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Effect of Aflatoxin B1 on farmed *Cyprinus carpio* in conjunction with bacterial infection

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ABSTRACT

Aflatoxin B_1 (AFB_1) is an immunosuppressive plant-origin toxin that reduces disease resistance of fish. A total number of 360 fries of the common carp, *Cyprinus carpio* (0.50 g/fish b.w.) were subdivided into 6 groups (G_1-G_6). Fish were fed on AFB_1-contaminated diet at a dose of 0.5 and 1.0 mg/kg fish feed in G_2 and G_3, respectively. AFB_1-contaminated diet plus nucleotides (NT) supplementation (at 0.2% instead of normal corn) were G_5 and G_6, respectively. Fish in G_1 were fed on diet free from AFB_1 and NT while in G_4, fish were fed on diet free from AFB_1 and supplemented with NT. Growth performance and liver enzymes (ALT, AST, and ALP) were adversely impacted by feeding on a contaminated diet and NT supplementation could ameliorate such withdraws. Anti-inflammatory IL-10 was increased in groups fed on contaminated diet indicating that inflammation had taken place. Feeding on AFB_1-contaminated diets resulted in degenerative changes (hepatic and spleen tissues), NT-supplementation could relieve such changes (low dose of AFB_1) however impacts of the high dose of AFB_1 were severe to a degree that NT-supplementation could not effectively diminish it. Regardless of feeding on contaminated diet, *C. carpio* supplemented with NT could resist *Aeromonas hydrophila* infection even those fed on contaminated diets, while un-supplemented groups showed higher mortality rates. *A. hydrophila* could isolate even after 35 days of experimental infection in *C. carpio* fed on AFB_1-contaminated diet (G_2 and G_3). It concluded that NT supplementation could ameliorate the adverse health impacts associated with feeding diet contaminated with AFB_1 in *C. carpio.*

INTRODUCTION

Aquaculture is an important participant of the global food supplies, fish and aquatic animal products are among the main sources of dietary protein in many countries worldwide **(FAO, 2018).** To minimize the costs of aquatic products, fish farmers have

added plant protein sources (**Anater *et al*. 2016**), the excessive usages of plant-based diets raise the potential exposure to aflatoxin B1 (AFB1) which is a foodborne toxin (**Hussain *et al*. 2017**). Aflatoxins have serious health hazards causing acute and chronic toxicity, AFB1 are substance with, carcinogenic, genotoxic, and immunosuppressive properties (**Flores-Flores *et al*. 2015**). AFB1 toxicity in silver catfish (*Rhamdia quelen*) associated with many non-specific signs such as immunosuppression, histological lesions, and behavioral changes, and biochemical, haematological, and histopathological parameters were the diagnostic tools to evaluate the health status (**Anater *et al*. 2020**; **Sherif *et al*. 2020a**). In aquatic experiments conducted on the toxicity of AFB1 in different fish spp, the main signs were gradual declining in growth performance, immune suppression, and fish quality (yellowing of the body surface) (**Deng *et al*. 2010**; **Mahfouz and Sherif, 2015 and Wang *et al.* 2016**). The susceptibility of fish to AFB1 is depending on several factors such as mycotoxins (type and amount), feeding period, fish (species, sex, age) (**Anater *et al*. 2016**). The impact of mycotoxins on fish production still needs to be more clear as it is in livestock (**Gonçalves *et al.* 2018**), also, the efficacy of the ameliorating agents needs to be evaluated before recommendation to the aquatic sector.

Nucleotides (NT) are low molecular weight intracellular compounds (Adenosine monophosphate (AMP), Cytosine monophosphate (CMP), guanosine monophosphate (GM), and uridine monophosphate (UMP)) which play major roles in many of the metabolic processes (**Gil, 2002**). Since the early 2000s, dietary NT was incorporated in fish feed as a growth promoter, and health enhancer (**Huu *et al*. 2012**), immunopromoter (diseases resistant), and gastrointestinal (physiology and morphology) in many aquatic animal species (**Burrells *et al*. 2001**). Several studies stated that adding NT to fish feed enhances the activity of the immune system and growth performance, 0.60% NT in juvenile hybrid tilapia (**Xu *et al*. 2015**), disease-resistant (*Aeromonas hydrophila*) in zebrafish was occurred by the direct action of the NT (**Guo *et al*. 2019**).

So, the purpose of this study was to evaluate the impacts of increasing levels of aflatoxin AFB1 in the fish feed on the immunity of common carp (*Cyprinus carpio)* fish and the potential role of NT to preserve normal immune status that resists the experimental challenge with *A. hydrophila* bacteria.

MATERIALS AND METHODS

1. Experimental Fish:

To achieve the purpose of the present study, a total of 360 common carp *(Cyprinus carpio)* fries were obtained from a private local farm in Kafr-Elsheikh, Egypt, with an average body weight of 0.50 g/fish. Fish were transported in a well-aerated tank to the laboratory of Animal Health Research Institute (AHRI) at Kafr-Elsheikh and then kept in glass aquaria. These aquaria were supplied with chlorine-free tap water. The aquaria were continuously aerated by an electric pump and were held at 28±2^{o}C. One third of the water was changed daily. Fish were acclimated for two weeks. During the acclimation period, fish were fed on the basal diet only.

2. Aflatoxin (AFB_1) preparation:

AFB_1 was produced through pellets fermentation using *Aspergillus parasiticus* NRRL 2999 according to the method described by **Abdelhamid and Mahmoud (1996).** Inclusion rate: 0.5 and 1.0 mg / kg diet. Determination of AFB_1 in ration was carried out

by quantitative thin layer chromatography TLC following the method described by **Eppley (1968)**.

3. Supplemented Nucleotides (NT):

The NT mixture contained 49% NT (12±1% of Adenosine monophosphate (AMP), 10±1% of Cytosine monophosphate (CMP), 16±1% of 5 – guanosine monophosphate (GM) and 11±1% of uridine monophosphate (UMP), 35% oligonucleotides, and 10% water, with the remainder comprised of proteins and polysaccharides. (Nanjing, China) Company: Saccharomyces cerevisiae-originated NT mixture from Biotogether, Batch No.: 2019041601.

Inclusion rate: 0.2 % instead of normal corn.

4. Experimental Design and Procedure:

Basal diet was formulated to meet the nutritional requirements of common carp according to **NRC (2011)**. Aflatoxin contaminated corn included in the basal diet instead of normal corn to add the required toxin level. **Table (1)** shows the physical and chemical composition of the experimental basal diet. The feed ingredients were thoroughly mixed, moisten with warm water (400 ml/kg) and then cold pressed and extruded to produce 2mm pellets. The diets were dried in an air convection oven set at 45 ^{0}C. After drying, the diets were stored in airtight bags prior to use.

Table (1): Ingredients composition and chemical analysis of the experimental diet.

Ingredients	Aflatoxin Level (mg/kg)			Chemical Analysis	
	0.0	0.5	1.0	Moisture%	11.09
Corn	15	14.65	14.3	CP%	42.05
Contaminated corn	0	0.35	0.7	Ether extract%	5.71
Soya (44%)	30	30	30	Ash%	7.23
Fish meal (60%)	25	25	25	Crude fiber%	2.63
Wheat flour	7	7	7	NFE [4]	35.29
DDGs[1]	5	5	5	DE (Kcal/Kg)[5]	2954
Corn gluten	15	15	15		
Soya oil	1.5	1.5	1.5		
MCP	1	1	1		
Salt	0.2	0.2	0.2		
Methionine	0.05	0.05	0.05		
Choline chloride	0.05	0.05	0.05		
Mineral premix [2]	0.1	0.1	0.1		
Vitamin premix[3]	0.1	0.1	0.1		

[1]DDGs = Dried distilled grains.

[2] Mineral premix: each one kg contain Manganese 60g, Copper 4 g, Zinc 50g, Iodine 1g, iron 80g, Cobalt 0.1g, Selenium 0.1g, calcium carbonate (CaCO3) carrier to 1000g.

[3] Vitamin premix: each one Kg contains vitamin A 12000000 IU, vitamin D3 2200000 IU, vitamin E 10 g, vitamin K3 2 g, vitamin B_1 1 g, vitamin B_2 5 g, vitamin B_6 1.5 g, vitamin B_{12} 0.01 g, vitamin C 250 g, Niacin 30 g, Biotin 0.050 g, Folic acid 1 g and Pantothenic acid 10 g and carrier to 1000 g.

[4]NFE= Nitrogen free extract.

[5] DE Kcal/kg = Digestible energy (DE) was calculated using formula based on chemical composition of feed stuffs nutrients according to **NRC (2011).**

Fish were randomly allotted into 6 equal groups (60 fishes per group) of 3 replicates (each aquarium measuring 80 x 40 x 40 cm, containing 20 fishes). **Table (2)** shows the

applied experimental design. Fish were fed to apparent visual satiation; by hand twice a day at 9:00 and 14:00. Extreme care was taken to assure that all supplied feed was consumed. Fish in each group were weighed at the beginning (W_0) and then biweekly weighed for a successive period of 8 weeks.

Table (2): Experimental design of fish groups.

Groups	Aflatoxin Level (mg/kg)			NT supplementation (0.2%)*
	0.0	0.5	1.0	
G1	--	--	--	--
G2	--	+	--	--
G3	--	--	+	--
G4	--	--	--	+
G5	--	+	--	+
G6	--	--	+	+

* NT (Nucleotides) supplemented at 0.2% instead of normal corn.

5 Growth performances and feed utilization:

Total weight gain and weight gain % were calculated as follows:

Total weight gain = Final body weight- Initial body weight

Weight gain % = Total weight gain/Initial weight X100

Specific growth rate (SGR) was calculated from the following equation:

Specific Growth Rate (SGR) = (ln W_f – ln W_i x100)/t

In W_f = the natural logarithm of the final weight

In W_i = the natural logarithm of the initial weight

t = time (days) between lnW_f and lnW_i

Feed Conversion Ratio (FCR) was calculated for each aquarium as follows:

FCR = Feed intake / total body weight gain

Protein Efficiency Ratio (PER) was calculated as follows:

PER = Weight gain/Protein intake

6. Gene expression of some cytokines of the experimental fish

To analyze the effect of AFB_1 and NT on gene expression of the pro-inflammatory cytokines interleukin IL-1β and tumor necrosis factor (TNF)-α and anti-inflammatory IL-10, the Reverse Transcription-PCR (RT-PCR) test was performed on tissue of head kidney of *C. carpio*. All primers are listed in **Table (3)**. The standard TRIzol extraction method (Invitrogen, Paisley, UK) was performed to extract the total RNA from 100 µg head kidney of *C. carpio*, RNA were stored at −80°C. By using the High-Capacity RNA-to-cDNA Kit (Applied Bio-systems, Carlsbad, CA, USA), 1 µg of the obtained RNA was reverse- transcribed into cDNA up to a total of 20 µl volume. The collected cDNA was directly used as a template for semi-quantitative PCR. β-actin amplification is constitutively expressed **(Choi *et al.*, 2004)** so, it was used as housekeeping gene in semiquantitative RT-PCR. After 19 cycles of amplification, the obtained products were within the linear range of signal amplification and allowed titration of the amount of the template to be subsequently used to obtain consistent

amounts of products, these products were electrophoretically separated, and on Gel Documentation (UVITEC, UK) the bands were distinguished.

Table (3): The cytokines primers used in the experiment.

Primer name	Direction (5′−3′)	Temperature (°C)	Size (pb)	Reference
IL−1β	F: GGATTCACAAGAACTAAGGAC R: ACTGTGATGTACTGCTGAAC	56 for 30 s	399	**Zou, *et al*. (1999)**
IL−10	F: ACCCCGTTCGCTTGCCA R: CATCTGGTGACATCACTC	56 for 30 s	70	**Buonocore *et al*. (2007**)
TNF-*α*	F: AGCATGGAAGACCGTCAACGAT R: ACCCTCTAAATGGATGGCTGCTT	56 for 30 s	131	**Laing *et al*. (2001)**
***β*-actin**	F: TGGCATCACACCTTCTACAACGA R: TGGCGGGGGTGTTGAAGGTCT	56 for 30 s	139	**Choi *et al*. (2004)**

7. Bacterial infection

After 8 weeks of feeding trial, fish (40 fish / group) was experimentally exposed to bacterial infection with *Aeromonas hydrophila* (AHRAS22). Fish was injected intraperitoneal at a dose of 10% of LD_{50} (3×10^5 CFU) according to methods reported by **Schaperclaus *et al.* (1992).** The number of dead fish was recorded for 14 days, and mortality rate during a specific period (MR) was measured using the following equation:

MR (%) = number of deaths ⁄ total fish number × 100

After 14 days, the survived fish was bacteriologically examined for *A. hydrophila*, three attempts of bacterial isolation were performed with a week-interval at days 21, 28, and 35 post-challenge. Bacterial isolation was done using randomly selected five fish from each treatment group and anaesthetized within 60 s using 50 mg/l tricaine mesylate. The fish abdomen aseptically opened (sterilized with methyl alcohol 70%), Specimens were taken from internal organs (**Amlacher, 1970)** and inoculated into Tryptic soy broth (Difco) and incubated at 28°C for 24 hr. Then the single pure isolated colonies were stored in cryovials containing 20%, 30% and 50% glycerol/broth at -20℃ for further identification by using API®20 E systems (BioMérieux, Mar-cy I‟Etoile, France) (**Austin & Austin (2012**).

8. Liver enzymes:

The activity of the liver enzymes in the experimental fish, Aspartate Amino Transaminase (AST) and Alanine Amino Transaminase (ALT) were determined in the serum to assess the impact of AFB_1 and the ameliorative role of NT according to the methods reported by **Reitman & Frankel (1957).** Levels of Alkaline phosphatase (ALP) activity were detected according to methods mentioned by **Rec (1972).** All kits and reagents were supplied by Diamond Diagnostic Co.

9. Histopathological examination:

Five fish of *C. carpio* from each group were randomly selected for histopathologically examination to detect the alterations in hepatic and splenic tissues. Tissue specimens were fixed with 10% neutral buffered formalin then processed and stained with haematoxylin and eosin; then examined using an Olympus BX51 light electric microscope according to previously methods described by **Roberts (2012).**

10. Statistical Analyses

To detected the impacts of AFB_1 and evaluate the ameliorating role of NT, the obtained results were analysed using SPSS software for windows, SPSS Inc., Chicago, IL, USA **(SPSS 2004)**, analysis of variance (ANOVA). All values were expressed as the mean ± SE (standard error). At a significance level of 0.05, the differences among groups were determined by using duncan's multiple range test **(Duncan, 1955)**.

11. Biosafety measures:

This study applied biosafety measures according to Pathogen safety data sheets: Infectious substances- *A. hydrophila*, Pathogen Regulation Directorate (**Public Health Agency of Canada, 2010).**

RESULTS

During the experimental period, the water parameter of the tank used showed: Temperature: 28°C±1°C, pH 7.8, salinity ≤ 0.3 g/I, and dissolved oxygen ≥ 5.5 mg/I. The level of AFB_1, in the experimental diets was 0.5 mg in groups G_2 and G_5 (with NT) and 1.0 mg in groups G_3 and G_6 (with NT) while diets of groups G_1 free from AFB_1 (without NT) and G_4 free from AFB_1 (with NT).

1. Clinical and post-mortem examination of *Cyprinus carpio*:

C. carpio in the group fed highly contaminated ration G_3 suffered from loss of appetite, lethargy, loss of reflexes at the end of the experiment **(Fig. 1B)**. Also, fish showed sluggish swimming, off feed, emaciated fish, dark skin, and loss of reflexes in G_3 than any other group. Moreover, fish in G_3 after bacterial infection showed dull appearance, emaciated fish **(Fig. 1C)**. On the other hand, the fish in G_4 appeared healthy **(Fig. 1A)**. Post examination presented revealed that *C. carpio* in the G_3 group which fed on a high AFB_1 diet exhibit slight pathological lesion as hepatopancreas discoloration, splenomegaly, and enlarged gall bladder than any other group. There is no change in the internal organs of fish in G_1 and G_4.

2. Effects of dietary aflatoxin on growth of *C. carpio*

2.1. Body weight development:

The result of body weight development is shown in **Table 4**. It was noted that the fish started with similar weights, but from the 2nd and 6th weeks control fish group was significantly higher bodyweight than the group fed on basal diet contaminated by aflatoxin B_1 at level 1.0 or 0.5 mg/kg diet respectively. Moreover, it was observed that NT supplementation in the fish diet without toxin contamination or with 0.5 and 1.0 mg AFB_1/kg diet increased final body weight by about 12.5%, 16.5%, and 48.9%, respectively, compared to fish group fed on the same diet without NT supplementation **(Table 4)**.

2.2. Growth performance and feed utilization parameters:

The total weight gain (TWG), daily weight gain (DWG), gain%, and specific growth rate (SGR) significantly reduced in the fish groups fed on AFB_1 contaminated diet at both concentrations compared to control. Furthermore, feed conversion ratio (FCR) and Protein efficiency ratio (PER) recorded a significant deterioration of fish group fed on AFB_1 contaminated diet at both concentrations compared to control **(Table 5)**. Moreover, NT supplementation in the fish diet without toxin contamination insignificantly improved all mentioned parameters compared to the fish group fed on the same diet without NT supplementation, while NT supplementation with AFB_1 contaminated diet at both concentrations significantly reduced all mentioned parameters compared to fish groups fed on the same diet without NT supplementation. Significantly improved all mentioned parameters compared to the fish groups fed on the same diet without NT supplementation.

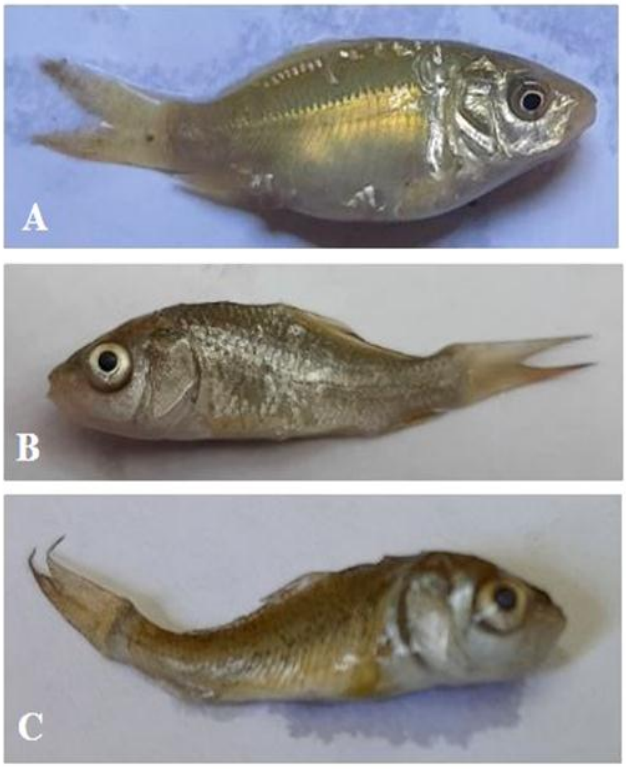

Fig. (1):

(A) *C. carpio* fed on NT-diets appears healthy.

(B) *C.carpio* fed on AFB1 intoxicated (G3) showing off feed, emaciated fish and dark skin.

(C) *C.carpio* fed on AFB1 intoxicated (G3) after bacterial infection, showing dull appearance, emaciated fish (big head)

Table(4):Body weight development (g/fish) of *C. carpio* fry as affected by dietary aflatoxin contamination without or with NT supplementation.

Experimental period (weeks)	Dietary aflatoxin contamination levels	NT supplementation			
		Without		With	
0	No contamination	G1	0.49±0.01^{ax}	G4	0.50±0.04^{ax}
	0.5 mg/kg diet	G2	0.51±0.01^{ax}	G5	0.51±0.01^{ax}
	1.0 mg/kg diet	G3	0.50±0.01^{ax}	G6	0.49±0.01^{ax}
2	No contamination	G1	0.74±0.04^{ax}	G4	0.78±0.07^{ax}
	0.5 mg/kg diet	G2	0.74±0.03^{ax}	G5	0.77±0.03^{ax}
	1.0 mg/kg diet	G3	0.65±0.01^{by}	G6	0.69±0.02^{bx}
4	No contamination	G1	1.13±0.11^{ax}	G4	1.21±0.13^{ax}
	0.5 mg/kg diet	G2	1.05±0.04^{ax}	G5	1.14±0.06^{ax}
	1.0 mg/kg diet	G3	0.84±0.01^{by}	G6	0.99±0.01^{bx}
6	No contamination	G1	1.73±0.14^{ax}	G4	1.91±0.20^{ax}
	0.5 mg/kg diet	G2	1.49±0.07^{bx}	G5	1.67±0.13^{bx}
	1.0 mg/kg diet	G3	1..13±0.02^{cy}	G6	1.49±0.02^{cx}
8	No contamination	G1	2.64±0.25^{ax}	G4	2.97±0.33^{ax}
	0.5 mg/kg diet	G2	2.12±0.09^{by}	G5	2.47±0.22^{bx}
	1.0 mg/kg diet	G3	1.45±0.03^{cy}	G6	2.16±0.07^{cx}

Data represented as means ± standard error. Mean values with different letters at the same column (a-c) or row (x–z) and period differ significantly at (P≤0.05).

Table(5):Growth performance and feed utilization parameters of *C. carpio* fries as affected by dietary aflatoxin contamination without or with NT supplementation.

Parameters	Dietary aflatoxin contamination levels	NT supplementation			
		Without		With	
Total weight gain, TWG (g/fish)	No contamination	G1	2..16±0.25^{ax}	G4	2.47±0.30^{ax}
	0.5 mg/kg diet	G2	1.61±0.08^{by}	G5	1.96±0.12^{bx}
	1.0 mg/kg diet	G3	0.96±0.05^{cy}	G6	1.67±0.08^{cx}
Daily weight gain, DWG (g/fish)	No contamination	G1	0.039±0.004^{ax}	G4	0.044±0.004^{ax}
	0.5 mg/kg diet	G2	0.029±0.003^{by}	G5	0.035±0.003^{bx}
	1.0 mg/kg diet	G3	0.017±0.001^{cy}	G6	0.029±0.001^{cx}
Weight Gain %	No contamination	G1	442.27±44.88^{ax}	G4	500.85±32.94^{ax}
	0.5 mg/kg diet	G2	312.87±5.42^{bx}	G5	378.70±26.53^{bx}
	1.0 mg/kg diet	G3	193.79±20.19^{cy}	G6	345.52±37.53^{bx}
Specific growth rate (SGR)	No contamination	G1	1.30±0.06^{ax}	G4	1.39±0.04^{ax}
	0.5 mg/kg diet	G2	1.09±0.01^{bx}	G5	1.21±0.04^{bx}
	1.0 mg/kg diet	G3	0.83±0.05^{cy}	G6	1.15±0.06^{bx}
Feed intake, FI (g/fish)	No contamination	G1	3.44	G4	3.89
	0.5 mg/kg diet	G2	3.18	G5	3.44
	1.0 mg/kg diet	G3	2.62	G6	3.08
Food conversion ratio (FCR)	No contamination	G1	1.62±0.11^{cx}	G4	1.49±0.05^{bx}
	0.5 mg/kg diet	G2	1.98±0.03^{bx}	G5	1.78±0.08^{ax}
	1.0 mg/kg diet	G3	2.76±0.21^{ax}	G6	1.87±0.14^{ay}
Protein efficiency ratio(PER)	No contamination	G1	1.63±0.10^{ax}	G4	1.76±0.06^{ax}
	0.5 mg/kg diet	G2	1.32±0.02^{ax}	G5	1.48±0.08^{bx}
	1.0 mg/kg diet	G3	0.96±0.06^{by}	G6	1.42±0.03^{bx}
Survival rate, SR (%) (n=60)	No contamination	G1	91.7	G4	86.7
	0.5 mg/kg diet	G2	90	G5	91.7
	1.0 mg/kg diet	G3	83.3	G6	88.3

Data represented as means ± standard error. Mean values with different letters at the same column (a - c) or row (x – z) and period differ significantly at (P≤0.05).

3. Effects of AFB_1 and NT on cytokines gene expression of *C. carpio*

AFB_1 impacted gene expression of pro-inflammatory (IL-1β, TNF-α) and anti-inflammatory IL-10 cytokines in the head kidney of *C. carpio* **(Table, 6)**. pro-inflammatory cytokines showed a declined gene expression with exposure to AFB_1 along with a significant increase of anti-inflammatory IL-10 (G_3 and G_2) 10.3 and 8.33 fold change, respectively. Compared with control supplementation of NT could ameliorate the immunosuppressive impacts of AFB_1 (G5 and G6).

Table (6): Gene expression of cytokines in head kidney of fish

Item	G1 (Control)	G2 (0.5 mg AFB_1)	G3 (1.0 mg AFB_1)	G4 (NT)	G5 (0.5 mg AFB_1+NT)	G6 (1.0 mg AFB_1+NT)
IL1β	3.33^{B} ± 0.3	2^{C} ± 0.58	1.67^{C} ± 0.33	4.67^{A} ±.33	3.67^{AB} ± 0.33	3.67^{AB} ± 0.3
IL10	4.67^{C} ± 0.7	8.33^{AB} ± 0.3	10.3^{A} ± 0.88	4.67^{C} ± 0.7	6.33^{B} ± 0.67	8.33^{AB} ± 0.3
TNF-α	7.33^{AB} ± 0.67	5.67^{CD} ± 0.3	4.33^{D} ± 0.3	8.33^{A} ± 0.3	7^{B} ± 0.18	6.67^{BC} ± 0.3

Data represented as mean±SE of 3 fish and group with different letters within the same row are significantly different at P≤.05. IL: interleukin; TNF; tumor necrosis factor.

4. Effect of dietary aflatoxin on liver enzymes:

After 12 weeks of fish feeding on AFB_1-contaminated diets, the activity of liver enzymes AST, ALT, and ALP were significantly raised in the serum of *C. carpio* in groups G_2 and G_3 (0.5 and 1.0 mg AFB_1/kg diets) than G_1 (Table 7). By adding the NT in the feed (0.5 g/kg diet), the levels of ALT, AST, and ALP in the serum of *C. carpio* of groups (G_5 and G_6) lowered only in comparison to fish contaminated with AFB_1 (G_2 and G_3).

Table (7): Liver enzymes in the experimental *C. carpio* fry.

Item	G1 (Control)	G2 (0.5 mg AFB_1)	G3 (1.0 mg AFB_1)	G4 (NT)	G5 (0.5 mg AFB_1+NT)	G6 (1.0 mg AFB_1+NT)
AST	44.67^{D} ± 1.76	62.3^{C} ± 1.45	116.7^{A} ± 2.6	43^{D} ± 1.7	47^{D} ± 1.7	88^{B} ± 6.8
ALT	20.3^{D} ± 0.88	41.3^{C} ± 4.8	65.7^{A} ± 2.3	19.3^{D} ± 0.3	23.67^{D} ± 1.2	54.3^{B} ± 2.6
ALP	25.7^{D} ± 1.2	29^{C} ± 2.6	41.7^{A} ± 1.45	23.7^{D} ± 0.88	26^{D} ± 2.1	32^{B} ± 1.5

Data represented as mean±SE of 3 fish; group with different letters within the same row are significantly different at P≤.05.

5. Histopathological examination of *C. carpio* intoxicated with AFB_1

In our study, the sensitivity of *C. carpio* to AFB_1, impacts of AFB_1 were determined in both hepatic and splenic parenchyma. Hepatic parenchyma in G_1 revealed normal architecture **(Fig. 2A)**; specimens of G_4 fed NT had hepatocytes with more eosinophilic cytoplasm **(Fig. 2B)**. Low-dose AFB_1 (G_2) exhibited the degenerative changes in form of hydropic degeneration **(Fig. 2C)**; admix NT to low-dose AFB_1 (G_5) led to improvement with restoring of hepatocytes to normal appearance **(Fig. 2D)**. More degenerative effect with recorded in high-dose AFB_1 (G_3) in form of severe and massive fatty changes, other area shows emboli **(Fig. 2E)**, admix of NT to high-dose AFB_1 (G_6) relieve (but not) diminish the degenerative effect from intoxicated hepatocytes **(Fig. 2F)**.

Splenic features detected the normal histopathological character of the fish spleen (G_1) with presence of melano-macrophage center in **(Fig. 3A)**; NT group (G_4) indicated to immune stimulation effect in form of multiple melano-macrophage centers **(Fig. 3B)**. Low-dose AFB_1 fed *C. carpio* surprisingly indicated the stimulation role in form of a large-sized melano-macrophage center **(Fig. 3C)**, this effect was markedly reduced in low-dose + NT fed *C. carpio* (G_5) **(Fig. 3D)**. Meanwhile, high-dose AFB_1 (G_3) markedly inhibited the melano-macrophage center stimulation with minute size **(Fig. 3E)**, admix of NT to high-dose AFB_1 (G_6) moderately improve the melano-macrophage status by restoring it to normal condition **(Fig. 3F)**.

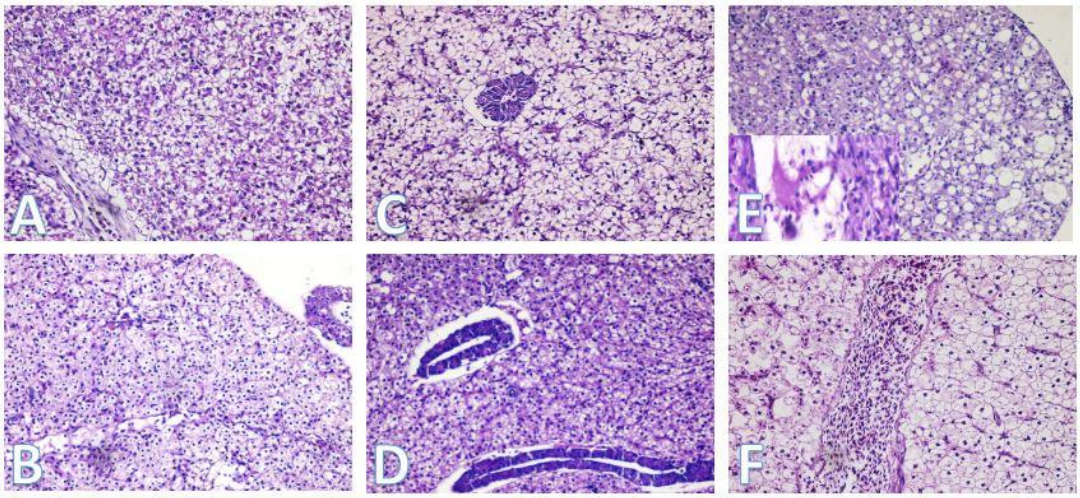

Fig. (2): Microscopic features of hepatic parenchyma in different investigated groups, indicating to normal hepatic character of control negative G_1 (A), more eosinophilic appearance of hepatocytes in NT G_4 (B), hydropic degeneration in low-dose AFB_1 G_2 (C), almost normal hepatocytes in low-dose AFB_1+ NT G_5 (D), marked hepatic degeneration in form of fatty changes in high-dose AFB_1 G_3 (E), persistence of hepatic degeneration in high dose AFB_1 + NT G_6 (F). H&E X 400

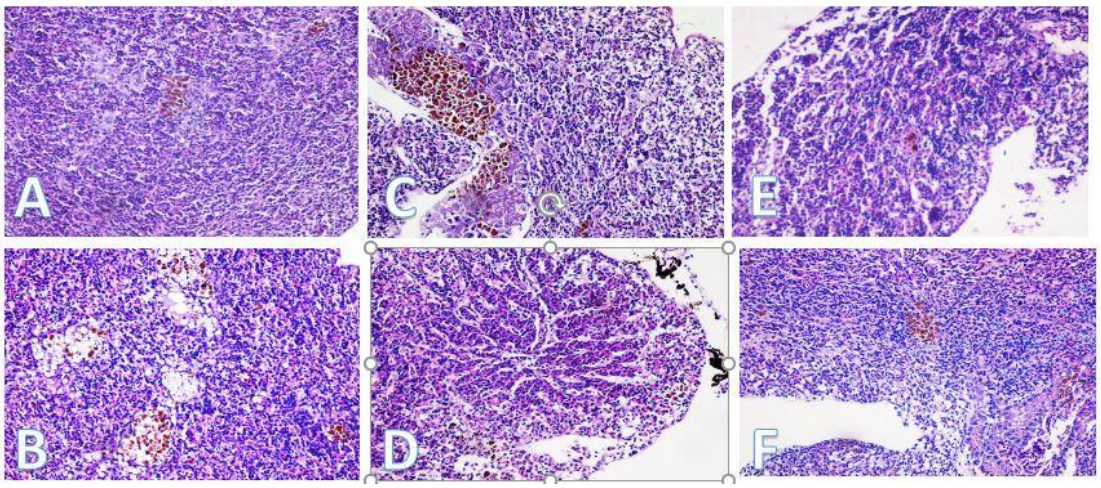

Fig. (3): Microscopic feature of splenic parenchyma in different investigated groups, indicating to normal splenic character of control negative G_1 (A), multiple melano-macrophage centers in NT treated G_4 (B), large –sized melano-macrophage centers in low-dose AFB_1 G_2 (C), almost absence of melano-macrophage centers in low-dose AFB_1+ NT G_5 (D), marked reduction in the size of melano-macrophage centers in high-dose AFB_1 G_3 (E), restoring of normal sized melano-macrophage centers of hepatic in high dose AFB_1 + NT G_6 (F). H&E X 400

6. *C. carpio* challenged against *A. hydrophila*:

All six fish groups, after 8 weeks of feeding trial, 40 fish of each group were experimentally infected with *A. hydrophila* bacteria **(Table 8)**. The ability of *C. carpio* to withstand the *A. hydrophila* showed a lower mortality rate of 7.5 % in a group G_4 which fed on (0.5 g of NT), 15% in group G_5 followed by group G_6 (17.5%). The highest MR% was showed in group G_3 then group G_2 (35 % and 20 % respectively).

On day 21, 28, and 35 post-challenge 5 survived *C. carpio* were bacteriologically examined for the occurrence of *A. hydrophila* infection. In **Table 9**, the presence of bacterial infection decreased and *A. hydrophila* could not be isolated from survived *C. carpio* at the day 35 post-challenge in groups supplemented with NT in diet except for G6 which fed on highly contaminated AFB_1 (I out of 5 fish). It was noticed that *C. carpio* intoxicated with AFB_1 still harbour *A. hydrophila.*

Table (8): Mortality rate in experimental fish challenged with *A. hydrophila*

Item	G1 (Control)	G2 (0.5 mg AFB_1)	G3 (1.0 mg AFB_1)	G4 (NT)	G5 (0.5 mg AFB_1+NT)	G6 (1.0 mg AFB_1+NT)
Challenged	40	40	40	40	40	40
Survived	36	32	26	37	36	33
Dead	4	8	14	3	4	7
MR%	10	20	35	7.5	15	17.5

G: Group; MR: mortality rate.

Table (9).Occurrence of *A. hydrophila* infection in the survived fish post-challenge. (n=5)

Item	G1 (Control)	G2 (0.5 mg AFB_1)	G3 (1.0 mg AFB_1)	G4 (NT)	G5 (0.5 mg AFB_1+NT)	G6 (1.0 mg AFB_1+NT)
Day 21	3	3	3	3	2	2
Day 28	1	3	3	0	1	1
Day 35	0	2	2	0	0	1

DISCUSSION

Comparing with the control, *C. carpio* in the group fed highly contaminated ration G_3 (1.0 mg AFB_1 / kg fish feed) for 60 days suffered from slight clinical and post-mortem lesions. Similar to some extent, fish artificially intoxicated with AFB_1 showed a variety of clinical signs such as retarded growth, skin lesions (yellowish discoloration and bleeding), and erratic swimming **(El-Sayed and Khalil, 2009** and **Mwihia *et al.*, 2018).** In accordance, no behavioral alterations were observed that fed gibel carps (*Carassius auratus gibelio*) fed on AFB_1-feed for 24 weeks such as loss of reflexes, sluggish movement, and dyspnea or rapid opercular movements **(Huang *et al.*, 2014)**.

The survival rate of *C. carpio* was badly impacted after 60 days of feeding the contaminated diet (G_3, 83.3%) and was enhanced by the NT supplementation (G_6, 88.3). Along with the obtained findings, after 12 weeks of exposure to AFB_1 (0, 50, 100, and 250 µg / kg), **Gonçalves *et al.* (2018)** observed different survival rates 99.0–99.7% in catfish, *Pangasius hypophthalmus*. While, Nile tilapia (freshwater fish) fed on AFB_1-

contaminated feed (2000 µg / kg) had a survival rate of only 82.2 %. According to the results achieved with NT supplementation, **Shiau *et al.* (2015)** fed Nile tilapia on diets supplemented with NT (≥120 mg/kg diet) had significantly improved survive.

After feeding of *C. carpio* on AFB_1-contaminated diets, the growth performance of fish was retarded in (G_2 and G_3) and the NT supplementation could keep normal growth behaviour (G_5 and G_6) compared to the control group (G_1). Generally, growth parameters gradually decreased when fish were fed on AFB_1-diet **(Deng *et al.* 2010).** Similarly, a decrease in the final weight, DWG, and FCR in Nile tilapia (*Oreochromis niloticus*) fed with a diet containing 2000 µg / kg of AFB_1 was observed by **Ayyat *et al.* (2018)**; as well **Mahfouz and Sherif (2015)** with 20 and 100 µg AFB_1 / kg feed for 12 weeks; and also **(Lopes *et al.* 2005)** with 41 to 204 µg AFB_1/ kg feed in silver catfish for 35 days. Controversy, **Anater *et al.* (2020)** conducted a study on different concentrations of dietary AFB_1 (0, 45, 90, and 180 µg/kg) fed to silver catfish, *Rhamdia quelen*, they observed that AFB_1 did not affect the growth performance linearly.

The improvements of growth parameters obtained in this study after NT supplementation are similar to many previous studies on different fish species. Administration of dietary NT has been reported to enhance the growth of red drum, *Sciaenops ocellatus* **(Li *et al.* 2007),** Caspain brown trout **(Kenari *et al.* 2013),** and Beluga sturgeon **(Abtahi *et al.* 2013).** Overall, high levels of NT are needed in periods of rapid growth in livestock animals **(Carver, 1994).** Dissimilar to this study, **Shiau *et al.* (2015)** stated that dietary NT supplementation had no growth-promoting effect in juvenile tilapia (0.15–5.1 g body weight). These opposite results could be due to the use of low fish meal (6%) diets in their experiment. Also controversy, NT supplementation did not promote the growth of hybrid striped bass **(Li *et al.* 2004),** barramundi **(Glencross and Rutherford, 2010),** channel catfish **(Welker *et al.* 2011)** and pikeperch **(Jarmolowicz *et al.*, 2012).** Different results could be due to fish species and supplementation period.

Pro-inflammatory cytokines (IL-1β and TNF-α) of the experimental *C. carpio* were significantly impacted with feeding on AFB_1 contaminated diet that indicating immunosuppressive status. The decrease of cytokines associated with pro-inflammatory and the increase of anti-inflammatory cytokines could decrease inflammatory responses in fish **(Wang and Secombes, 2013; Sherif and Mahfouz, 2019** and **Sherif *et al.* 2020b).** Generally, the occurrence of chronic stress could suppress the growth performance **(Pickering, 1993).** The low dose of AFB_1 slightly decrease both mRNA and protein levels of lymphocytic IL-2, IFNγ and it preferentially affects macrophage functions as well as IL-1α, IL-6, and TNF production by these cells as it decouples the close correlation usually observed between transcriptional and translational controls of IL-1α, IL-6 and TNF production by these cells **(Giambrone *et al.*, 1978** and **Dugyala & Sharma 1996).**

In accordance,the silver catfish, *Rhamdia quelen* fed on AFB_1-contaminated feed had hematological and biochemical alterations associated with immunosuppression and hepatoxicity, which are commonly observed with AFB_1-exposure in animals **(Sahoo *et al.*, 2003** and **Anater *et al.*, 2020)**. On the contrary, Nile tilapia exposed to AFB_1 had no significant differences in serum total protein and albumin **(Saei *et al.*, 2017)**.

The present results showed that the supplementation of NT to fish feed could normalize the immune status of *C. carpio* exposed to AFB_1 toxicity. **Asaduzzaman *et al.* (2017)** reported that gene expression of IGF-1 could be up-regulated in hepatic of Nile

tilapia which was supplemented with one of the NT (IMP). Accordingly, **Shiau *et al.* (2015)** claimed that immune responses and disease resistance of juvenile hybrid tilapia, *Oreochromis niloticus* × *Oreochromis aureus* supplemented at 120–240 mg NT/kg in diet were enhanced as well as survival rate and plasma lysozyme activity. Along with findings of this study, grass carp showed that optimum NT supplementation improved non-specific immune responses **(Tie *et al.* 2019),** Nile tilapia **(Ramadan *et al.*, 1994),** hybrid striped bass **(Li *et al.* 2004),** and red drum, *Sciaenops ocellatus* **(Li *et al.* 2007).**

Aflatoxin detoxification in fish takes place in the hepatopancreas tissue, which is considered to be the main impacted organ **(Santacroce *et al.*, 2008),** and liver enzymes including ALT, AST, and ALP activities are good bioindicators for tissue damage and dysfunction **(Hassaan *et al.*, 2014).** A high level of serum ALP was detected after metabolizing large amounts of toxins by hepatopancreas **(Thrall *et al.* 2007).**

After 12 weeks of feeding AFB_1, the activity of liver enzymes AST, ALT, and ALP were significantly increased in fish fed on the highly contaminated diet (G_3) and supplementation with NT resulted in an enhancement that still significantly higher than control (G_6). The higher activities of ALT and AST in this study are confirmed with those found in *Oreochromis mossambicus* subjected to AFB_1 which alter the integrity of the lysosomal membrane resulting in hepatic impairment **(Varior and Philip 2012).** These impairments could be assessed by measuring the activity of AST and ALT **(Gonçalves *et al.* 2018** and **Sherif *et al.* 2020b).** Different findings obtained by **(Deng *et al.* 2010)** who did not found significant differences in the activities of ALT and AST in Nile tilapia fed on 1,641 μg AFB_1/ kg feed and in Gibel carps fed on diets containing up to 1,000 μg AFB_1/ kg feed. Close to the obtained results, Nile tilapia ingested AFB_1-contaminated diets 20 and 100 μg AFB_1/ kg feed for 12 consecutive weeks had a significant increase of AST and ALT **(Mahfouz and Sherif, 2015)**. Disagreeing with the present results, rainbow trout fingerlings exposed to AFB_1 recorded insignificant differences in ALP activity **(Saei *et al.* 2017).** Furthermore, **Shehata *et al.* (2009)** measured AST and ALT in the serum of Nile tilapia fed a diet containing 100 μg/kg of AFB_1 and found that enzyme activity had decreased. These differences could be due to aflatoxin has exhausted the hepatic cells along with severe damages of hepatopancreas tissue.

Our results showed improvements in liver condition as the activity of AST, ALT, and ALP were significantly normalized in fish supplemented with NT. Likewise, supplemental NT was found to reduce both ALP and ALT activities in serum of rainbow trout **(Tahmasebi-Kohyani *et al.* 2012),** reducing ALP activity as observed in weaned pigs when dietary NT and organic acids were combined **(Lee *et al.* 2007).** Variations of liver enzymes were dose-dependent in the plasma of female Nile tilapia **(de Lima *et al.* 2020).**

The present study indicated hepatic degeneration in form of hydropic degeneration in the AFB_1 low-dose group (G_2) that progresses to fatty degeneration in the AFB_1 high-dose group (G_3), this indicated the sensitivity of *C. carpio* to AFB_1. In agreement, **Al-Azri, *et al.* (2015)** noticed a correlation between the occurrence of vacuolar degeneration to fatty changes in killifish, *Aphanius Dispar* AFB_1 exposure (dose and/or period). **Batatinha, *et al.* (2008)** added that these pathological alterations were attributed with biodistribution of AFB_1 (via gastrointestinal tract) reaching the hepatic tissue; furthermore, the severity of these alterations was correlated with the dose of AFB_1,

and may led to necrosis via binding of AFB_1 metabolites to DNA and RNA of hepatic cells. Other histopathological features were observed; it varied from nuclear and cellular hypertrophy **(Chavez-Sanchez *et al*. 1994)** to irregular cords of hepatocytes (large, dark, basophilic with large hyperchromatic nuclei) in rainbow trout **(Mwihia *et al.* 2018).** Nucleotides are low molecular weight agents used in clinical nutrition as an exogenous supplement **(Carver and Walker, 1995)** to improve some physiological functions **(Quan, 1992).** In this study, improvements of hepatic status were detected in low dose intoxicated group (G_5), while in high-dose intoxicated fish (G_6) there was a lower stimulation effect of NT on melano-macrophage centers. The recorded alleviation effect of NT supplementation on AFB_1 could be attributed to its ameliorating effect on stress factors **(Lin, *et al*. 2009).** NT supplement enhance the anti-oxidant state of the hepatic tissue **(Xu, *et al*. 2015).** So, our findings agree with those obtained by **Welker *et al.* (2011)** who claimed that NT has a potential role that could ameliorate the immunosuppressive impacts of stressful factors on the non-specific immunity.

In aquatic pathologists have considered *A. hydrophila* as one of the most fish pathogens causing severe morbidity and high mortalities which accompanied by economic losses **(Guo *et al*. 2019** and **Sherif *et al*. 2020b).** In this study, diseases resistance was improved in *C. carpio* received NT could be explained by the findings of **Xu *et al.* (2016)** who noted that the growth performance in fish may have been closely related to the infectious diseases resistance, which has been proved to be closely correlated with the immune function in grass carp, *Ctenopharyngodon idella*. In the present study, fish were subjected to experimental infection with *A. hydrophila* bacteria, the highest mortality rate (%) was showed in group G_3 then group G_2 (0.5 and 1 mg of AFB_1 respectively). After 21 days post-challenge, survived *C. carpio* still harbour *A. hydrophila* bacteria in groups fed on contaminated AFB_1-diets (G_2 and G_3). These findings indicated that fish were immune-compromised and fish are still vulnerable for bacterial infection relapse. Along with our results, **Oliveira *et al.* (2013)** noticed a synergistic action between AFB_1 and *A. hydrophila* infection in *O. niloticus* fed on AFB_1 contaminated diet at 1.177 mg/kg that survived fish had significant differences with the control group for feed conversion and total length. Fishes fed on an aflatoxins-contaminated diet became vulnerable to stress due to microbial factors such as viruses and bacteria **(Rosmaninho *et al.* 2001** and **Lopes *et al*. 2005).** Aflatoxin suppressed both humoral and cell-mediated immune responses; leading to increase vulnerability to secondary bacterial and viral infections which could raise the MR% in broiler chicks **(Yarru *et al.* 2009).**

C. carpio lower mortality rate in a group G_4 which fed on the supplemented diet (0.5 g NT) and these findings match with the succeeded results of growth parameters and cytokines that showed that AFB_1 had dramatically impacted the immune status of *C. carpio*. In accordance, **Shiau *et al.* (2015)** found that fish fed on diets supplemented with NT could resist bacterial infection of *Streptococcus iniae* and survival rate were increased (>80%) compared with the control group (56.7%), and in rainbow trout, *Oncorhynchus mykiss* **(Tahmasebi-Kohyani *et al.* 2011).** Similarly, **Guo *et al*. (2019)** recorded that zebrafish fed on 0.1% NT-supplemented diets for 4 weeks could resist *A. hydrophila* NJ-1infection; also they explained their findings as dietary NT enhanced the physical barrier and mucosal immunity in the intestine of zebrafish. On the other hand, NT did not alter

immune response or disease resistance in red drum, *Sciaenops ocellatus* **(Li *et al.* 2007),** these differences could be due to fish species and/or dose-depended.

CONCLUSION

It is concluded that *C. carpio* is sensitive to AFB_1, immune status (cytokines and challenge against *A. hydrophila*) is greatly influenced particularly in fish received a dose of 1 mg AFB_1/kg fish feed. Despite that fish supplemented NT could restore normal health status, those fed on higher dose of AFB_1 dose not fully. Concerning liver enzymes and histopathological features, intoxicated *C. carpio* still harbour *A. hydrophila* infection even after 35 days post-challenge.

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