3 program (accessible at http://www.genome.wi.mit.edu/cgi-bin/primer/primer3www.cgi) on the base of its nucleotide sequences available on GenBank (GQ853451). Due to its constitutive expression, *β-actin* amplification (Choi et al., 2004) was used as the housekeeping gene in semi-quantitative RT-PCR analysis. Products obtained using 19 cycles of amplification were within the linear range of signal amplification and allowed titration of the amount of template to be subsequently used in order to obtain consistent amounts of products between samples. The adjusted cDNA volumes were then used in the succeeding PCR reactions with gene specific primers for *GPx* and *GST*. All primers were synthesized commercially by Metabion International AG (Martinsried/Deutschland). The specificity of the amplification products was confirmed by size estimations on a 1.2% (w/v) agarose gel.

The PCR reaction mixture was set up in a total volume of 20 µl, containing 1.5 µl cDNA (~0.1 µg), 2 µl dNTPs (2.5 mM each), 2 µl 10× PCR Buffer, 0.5 µl (5u/µl) TaqTM DNA polymerase (Intron biotechnology, Korea), 1 µl (10 pmoles) forward primer, 1 µl (10 pmoles) reverse primer and 12 µl sterilized distilled water. The PCR parameters were: 35 cycles at 94 °C for 1 min, 58 °C (in case of *GST* and *β-actin*) or 60 °C (in case of *GPx*) for 1 min, and 72 °C for 1 min, with an additional initial 2 min denaturation at 94 °C and 5 min final extension at 72 °C. DNA amplifications were carried out on a Techene, TC3000 thermal cycler (UK). Primers sequences, expected amplicon size and annealing temperatures are listed in Table 1.

Semi-quantitative analysis of gene expression

Following amplification, a 10-µl aliquot of each PCR product electrophoresed on 1.2% agarose gels containing 0.005% ethidium bromide at 90 V and DNA bands were visualized using long wavelength UV illumination and photographed using a gel documentation system (UV Products, Ltd, Cambrige, UK). The level of expression of different bands was densitometry analyzed by an ImageJ gel analysis program (Abramoff et al., 2004). This relies on comparing the density of each target gene band with that of the corresponding *β-actin* band. Analysis was performed in triplicate, and the means of three values are presented.

Statistical analysis

Data were processed in order to investigate the effect of cumulative impacts of aflatoxin on *O. niloticus* health using factor of time (weeks). Results are presented as mean ± standard deviation (SD). Graph Pad Prism 5.0 software (Graph Pad Software, Inc., San Diego, CA, USA) was used for the statistical analyses in this study. The statistical evaluation of all data was done using a one-way analysis of variance (ANOVA) to check the effect(s) of aflatoxin. The significance of difference owing to this effect was evaluated using multiple comparisons Dunnett's test (compare all versus controls). P values $\leqslant 0.05$ were regarded as statistically significant.

Results

Growth indices and survival rate

The results of growth and survival rate of *O. niloticus* exposed to either 20 or 100 ppb AFB1 for 6 or 12 weeks (identified as 2nd 6 weeks) are presented in Table 2. During the course of experiment, no mortality was observed and the survival rate was 100% in 20 ppb AFB1 exposure group (group 1) after 6 weeks and all fish appeared healthy, while the survival rate was lowered (96%) after 12 weeks. This group showed a significant ($P < 0.05$) increase in growth indices as indicated by the average daily growth (ADG), relative growth rate (RGR), and total weight gain (TWG). In the second group; fish were fed contaminated diet with AFB1 100 ppb, survival rate recorded 96% after 6 or 12 weeks. Furthermore, the fish suffered from significant decrease in growth indices relative to group 1. For example, TWG recorded 59% and 35% increase after the 1st and 2nd 6 weeks of toxin exposure, respectively, in the fish fed 20 ppb AFB1, while it verified 49% and 23% after the same exposure periods in the fish fed on the higher AFB1 concentration (100 ppb).

Hematological and biochemical analyses

All the examined blood parameters; RBCs, WBCs, PCV, Hb and MCV, except MCH and MCHC showed a significant

Table 1 List of all primers used, together with PCR amplification conditions and amplicon size.

Target	Accession no. (GenBank)	Sequence (5′-3′) (*S: Sense, A: Anti-sense*)	Expected amplicon size	Source	Annealing temperature (°C)
GST	EU107284	S: CTTCACTCTCAGTTGTAAG A: TTGAATGTTGGAAGCTGTC	217 bp	Cheng et al. (2012)	58
GPx	GQ853451	S: CTCTGAGGAACGACAACCAGG A: CGTCAGGACCAACCAGGAAC	376 bp	This study	60
β-actin	EU887951	S: TGGCATCACACCTTCTACAACGA A: TGGCGGGGGTGTTGAAGGTCT	139 bp	Choi et al. (2004)	58

Table 2 Effects of different AFB1 on growth indices and survival rate of *O. niloticus* (mean ± SE).

Groups	IW (g)	FW (g)	TWG (g)	ADG (g)/day	Feed intake	FCR (g)/day	RGR (%)	SR (%)
1 (20 ppb)								
1st 6 weeks	35.80 ± 0.5	56.14 ± 0.1*	21.1 ± 0.14*	0.50 ± 0.01*	77.27 ± 0.14*	3.66 ± 0.01*	60.4 ± 0.4*	100.0 ± 0.0*
2nd 6 weeks	56.14 ± 0.1	75.9 ± 0.6*	19.70 ± 0.6*	0.47 ± 0.01*	95.6 ± 0.6*	4.85 ± 0.03*	42.6 ± 1.7*	96.7 ± 0.33*
2 (100 ppb)								
1st 6 weeks	35.90 ± 0.7	51.10 ± 0.4*	16.1 ± 0.4*	0.38 ± 0.01*	67.21 ± 0.7*	4.17 ± 0.05*	46.0 ± 1.8*	96.7 ± 0.33*
2nd 6 weeks	51.10 ± 0.4	62.8 ± 0.8*	11.8 ± 1.1	0.28 ± 0.02*	74.6 ± 0.7*	6.40 ± 0.14*	23.1 ± 1.2	96.7 ± 0.33*

Each reading represents mean ± SD of 3 fish and asterisks indicate significant change ($^*P \leqslant 0.05$). IW initial weight, FW final weight, TWG total weight gain, FCR feed conversion ratio, ADG average daily gain, RGR relative growth rate, SR survival rate.

decrease in their values in fish exposed to a higher AFB1 contaminated diet (group 2–100 ppb) in comparison to group 1 (Table 3). In this group, *O. niloticus* suffered from anemic condition as revealed by a marked decrease in RBC and Hb values. This pattern was not only concentration dependent, but it was also time dependent as it appeared that 12 weeks of exposure in both groups 1 and 2 demonstrated lower blood parameter values than 6 weeks of exposure. Meanwhile, WBC differential count (Table 4) showed a non-significant decrease in monocytes as well as esinophils, a significant decrease in lymphocytes and a significant increase in neutrophils, while no significant change in basophils was recorded.

As presented in Table 5, the activity of serum AST, ALT and ALP liver enzymes significantly increased in *O. niloticus* exposed to 100 ppb AFB1 (group 2) relative to that exposed to 20 ppb AFB1 (group 1). Also, these values were significantly increased within each group with extended exposure time; from 6 to 12 weeks of exposure. On the other hand, HSI showed a significant decrease in its value with high AFB1 in diet.

AFB1 residues determination in liver and musculature

The results of AFB1 residue determination in the liver and musculatures of fish in the treated *O. niloticus* groups are shown in Table 6. Toxin residues were detected only in the liver in fish exposed to 20 ppb AFB1 (group 1) after 6 and 12 weeks. In group 2, fish exposed to 100 ppb AFB1 showed an increase in AFB1 residues in the liver after 6 weeks of exposure, which continued to raise to its highest level after 12 weeks. On the other hand, no toxin residues were detected in fish musculatures after 6 weeks while after 12 weeks of exposure, AFB1 residues were detected (5 μg/kg).

Challenge test

Both fish groups, fed 20 and 100 ppb AFB1, were subjected to bacterial infection with *A. hydrophila* (Table 7). The ability of *O. niloticus* to withstand the pathogen showed a lower efficiency in fish fed with a higher level of AFB1 (100 ppb) as indicated by a higher mortality rate percentage (100% versus 70%). Meanwhile, no mortality was recorded in the negative control fish injected with pure saline solution in any of the treated groups.

Histopathological examinations

After 6 weeks of exposure to 20 ppb AFB1 in diet, no remarkable histopathological changes were developed in the liver (Fig. 1a). Hepatocytes were polygonal in shape, arranged in several cellular layers and surrounded by sinusoids. Following 12 weeks of exposure, microscopical examinations showed irregular arrangements of hepatocytes, mild hepatocellular vacuolation and pyknosis with moderate fatty changes of

Table 3 Blood parameters of *O. niloticus* fed AFB1 contaminated diet.

Group	Period	RBCs × 10^6	WBCs × 10^3	PCV (%)	Hb (g/dL)	MCV (m/mm^3)	MCH (m/mm^3)	MCHC (%)
G1 (20 ppb)	6 w	4.23 ± 0.14*	35.4 ± 0.5*	33.7 ± 1.5*	13.0 ± 0.6*	79.5 ± 0.8*	31.2 ± 0.4*	39.3 ± 0.2*
	12 w	4.1 ± 0.1*	34.9 ± 0.8*	31.8 ± 0.6*	12.4 ± 0.18*	78.1 ± 0.5*	30.6 ± 0.2*	39.1 ± 0.22*
G2 (100 ppb)	6 w	3.35 ± 0.2*	33.8 ± 0.2*	25.0 ± 1.3	9.8 ± 0.4*	74.6 ± 0.8*	29.1 ± 0.7*	39.3 ± 0.4*
	12 w	3.0 ± 0.1*	32.3 ± 0.8*	22.1 ± 0.85*	9.0 ± 0.25*	72.8 ± 0.9*	29.7 ± 0.14*	40.6 ± 0.6*

Each reading represents mean ± SD of 3 fish and asterisks indicate significant change (*$P \leqslant 0.05$). w = week.

Table 4 WBC differential count percentage of *O. niloticus* fed AFB1 contaminated diet.

Group	Exposure time (weeks)	Neutrophis (%)	Monocytes (%)	Esinophils (%)	Basophils (%)	Lymphocytes (%)
G1 (20 ppb)	6 w	36.67 ± 1.4*	4.30 ± 0.6	5.70 ± 0.6	0.33 ± 0.6	53.00 ± 1.0*
	12 w	39.67 ± 0.9*	4.00 ± 1.0	5.00 ± 1.0	0.33 ± 0.6	51.00 ± 1.0 *
G2 (100 ppb)	6 w	39.70 ± 1.2*	4.00 ± 1.0	5.00 ± 2.0	0.33 ± 0.5	51.3 ± 1.2*
	12 w	45.70 ± 2.2*	3.70 ± 0.6	4.50 ± 0.7	0.33 ± 0.6	45.7 ± 3.2

Each reading represents mean ± SD of 3 fish and asterisks indicate significant change (*$P \leqslant 0.05$). w = week.

Table 5 Liver enzymes and HSI of *O. niloticus* fed AFB1 contaminated diet.

Group	Exposure time (weeks)	ALT (U/L)	AST (U/L)	ALP (U/L)	HSI (%)
G1 (20 ppb)	6 w	17.90 ± 0.20*	74.00 ± 1.00*	23.30 ± 0.60*	2.40 ± 0.06*
	12 w	19.80 ± 0.17*	82.30 ± 3.80*	24.90 ± 1.30	2.24 ± 0.15
G2 (100 ppb)	6 w	22.80 ± 1.30	93.70 ± 5.10	26.50 ± 0.70*	2.00 ± 0.08*
	12 w	24.80 ± 3.10	94.70 ± 3.20*	27.90 ± 1.90	1.42 ± 0.07*

Each reading represents mean ± SD of 3 fish and asterisks indicate significant change (*$P \leqslant 0.05$). w = week.

Table 6 Residues (μg/kg-ppb) of AFB1 in *O. niloticus* liver and musculature following AFB1 contaminated diet.

Group	Expoure time (weeks)	Musculature	Liver
G1 (20 ppb)	6 w	ND	5
	12 w	ND	8
G2 (100 ppb)	6 w	ND	10
	12 w	5	15

w = week and ND = not detected.

Table 7 Challenge of *O. niloticus* fed AFB1 contaminated diet against *A. hydrophila*.

Group	Total no.	Survival	SR (%)	Mortality	MR (%)
G1 (20 ppb)	10	3	30	7	70
G2 (100 ppb)	10	0	0	10	100
Control G1 (saline)	3	3	100	0	0
Control G2 (saline)	3	3	100	0	0

G = group, Total no. = total number of *O. niloticus*, SR = survival rate and MR = mortality rate.

hepatocytes. Following exposure to 100 ppb AFB1 for 6 weeks, pronounced fatty changes of hepatocytes were evident, while after 12 weeks severe vacuolation and loss of hepatic cellular structure, in addition to pyknosis were displayed.

Gene expression of GPx and GST

Semi-quantitative RT-PCR method was used to detect the expression of *GPx* and *GST* of *O. niloticus* that reflects the changes in transcription levels of these genes in the liver after exposure to 20 or 100 g/kg AFB1. When the viability of the purified RNA samples was analyzed via the amplification of *β-actin*, all samples from control as well as treated fish presented detectable quantities of *β-actin* mRNA (139 bp fragment) showing an acceptable integrity to amplification as well as a successful first-strand cDNA preparation. Furthermore, no amplification product could be detected from any of the negative control specimens, which demonstrated that any contaminating DNA did not amplify using the above-mentioned pairs of primers.

Changes in the transcription levels of *O. niloticus GPx*, *GST* and *β-actin* genes in the liver following feeding on AFB1 contaminated diet are presented in Figs. 2 and 3. In group 1 (20 ppb AFB1), after 6 weeks of exposure, the mRNA expression of *GPx* recorded a significantly high level of expression (170%) relative to that of the control *β-actin* housekeeping gene. Following 12 weeks, the expression was still up-regulated but to a lesser extent (140%). On increasing AFB1 diet contamination to 100 ppb (group 2), *GPx* was significantly down-regulated (70%) after 6 weeks and its expression level reached its minimum level (50%) after 12 weeks of exposure. A similar pattern was revealed for the expression of *GST*. The gene showed a significant high expression level in the group 1 after 6 and 12 weeks recording 130% and 110%, respectively. In group 2, *GST* was significantly down-regulated revealing 40% and 20% following 6 and 12 weeks of AFB1 exposure in diet, respectively.

Discussion

In the present study, two AFB1 concentrations were employed for *O. niloticus* diet contamination. Results showed differential response dependence on the toxin concentration and duration of exposure. Exposure to AFB1 at 100 ppb for 6 or 12 weeks has significantly reduced growth indices including total weight gain, average daily gain and relative growth rate but not the survivability, in comparison to that at 20 ppb. These results agree with those proving that ingestion of low to moderate doses of AFB1 over a long period of time caused significant growth depression and produced a reduction in weight gain and feed efficacy in a concentration-dependent manner (Tuan et al., 2002; Cagauan et al., 2004; Abdelhamid, 2008; Selim et al., 2013; Mahfouz, 2015). This was attributed to expelling the feed from the mouth of fish after ingestion (Nguyen et al., 2002) and inhibition of DNA, RNA and protein synthesis (Abdelhamid, 2008). However, contradicting results regarding the reduction of survivability of tilapia by AFB1 have been reported. Although a 200 μg/kg AFB1 in diet, fed for 10 weeks or 16 weeks, showed 34.34% and ~30% mortality rate in Nile tilapia, respectively (Selim et al., 2013; Mahfouz, 2015), exposure to 53.02-115.34 μg/kg for 120 days induced 67% mortality (Cagauan et al., 2004). Meanwhile, lower doses of AFB1 (30 μg/kg or less) did not induce mortalities in the same species (Tuan et al., 2002) or in hybrid tilapia (*O. niloticus* × *O. aureus*) (Deng et al., 2010), while 50-1000 μg/kg AFB1 displayed no mortality in red tilapia (*O. niloticus* × *O. mossambicus*) (Usanno et al., 2005). Differences may be attributed to fish species, experimental conditions or duration of AFB1 exposure. On the other hand, the results herein indicated that fish feed intake and FCR, frequently used as quality indicators for fish growth, were significantly decreased and increased, respectively, with elevating AFB1 concentrations, which implies the increase in feed consumption for weight gain. The negative effect of AFB1 on both parameters seems to be correlated to the amount of toxin and duration of exposure. Similar results were demonstrated for the

effect of AFB1 in Beluga (*Huso huso*) (Sepahdari et al., 2010) and broiler chickens (Manafi et al., 2014).

In the present study, most of the investigated hematological profiles were influenced by the dose and duration of exposure to AFB1 contaminated diet. RBC, WBC, PCV, Hb and MCV levels were significantly ($P \leqslant 0.05$) decreased in the high AFB1 exposed group compared with the other group. Meanwhile, differential RBC count demonstrated that monocytes, esinophils, and lymphocytes were lowered while neutrophils were significantly increased. The decrease in RBCs, PCV% and Hb% indicated anemia, possibly due to erythropoiesis, hemosynthesis and osmoregulatory dysfunction that occur owing to inhibiting the activities of several enzymes involved in heme biosynthesis (ATSDR, 2005) or due to an increase in the rate of erythrocyte destruction in hematopoietic organs (Jenkins and Smith, 2003). Meanwhile, the reduction in WBC count may be due to the release of epinephrine during stress, which is capable of causing the contraction of spleen and a decrease of leucocytes count, which accordingly results in the weakening of the immune system (Witeska, 2003). This, in turn, renders the fish vulnerable to infection. Besides, the release of neutrophils into the blood is known to occur as a non-specific response to a variety of stress stimuli in mammals and fishes (Singh et al., 2008). These changes are similar to those reported for fish aflatoxicosis in *O. niloticus* (e.g. Tuan et al., 2002; Selim et al., 2013), *O. aureus* (Rizkalla et al., 1997) and *labeo rohita* (Mohapatra et al., 2011) as well as other animals including broilers (Raju and Devegowda, 2000), rabbits (Donmez and Keskin, 2009) and Merino rams (Donmez et al., 2012).

One of the morphological parameters that are often determined in field research is the HSI, to identify the condition of the liver and potential liver diseases/damage (de Ben Ameur et al., 2012). It can provide information on potential pollution impacts. In the present work, the HSI of fish exposed to the higher AFB1 concentration (100 ppb) was found to be significantly lower than those exposed to lower concentration (20 ppb) and the longer the exposure (12 weeks), the lower the index. This is in agreement with the reported effect of AFB1 in sex reversed red tilapia (*O. niloticus* Linn. × *O. mossambicus* Peters) (Usanno et al., 2005), hybrid tilapia (Deng et al., 2010) and in the Red Drum (*Sciaenops ocellatus*) (Zychowski et al., 2013). The overall decrease in HSI was attributed to an indication of hepatic disorder or liver degeneration (Deng et al., 2010; Zychowski et al., 2013).

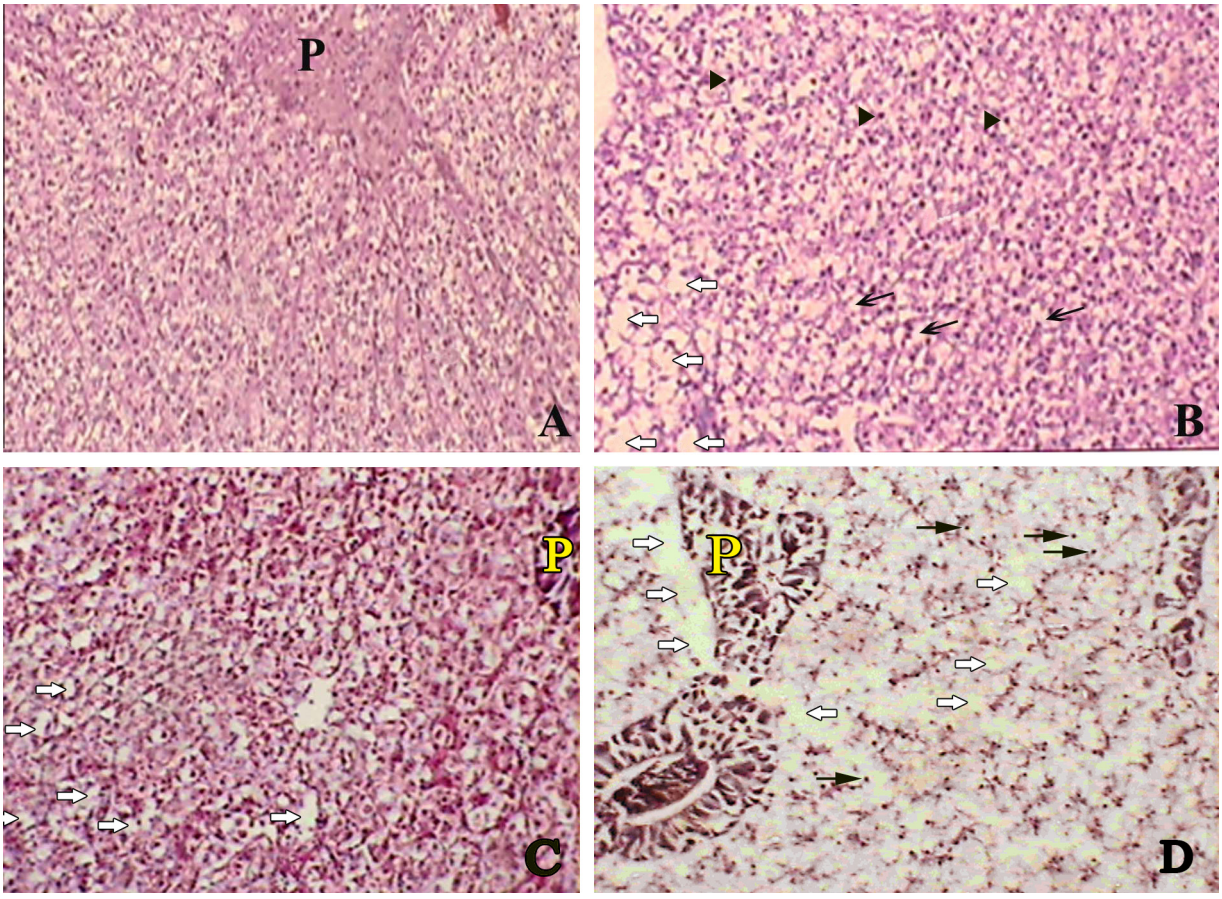

Figure 1 Photomicrographs of transverse sections of *O. niloticus* liver for fish fed AFB1, stained with hematoxylin and eosin, P indicates pancreatic tissue scattered throughout the liver (×100). (A) Apparently normal liver of fish exposed to 20 ppb AFB1 for 6 weeks. (B) Fish exposed to 20 ppb AFB1 for 12 weeks with mild hepatocytes vacuolation (black arrows), pyknosis (arrows heads) and moderate fatty changes of hepatocytes (white arrows). (C) Fish exposed to 100 ppb AFB1 for 6 weeks showing pronounced fatty changes of hepatocytes (white arrows). (D) Fish exposed to 100 ppb AFB1 for 12 weeks presenting severe vacuolation (white arrows) and pyknosis (black arrows), indicating liver degeneration.

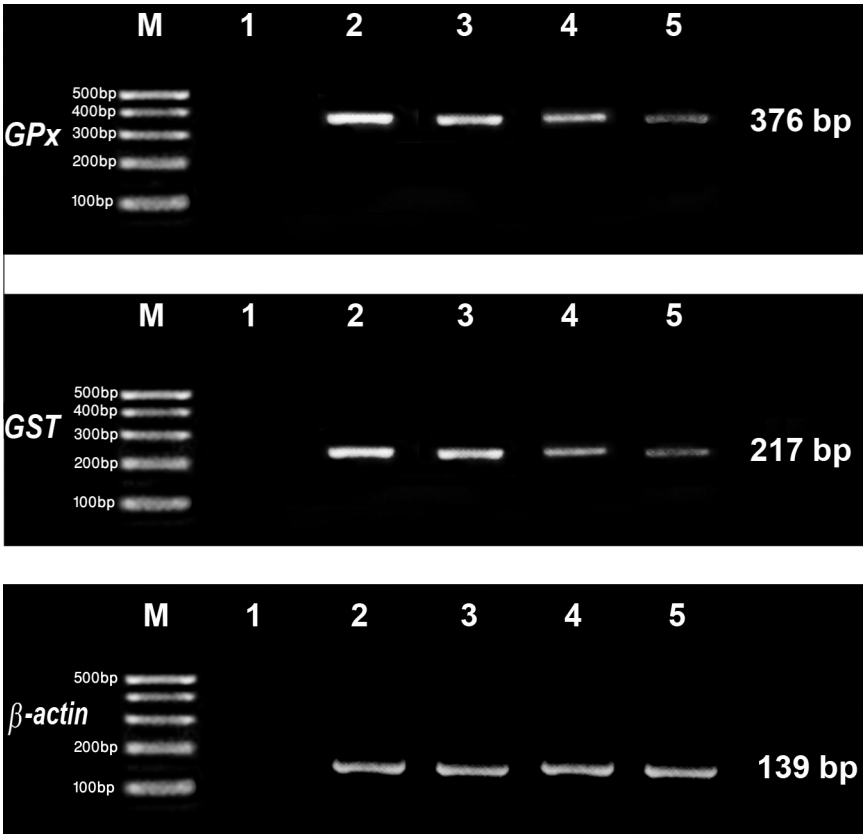

Figure 2 *GPx*, *GST* and *β-actin* mRNA expression in *O. niloticus* fed AFB1 contaminated diet. Electrophoresis of RT-PCR products of gene mRNA was performed in ethidium bromide-stained agarose gel (1.5%). Shown are amplicons: **M**, 100-bp marker; **1**, −ve control; **2**, 6 w-20 ppb; **3**, 12 w-20 ppb; **4**, 6 w-100 ppb; **5**, 12 w-100 ppb.

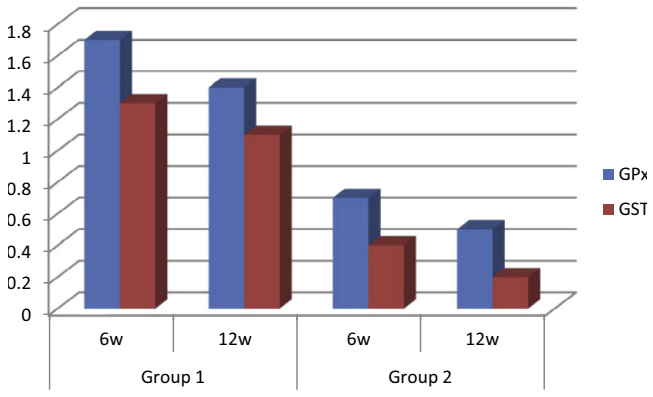

Figure 3 Levels of *GPx* and *GST*-gene expression in relation to the expression of *β-actin*. Group 1 and group 2 were exposed to 20 and 100 ppb AFB1, respectively. W denotes to weeks of exposure.

Alterations in enzyme activities of fish resulting from toxicants or contaminant affecting various cells, immune system, tissues and organs of fish have been reported (Gabriel and George, 2005; Ruas et al., 2008). AST and ALT, function as a link between carbohydrate and protein metabolism; their activities might be altered by a variety of chemical, biological and physiological factors or by a disturbance in Kreb's cycle, while ALP is a membrane bound enzyme and its alteration is likely to affect the membrane permeability and produce derangement in the transport of metabolites (Hagerstrand, 1975). Herein, the mean serum AST, ALT and ALP activities were significantly increased following exposure for 6 or 12 weeks to 100 ppb AFB1. An increase of these enzyme activities in the extracellular fluid or serum is a sensitive indicator of even minor cellular damage (Palanivelu et al., 2005) in which cellular enzymes are released from the cells into the blood serum, which in turn indicates stress-based tissue impairment. Varior and Philip (2012) reported that AFB1 significantly changed the stability of the lysosomal membrane, leading to a disorder of hepatocyte permeability and pathological changes in the liver of *O. mossambicus* that was confirmed by high levels of ALT and AST enzymes in the blood. Similarly, Selim et al. (2013) realized a significant increases in serum ALT and AST, following exposure of *O. niloticus* to 200 ppb AFB1 for 6–10 weeks, signifying hepatotoxicity. Also, Kheir Eldin et al. (2008) verified that AST, ALT and ALP were significantly increased in the serum of rats administered AFB1 (250 μg/kg/day for 2 weeks) indicating the presence of damaged and dysfunctional liver cells.

Due to human consumption of contaminated food, dietary contamination of aflatoxins poses a big risk to human health in different regions of the World particularly Asian and African countries (Wild and Gong, 2010). The present results demonstrated that concentration of AFB1 residues in the liver

increased with increasing toxin dietary concentration and duration of exposure to the toxin and reached its highest level after 12 weeks of feeding AFB1 contaminated ration. This observation suggested that fish could not develop an efficient mechanism of metabolizing AFB1 with time progression and an increase of AFB1 retention has happened. This is in accordance with the findings of Abdelhamid et al. (2004), Deng et al. (2010) and Selim et al. (2013). On the other hand, AFB1 residues in fish edible tissue (musculature) were not detected except after 12 weeks in the high toxin dietary concentration, although its level (5 ppb) was lower than that observed in the liver tissue, which was below the AFB1 safe/permissible level for human consumption (Radhika, 2006; Raghavan et al., 2011). A lower muscle AFB1 residual level in comparison with the liver was also reported by Begum et al. (2001), Bintvihok et al. (2002) and Kenawy et al. (2009).

Histopathological changes have been widely used as biomarkers in the evaluation of the health of fish exposed to pollutants/contaminants, both in the laboratory (Thophon et al., 2003) and field studies (Teh et al., 1997). Mycotoxins, including AFB1, exhibit a variety of toxic effects in animals, primarily in the kidney and liver (Kovacs, 2004); the latter is the major detoxifying organ directly receiving the materials from the intestine. In the current work, several histopathological alterations were observed in *O. niloticus* liver, which were dose and time dependent; most of which were more pronounced in fish fed 100 ppb AFB1 for 12 weeks. The liver showed extensive vacuolation corresponding to relatively higher lipid contents and signs of degeneration. These observations are comparable with previous reports (Cagauan et al., 2004; Lewis et al., 2005; Kenawy et al., 2009). Also, hepatocellular lipid deposition is a well-documented classical sign of aflatoxicosis in fish (Zychowski et al., 2013).

A previous study has suggested that AFB1 studies should pair liver histological evaluation with molecular markers to confirm liver damage (Zychowski et al., 2013). Intake of AFB1 diet was hypothesized to result in changes in liver gene expression, which represent characteristic pathophysiology that associated with aflatoxicosis (Yarru et al., 2009). Pollutants, including AFB1, exert their toxic effect by generating ROS, causing oxidative stress, leading eventually to many chronic diseases as well as cancer (Kotan et al., 2011) due to cytotoxicity, DNA damage, impairment of protein function and peroxidation of lipids (Hayes and McLellan, 1999). Oxidative stress occurs when the rate of ROS generation exceeds the antioxidant defense system (de Ben Ameur et al., 2012). GPx, an essential component of the cellular detoxification system, protects the cell and hypersensitive molecules from the attack of free oxygen radical (Choi et al., 2008; Li et al., 2008), while GST, the most important antioxidant enzyme in the detoxification system, belongs to a ubiquitous class of enzymes, which catalyze the conjugation of the tripeptide glutathione to electrophilic compounds thus favoring their excretion (Choi et al., 2008). Through the action of GST, the metabolites of AFB1 are mainly conjugated with glutathione hormone before being excreted (Bernabucci et al., 2011). In the present study, the expression of both *GPx* and *GST* antioxidant genes was remarkably down-regulated in the liver of fish fed high AFB1 (100 ppb) compared with controls. This reduction, possibly the result of liver damage, could hamper the fish's ability to protect itself from oxidative damage. A similar finding was demonstrated in birds subjected to AFB1, which contributed to the toxicological and pathological effects of the toxin (Yarru et al., 2009). Consistent with our results, several reports have demonstrated that the toxic effects of AFB1 involved decreased antioxidant capacity (Türkez et al., 2011), for example, AFB1 significantly decreased the activities of antioxidase, including GPx, in the broiler spleen (Wang et al., 2013), in rat liver, and kidney (Ravinayagam et al., 2012; Sivanesan and Hazeena Begum, 2014) and in laying hen's liver (Ma et al., 2012).

The ability of *O. niloticus* to withstand pathogen infection showed a lower efficiency in fish fed with a higher level of AFB1 (100 ppb) as indicated by a higher mortality rate percentage (100% versus 70%). The lowering of the fish ability to withstand *A. hydrophila* infection coincides with the low WBC count and down-regulation of *GPx* and *GST*. Consistent with our results, previous reports have suggested that aflatoxin at dietary concentrations of 1 mg/kg or more causes a significant reduction in the immune response of broilers and chicks (Shivachandra et al., 2003; Verma et al., 2004). These results indicate an immunosuppressive effect of aflatoxin on both humoral and cell-mediated immune responses that lead to increasing the susceptibility to secondary bacterial and viral infections, which could cause detrimental effects and even lead to death (Yarru et al., 2009).

In conclusion, this work provides an insight into a wide rage plateau about the internal environment of *O. niloticus* following exposure to the elevated AFB1 concentration. The impairment in fish health was evidenced by reduced growth indices, hematological profiles and fish immune status. Liver histopathological alterations, beside HSI decrement and liver enzymes activity elevation are speculated to be due to the AFB1 residual deposition in the liver that induced an oxidative stress, which in turn led to *GPx* and *GST* gene down-regulation.

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Further reading

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