

## Protective effect of dietary vitamin E against fungicide copperoxychloride stress on Nile tilapia, *Oreochromis niloticus* (L.) fingerlings

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**Abstract** A  $2 \times 3$  factorial experiment was designed to study the effect of sublethal toxicity of technical grade copperoxychloride, dietary vitamin E and their interactions on growth performance, some blood parameters, DNA fragmentation and histopathological lesions of Nile tilapia (*Oreochromis niloticus*). The 96 h  $LC_{50}$  of copperoxychloride was found to be 129.21 mg/l. Four hundred and fifty fingerlings with an average initial body weight ranged from 1.92 to  $2.04 \pm 0.11$  g were randomly distributed equally in six treatments (T1–T6) with each of triplicates. T4, T5 and T6 were exposed to sublethal concentration 0.25  $LC_{50}$  of copperoxychloride. Fish of T1, T2 and T3 fed diet supplemented with different levels of vitamin E (0, 1 and 2 g/kg diet), respectively. While fish of T4, T5 and T6 fed diet supplemented with different levels of vitamin E (0, 1 and 2 g/kg diet), respectively. At the end of the experiment (90 days) the results indicated that fish exposed to 0.25  $LC_{50}$  of copperoxychloride and fed diet with normal level of vitamin E, showed significantly lower values ( $P < 0.01$ ) of all growth and feed utilization parameters, white blood cells and immunoglobulin M, but they showed significantly increased ( $P < 0.05$ ) values of alanine aminotransferase, aspartate aminotransferase, hematocrit, hemoglobin, uric acid and creatinine. Fish fed with varied concentrations of vitamin E neutralized the toxic effect of copperoxychloride as well as vitamin E, significantly lowered the hematological and biochemical response and enhanced the growth parameters and feed utilization. The toxic effect of copperoxychloride exposure and vitamin E feeding on histopathological alterations of liver and gill of the same studied fish will be taken in concern. The results showed improvement in samples treated with vitamin E. This study suggests that vitamin E can be effectively used to decrease the toxic effect of copperoxychloride on *O. niloticus* and its amelioration through dietary vitamin E supplementation.

**Keywords** Copperoxychloride · Vitamin E · Growth · IgM · DNA fragmentation histopathology · Nile tilapia

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## Introduction

Today, water quality management faces greater problems than at any time in its history. In addition to natural pollutants, varied contaminants exist in surface waters including multiple chemical compounds and different products of industrial and agricultural revolution. Pollution of the aquatic environment by toxic substances is a cause of growing concern throughout the world, especially in developing countries. The immediate concern is human health and welfare, but the effect of pollution on aquatic organisms also has ecosystem wide consequences (Alam and Maughan 1992). Many of the toxic substances are lipophilic and are not adversely affected by water. These substances accumulate in fish fatty tissues or become protein bound, so it is of importance to know the critical concentration above which humans are affected and the commercial fish species become unsuitable as food (El-Ezaby 1994).

There are many pathways by which insecticides leave their sites of application and distribute throughout the environment and enter the aquatic ecosystem. The major route of insecticides to water ecosystems in urban areas is through rainfall runoff and atmospheric deposition. Another source of water contamination by pesticide is from municipal and industrial dischargers. Most pesticides ultimately find their way into rivers, lakes and ponds (Bagheri 2007; Vryzas et al. 2009) and have been found to be highly toxic to non-target organisms that inhabit natural environments close to agricultural fields. One especially important concern is the large amounts of colloid-size copperoxychloride-based fungicide still freely used in organic farming in Europe (EU directive CE 889/2008). Copperoxychloride [ $\text{Cu}_2\text{Cl}(\text{OH})_3$ ]-based fungicides are in fact widely used against fungal diseases in crops. Copper can enter soil by off-target deposition and foliage wash-off, which lead to its gradual accumulation (Fernández-Calviño et al. 2008; Komárek et al. 2009). The toxicity may be lethal to fish or may lead to stress which in turn causes immuno suppression and susceptibility to secondary infection. The effects of pesticide pollution on non-target organisms in the environment can be studied by detecting changes in organisms at the physiological, biochemical or molecular levels, providing early warning tools in monitoring environment quality (Crane and Maltby 1991; Miren et al. 2000). These sensitive early warning biomarkers can measure the interaction between environmental xenobiotics and biological effects. Inhibition and induction of these biomarkers comprise a good approach to measure potential impacts of pollutants on environmental organisms (Rendon-von Osten et al. 2005). The analysis of hematological and biochemical parameters in fish can contribute to the assessment of the animal's health and also the habitat conditions (Thrall 2004).

Vitamin E ( $\alpha$ -tocopherol) is essential for maintaining the normal growth and metabolic function of fish. It acts as a fat-soluble antioxidant in biological membranes and it has been demonstrated to be an essential nutrient for all fish studied (NRC 1993). As well as, its main function is to protect unsaturated fatty acids against free radical-mediated oxidation (Hamre et al. 1998). In addition to that, high dietary supplementation of vitamins E showed positive effects on growth and immune response in channel catfish (Li et al. 1993). Several studies have been carried out on the effect of different fungicides on hematological and biochemical aspects of fish and other aquatic animals, but very few on its amelioration through dietary vitamin E. Considering the commercial value of *O. niloticus*, it is necessary to obtain scientific information about the effect of copperoxychloride on *O. niloticus* to improve risk-assessment studies and its attenuation with the help of dietary vitamin E. This study aimed to assess the effect of the sublethal concentration of copperoxychloride over a long exposure period (90 days) on growth, hematological, histological and biochemical parameters of *O. niloticus* and its amelioration through a gradient of dietary vitamin E.

## Materials and methods

Fingerlings of Nile tilapia, *O. niloticus*, were obtained from Abassa, Abo-Hamad, Sharkia Governorate, Egypt. Fish were acclimatized for 2 weeks for the lab condition prior to start the experiment; during this period fish were fed a basal diet containing 30 % crude protein. Chlorine-free tap water was used throughout the experiment.



### Lethal concentration 50 of copperoxychloride

Stock solution of copperoxychloride was prepared by dissolving analytical grade copperoxychloride (from Merck) in distilled water. The lethal concentration 50 (LC<sub>50</sub>) value of copperoxychloride fungicide was determined in the laboratory starting with range finding test to acute toxicity trials according to Reish and Oshida (1987). Three hundred and fifteen fish were randomly distributed in twenty-one glass aquarium (180 l) and exposed to different concentrations of copperoxychloride (0, 25, 50, 75, 100, 125 and 150 mg/l) in triplicates. The mortality was recorded for 96 h at regular intervals of 12 h. The LC<sub>50</sub> value of copperoxychloride was calculated with the help of probit analysis using SPSS software (SPSS for Windows 14.0; SPSS Inc., Chicago, IL, USA, 2005).

### Experimental diet

The basal diet (30.83 % crude protein and 19.39 gross energy Mj/kg) was formulated (Table 1). Vitamin E (DL- $\alpha$ -tocopheryl acetate) was supplemented to the basal diet to achieve three levels (0, 1 and 2 g/kg diet). All feed ingredients were mixed thoroughly, and dough was prepared with required amount of water. Then dough was conditioned for 30 min followed by steaming for 1 min. The dough was pressed through a hand pelletizer (2 mm). Pellets were sun dried for 48 h and stored at 4 °C. The chemical composition of the diet was determined according to procedures of AOAC (1990). Dry matter was determined after drying the samples in an oven (105 °C) for 24 h. Ash by incineration at 550 °C for 12 h. Crude protein was determined by micro-Kjeldhal method, N%  $\times$  6.25 (using Kjeltach autoanalyzer, Model 1030, Tecator, Höganäs, Sweden) and crude fat by Soxhlet extraction with diethyl ether (40–60 °C).

### Experimental design and procedures

A 2  $\times$  3 factorial experiment was designed to study the main effect of fungicide level, vitamin E and their interaction on growth performance, blood parameters, DNA fragmentation and histopathological lesion of Nile tilapia. Eighteen aquarium (100  $\times$  40  $\times$  50 cm, 180 l each) triplicates for each treatment were used and

**Table 1** Feed ingredients and proximate analysis of the basal diet

Ingredients	%
Fish meal (60 %)	16
Soybean meal (44 %)	40
Yellow corn	28
Wheat bran	10
Corn oil	4
Vit and min. mix <sup>a</sup>	2
Total	100
Proximate analysis	
Dry matter (DM)	89.56
Crude protein (CP)	30.83
Ether extract (EE)	5.79
Ash	6.16
Nitrogen free extract (NFE) <sup>b</sup>	57.22
(Gross energy Mj/kg diet) <sup>c</sup>	19.39

<sup>a</sup> Vitamin and mineral mix (per kg of diet): MnSO<sub>4</sub>, 40 mg; MgO, 10 mg; K<sub>2</sub>SO<sub>4</sub>, 40 mg; ZnCO<sub>3</sub>, 60 mg; KI, 0.4 mg; CuSO<sub>4</sub>, 12 mg; Ferric citrate, 250 mg; Na<sub>2</sub>SeO<sub>3</sub>, 0.24 mg; Co, 0.2 mg; retinol, 40,000 IU; cholecalciferol, 4,000 IU;  $\alpha$ -tocopheryl acetate, 400 mg; menadione, 12 mg; thiamine, 30 mg; riboflavin, 40 mg; pyridoxine, 30 mg; cyanocobalamin, 80 mcg; nicotinic acid, 300 mg; folic acid, 10 mg; biotin, 3 mg; pantothenic acid, 100 mg; inositol, 500 mg; ascorbic acid, 500 mg

<sup>b</sup> NFE = 100 – (CP + EE + Ash)

<sup>c</sup> Calculated using gross calorific values of 23.63, 39.52 and 17.15 kJ/g for protein, fat and carbohydrate, respectively, according to Brett (1973)

were continuously aerated with air pumps. Four hundred and fifty fingerlings with an average initial body weights ranged from 1.92 to 2.04 ± 0.11 g were randomly distributed equally in six treatments (T1, T2, T3, T4, T5 and T6) with each of triplicates. T4, T5 and T6 were exposed to sublethal concentration 0.25 LC<sub>50</sub> of copperoxychloride (32.3025 mg/l) and fed diet supplemented with different level of vitamin E (0, 1 and 2 g/kg diet), respectively. Fish in T1, T2 and T3 fed diet supplemented with different level of vitamin E (0, 1 and 2 g/kg diet) respectively. Fish were fed to satiation, three times a day (09:00, 12:00 and 16:00 h), 6 days a week for 90 days.

Water samples for chemical analysis were monitored weekly during the experimental period. Dissolved oxygen and temperature were measured on site with a YSI model 58 oxygen meter (Yellow Spring Instrument Co., Yellow Springs, OH, USA). Total ammonia was measured using DREL/2 HACH kits (HACH Co., Loveland, Co. USA). pH was measured using a pH meter (Digital Mini-pH Meter, model 55, Fisher Scientific, USA). Total alkalinity and total hardness were measured by titration as described by Boyd (1984).

#### Growth performance and feed utilization indices

At the end of the experiment fish were collected and weighed. The different parameters of growth and feed utilization were calculated as follows:

$$\text{Weight gain (WG) g} = \text{Final body weight (FBW) g} - \text{Initial body weight (IBW) g.}$$

$$\text{Specific growth rate (SGR)} = [(\text{LnW2} - \text{LnW1})/T] \times 100,$$

where: Ln = the natural log; W2 = FBW at T2 (g); W1 = IBW at T1 (g); T = period of the experimental study, T2–T1 (days).

$$\text{Survival rate (SR\%)} = [(\text{total number of fish} - \text{dead number of fish})/\text{total number of fish}] \times 100.$$

$$\text{Feed conversion ratio (FCR)} = \text{Feed intake (FI) g}/\text{WG g.}$$

$$\text{Protein efficiency ratio (PER)} = \text{WG g}/\text{crude protein intake (g)}.$$

#### Hematological and biochemical parameters

At the end of the experiment blood samples were collected from the caudal vein of fish of all treatments and were divided into two portions. The first portion was collected with anticoagulant 10 % EDTA (ethylenediaminetetraacetate) to measure hematocrit (Ht), hemoglobin (Hb) and white blood cells (WBCs). Ht was determined as described by Reitman and Frankel (1957), hemoglobin (Hb) was determined by the hemoglobin kits which is a standardized procedure of the cyanomet hemoglobin method as well as the total count of white blood cells (WBCs) was carried out by the indirect method (Martins et al. 2004). The second portion of the blood sample was allowed to clot overnight at 4 °C, centrifuged at 3,000 rpm for 10 min. The non-hemolysed serum was collected and stored at –20 °C until use. Levels of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) according the method described by Reitman and Frankel (1957) and serum creatinine was measured by calorimetric method and enzymatic determination methods as described by Henry (1974).

Serum total immunoglobulin M (IgM) levels were measured by an Enzyme-Linked Immunosorbent Assay (ELISA) using a kit (Cusabio, Wuhan, Hubei, China).

#### DNA fragmentation

DNA fragmentation was determined using Pernadones methods (Pernadones et al. 1993): liver tissues were dissociated in 400 µl hypotonic lysis buffer (0.2 % Triton X-100, 10 mM Tris and 1 mM EDTA, pH 8) and centrifuged 15 min at 13,800 × g. The supernatant (SN) containing small DNA fragments was separated immediately as well as the pellet containing large pieces of DNA and cell debris was used for the

diphenylamine assay (DPA). DPA reaction: perchloric acid (0.5 M) was added to the pellets containing uncut DNA (resuspended with 200  $\mu$ l of hypotonic lysis buffer) and the SN followed by 2 volume of a solution containing (0.088 M DPA, 98 % v/v glacial acetic acid, 1.5 % v/v sulfuric acid and 0.5 % v/v of 1.6 % of acetaldehyde solution). The samples were stored at 4 °C for 48 h. The colorimetric reaction was quantitated spectrophotometrically at 575 nm optical density (OD) using an ELISA reader (SLT Labinstruments A-5082, Austria). The percentage of DNA fragmentation was expressed by the formula:

$$\% \text{ DNA} = 100 \times \frac{\text{OD SN}}{\text{OD SN} + \text{OD pellet}}$$

### Histopathological studies

Liver and gill samples of the same collected fish were carefully removed and fixed in 10 % formalin, dehydrated in ascending grades of alcohol and cleared in xylene. The fixed tissues were embedded in paraffin wax and sectioned into five micrometers in thickness by Euromex Holland microtome. Section was stained according to Harris hematoxylin and eosin method. Consequently, these sections were examined microscopically and their photos were taken using a microscopic camera (Bernet et al. 1999).

### Statistical analysis

All data were analyzed by software SAS (Statistical Analysis System 1993) ANOVA procedure. One-way analysis of variance (One-way ANOVA) was used to determine whether significant variation existed between the treatments. Two-way ANOVA was used for analyzing the individual effects of fungicide levels and vitamin E, and the interaction between them. Duncan's multiple range test (Duncan 1955) was used to compare differences among individual means. All differences were considered significant at  $P < 0.05$ . All percentage data were arc-sin transformed prior to analysis (Zar 1984); however, data are presented untransformed to facilitate comparisons.

## Results

In the present study, the 96 h  $LC_{50}$  of copperoxychloride was found to be 129.21 mg/l.

During the 12 weeks the water temperature was  $26.17 \pm 0.8$  °C: dissolved oxygen,  $5.6 \pm 0.8$  mg/l: total ammonia,  $0.18 \pm 0.12$  mg/l and pH  $8.52 \pm 0.3$ .

Effect of vitamin E (vit.E) and copperoxychloride and their interactions on growth performance and feed utilization of treatment groups are presented in Table 2. Results indicates that FBW, WG, SGR, FI and PER were significantly higher ( $P < 0.05$ ) in T2 and T3 compared to all treatments. The significant ( $P < 0.05$ ) value lowest of FBW, WG, SGR, FI and survival rate (SR) were recorded in T4, whereas T5, which was exposed to 0.25  $LC_{50}$  copperoxychloride and fed diet supplemented with 1 g vit.E/kg showed similarity with T1 in FBW, WG, SGR and SR and the differences among treatments were not significant ( $P > 0.05$ ). In the same respect, FCR was significantly ( $P < 0.01$ ) lowest (better) in T2 and T3 in comparison to the other groups.

The changes in some hematological and some biochemical parameters of Nile tilapia were affected significantly ( $P < 0.05$ ) by sublethal fungicide level and vit.E and their interactions (Table 3). AST, ALT, uric acid and creatinine were significantly higher ( $P < 0.01$ ) in fish T4 which was exposed to 0.25  $LC_{50}$  and fed diet unsupplemented with vitamin E in comparison to the other treatments, but the lowest was one obtained by fish of T3 which were not exposed to fungicide and fed 2 g vit.E/kg diet. Moreover, fish of T6 which were exposed to 0.25  $LC_{50}$  of copperoxychloride and fed diet supplemented with 2 g vitamin E/kg showed similarity with T1 in respect to values of AST, uric acid and creatinine. Ht and Hb showed the opposite trend of biochemical parameters, with the highest Ht and Hb values recorded by fish of T3 and the lowest one obtained by fish of T4.

WBCs and IgM were significantly ( $P < 0.05$ ) affected by sublethal fungicide and level of supplemented vitamin E and their interactions (Table 4). High WBCs and IgM were recorded in T2 and T3, but the lowest values of WBCs and IgM were recorded by fish of T4 which exposed to 0.25  $LC_{50}$  of copperoxychloride.



**Table 2** Effect of fungicide and vitamin E-supplement levels and their interactions on body weight, weight gain, specific growth rate, survival rate, feed intake, feed conversion ratio and protein efficiency ratio of *O. niloticus*

T	Fungicide	Vit.E (g/kg diet)	Body weight		WG	SGR	FI	FCR	PER	SR%
			IBW	FBW ± SE						
T1	0	0	1.94	19.50 ± 0.73 <sup>ab</sup>	17.27 <sup>b</sup>	2.41 <sup>c</sup>	30.93 <sup>b</sup>	1.79 <sup>ab</sup>	1.85 <sup>b</sup>	91.66 <sup>a</sup>
T2	0	1	2.04	21.94 ± 0.73 <sup>a</sup>	19.73 <sup>a</sup>	2.55 <sup>b</sup>	31.48 <sup>a</sup>	1.62 <sup>c</sup>	2.05 <sup>a</sup>	93.33 <sup>a</sup>
T3	0	2	1.98	21.20 ± 0.71 <sup>a</sup>	20.11 <sup>a</sup>	2.62 <sup>a</sup>	32.15 <sup>a</sup>	1.60 <sup>c</sup>	2.08 <sup>a</sup>	95.00 <sup>a</sup>
T4	0.25	0	1.96	18.56 ± 0.75 <sup>b</sup>	16.20 <sup>d</sup>	2.29 <sup>d</sup>	29.15 <sup>d</sup>	1.82 <sup>a</sup>	1.85 <sup>b</sup>	85.00 <sup>b</sup>
T5	0.25	1	1.92	19.66 ± 0.73 <sup>ab</sup>	17.45 <sup>b</sup>	2.43 <sup>c</sup>	29.72 <sup>c</sup>	1.77 <sup>ab</sup>	1.88 <sup>b</sup>	91.66 <sup>a</sup>
T6	0.25	2	1.95	18.83 ± 0.71 <sup>b</sup>	16.76 <sup>c</sup>	2.45 <sup>c</sup>	29.77 <sup>c</sup>	1.71 <sup>b</sup>	1.95 <sup>b</sup>	92.50 <sup>a</sup>
Pooled SE			0.11		0.163	0.021	0.273	0.028	0.030	1.29
Two-way ANOVA ( <i>P</i> value)										
Fungicide				0.0030	0.001	0.001	0.001	0.025	0.0241	0.0054
Vitamin E				0.0336	0.001	0.001	0.0001	0.043	0.0471	0.0056
Fungicide × Vit.E				0.0004	0.001	0.001	0.0001	0.018	0.0189	0.0074

Data are presented as mean ± standard error (SE). Means followed by different letters in each column are significantly ( $P < 0.05$ ) different

IBW initial body weight (g), FBW final body weight (g), WG weight gain, SGR specific growth rate, SR survival rate, FI feed intake (g), FCR feed conversion ratio, PER protein efficiency ratio

**Table 3** Effect of fungicide and vitamin E-supplement levels and their interactions on alanine aminotransferase, aspartate aminotransferase, hematocrit, hemoglobin, uric acid and creatinine levels of *O. niloticus*

T	Fungicide	Vit.E (g/kg diet)	ALT (IU/l)	AST (IU/l)	Ht (%)	Hb (g/dl)	Uric acid (mg/100 ml)	Creatinine (mg/100 ml)
T1	0	0	19.96 <sup>d</sup>	93.62 <sup>b</sup>	21.38 <sup>ab</sup>	11.33 <sup>bc</sup>	11.13 <sup>cd</sup>	0.657 <sup>c</sup>
T2	0	1	20.17 <sup>d</sup>	93.67 <sup>b</sup>	22.33 <sup>a</sup>	11.48 <sup>ab</sup>	11.03 <sup>cd</sup>	0.623 <sup>c</sup>
T3	0	2	19.89 <sup>d</sup>	92.10 <sup>b</sup>	22.81 <sup>a</sup>	11.70 <sup>a</sup>	10.80 <sup>d</sup>	0.617 <sup>d</sup>
T4	0.25	0	47.94 <sup>a</sup>	99.02 <sup>a</sup>	19.13 <sup>c</sup>	10.02 <sup>d</sup>	16.11 <sup>a</sup>	0.920 <sup>a</sup>
T5	0.25	1	33.88 <sup>b</sup>	95.51 <sup>ab</sup>	20.29 <sup>bc</sup>	11.10 <sup>c</sup>	12.24 <sup>b</sup>	0.810 <sup>b</sup>
T6	0.25	2	32.55 <sup>c</sup>	94.69 <sup>b</sup>	21.27 <sup>ab</sup>	11.13 <sup>c</sup>	11.87 <sup>bc</sup>	0.660 <sup>c</sup>
Pooled SE			0.328	1.14	0.477	0.086	0.263	0.012
Two-way ANOVA ( <i>P</i> value)								
Fungicide			0.0001	0.0055	0.0005	0.0001	0.0001	0.0001
Vit.E.			0.0001	0.0078	0.0122	0.0001	0.0001	0.0001
Fungicide × Vit.E			0.0001	0.0230	0.0030	0.0001	0.0001	0.0001

Data are presented as mean ± standard error (SE). Means followed by different letters in each column are significantly ( $P < 0.05$ ) different

ALT alanine aminotransferase, AST aspartate aminotransferase, Ht hematocrit, Hb hemoglobin

Whereas, there were no significant ( $P > 0.05$ ) differences between T5, T6 exposed to 0.25 LC<sub>50</sub> and fish fed diet supplemented with 1 or 2 g/kg diet, respectively.

The DNA fragmentation was significantly ( $P < 0.05$ ) affected by sublethal fungicide and vitamin E supplementation and their interactions; it was increased significantly with fungicide exposure and a positive trend was shown in diet supplemented with vitamin E. The highest DNA fragmentation was obtained by fish of T4 which were exposed to 0.25 LC<sub>50</sub> of copperoxychloride and fed diet unsupplemented with vit. E. T5 and T6 showed the same trend of DNA fragments T1 group without any significant differences between T1, T5 and T6.

Liver histology from control and exposed Nile tilapia is briefly illustrated in Fig. 1a–f. In control group, the liver of many samples exhibited a normal architecture and there were no pathological abnormalities, with hepatocytes presenting a homogenous cytoplasm, and a large central or subcentral spherical nucleus, only

**Table 4** Effect of fungicide and vitamin E-supplement levels and their interactions on white blood cells (WBCs), immunoglobulin M (IgM) and DNA fragmentation of *O. niloticus*

T	Fungicide	Vit.E (g/kg diet)	WBCs ( $\times 10^3 \text{ mm}^{-3}$ )	IgM ( $\mu\text{g/ml}$ )	DNA fragmentation (%)
T1	0	0	15.06b	159.79b	5.57b
T2	0	1	16.85a	164.41a	5.63b
T3	0	2	17.74a	165.91a	5.39b
T4	0.25	0	10.70c	143.28c	9.68a
T5	0.25	1	15.32b	159.10b	6.69b
T6	0.25	2	15.81b	160.33b	5.59b
Pooled SE			0.309	0.747	0.480
Two-way ANOVA ( <i>P</i> value)					
Fungicide			0.0001	0.0001	0.0011
Vit.E.			0.0001	0.0001	0.0035
Fungicide $\times$ Vit.E			0.0001	0.0001	0.006

Data are presented as mean  $\pm$  standard error (SE). Means followed by different letters in each column are significantly ( $P < 0.05$ ) different

some samples showed fatty degeneration (Fig. 1a). The hepatic parenchyma of fish fed a diet supplemented by 1 g Vit.E showed degeneration (Fig. 1b). While fish fed a diet supplemented by 2 g vitamin E showed pyknosis (Fig. 1c). Additionally, *O. niloticus* exposed to copperoxychloride cleared, hemorrhage in their hepatic parenchyma (Fig. 1d). However, the hepatic parenchyma of fish exposed to copperoxychloride and fed 1 g vit.E suffer only from focal necrosis (Fig. 1e). Finally, *O. niloticus* exposed to copperoxychloride and fed 2 g vit.E showed congestion in blood sinusoid (Fig. 1f).

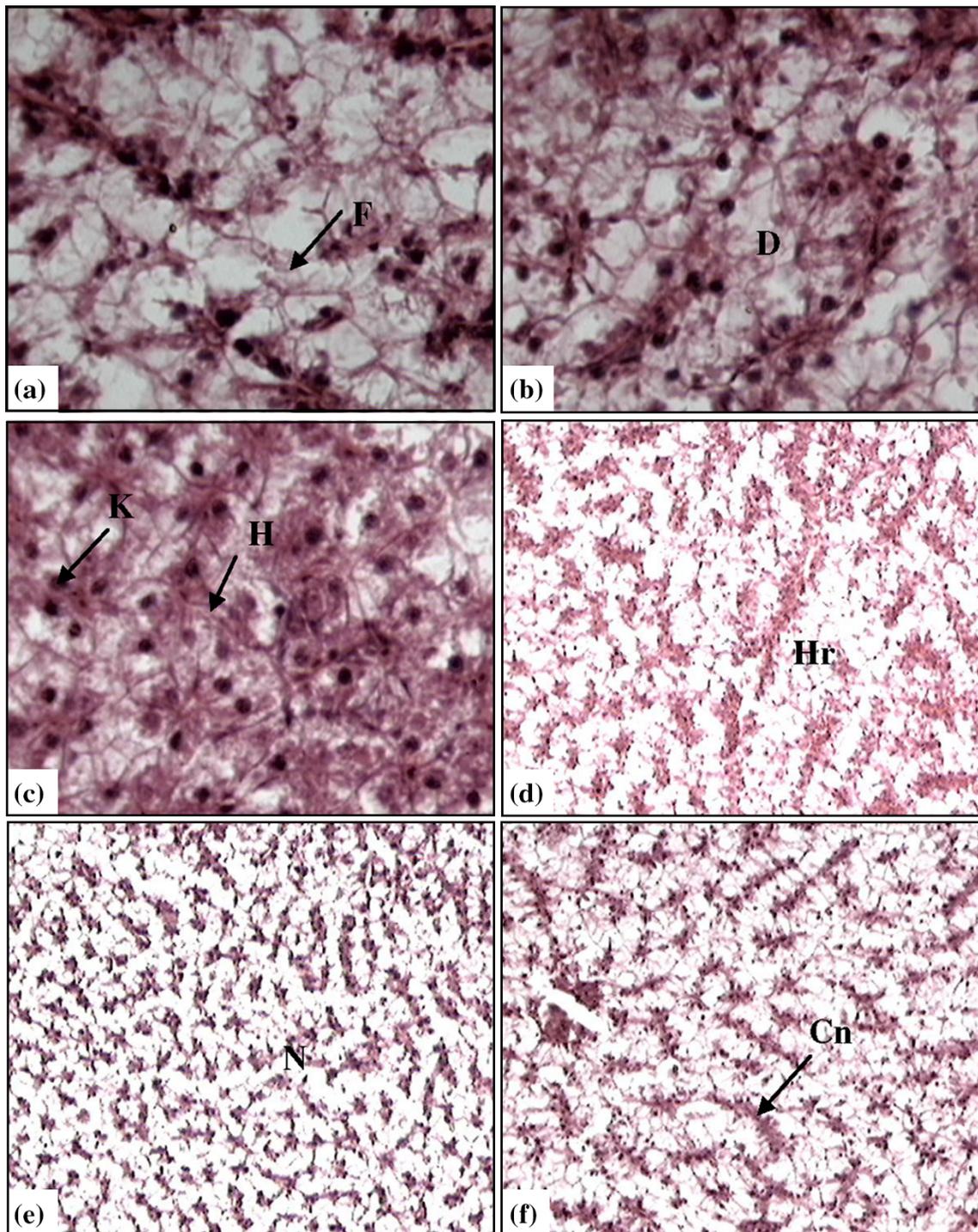
Gill, histology from control and exposed fish is briefly illustrated in Fig. 2a–f. The gill morphology of the control tilapia was similar to that of other teleost fish species (Wilson and Laurent 2002). In fish fed diet with vit.E and not treated with fungicide, the gill was made up of double rows of filaments (primary lamellae) from which arise perpendicularly the lamellae (secondary lamellae). The lamellae are lined by a squamous epithelium composed by pavement and non-differentiated cells. Gill obtained from fish fed diet supplemented with 1 and 2 g vitamin E show normal appearance of gill primary and secondary lamellae. On the other hand, only some samples showed cell degeneration (Fig. 2a–c). While fish exposed to copperoxychloride showed hemorrhage in primary lamellae and separation in epithelial cells of secondary lamellae (Fig. 2d). However, fish exposed to copperoxychloride and fed 1 g vit.E suffer from necrosis and curling in their gill epithelial cells of secondary lamellae (Fig. 2e). Finally, fish exposed to copperoxychloride and fed 2 g vit.E showed separation in epithelial cells of secondary lamellae (Fig. 2f).

## Discussion

The obtained result indicated a significant decrease in the growth performance of *O. niloticus* after exposure to sublethal dose of copperoxychloride fungicide. In *Channa punctatus* the most important factors decreasing growth consist of disorder in feeding behaviors, decrease in feeding rate, dysfunction in metabolism process and waste of energy to overcome the stress caused by insecticide exposure (Tripathi et al. 2003). The reduced growth is in agreement with the results of Sweilum (2006) who found that the growth of *O. niloticus* decreased due to the exposure to dimethoate (20, 10 and 5 mg/l) and malathion (2, 1 and 0.5 mg/l). Similarly, Mousa et al. (2005) reported that Nile tilapia exposed to basegran herbicide at field concentration (0.004 mg/l = 1/100 LC<sub>50</sub>) and at half of field concentration showed significant ( $P < 0.05$ ) reduction in growth with increasing the level of pesticide compared with the control fish. Moreover, Jiann and Chia (2001) revealed that the mortality rates of *Penaeus monodon* juveniles following exposure to 0 (control), 0.45, 0.90, 1.80 and 4.50 mg/l copper concentrations after 30 days were 0, 5.6, 22.2 and 55.6 %, respectively.

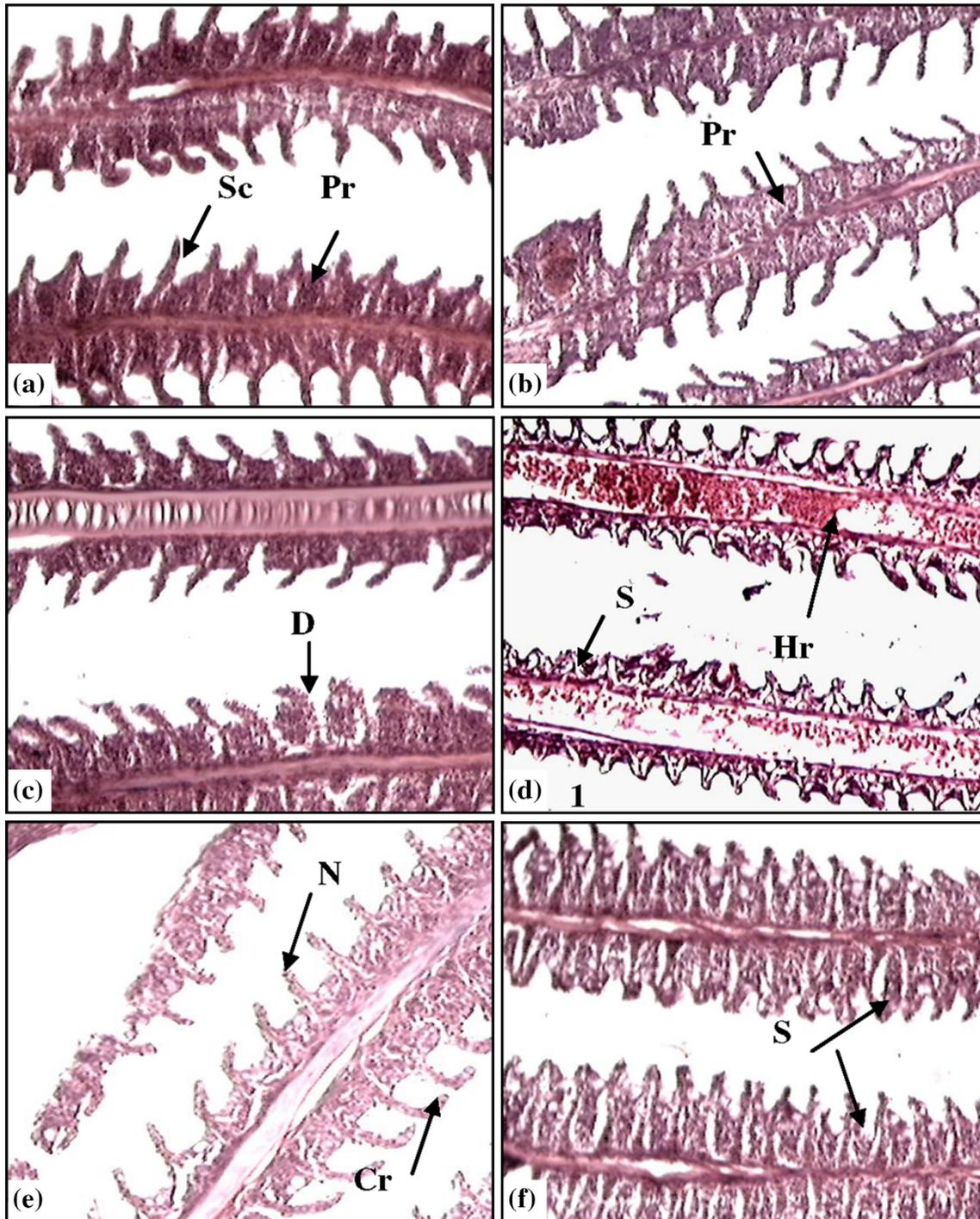
Irrespective of copperoxychloride levels, the diet supplemented with 1 and 2 g vit.E/kg diet had better results of growth performance and feed utilization than those of fish fed diets unsupplemented with vit.E.





**Fig. 1** Liver sections of *O. niloticus* fish stained with H&E. **a** Fatty degeneration (normal), **b** degeneration (D) (1 g vit.E), **c** hepatic cell (H) and pyknosis (K) (2 g vit.E), **d** severe hemorrhage (Hr) (copperoxychloride), **e** focal necrosis (N) (copperoxychloride and 1 g vit.E), **f** congestion in blood sinusoid (Cn) (copperoxychloride and 2 g vit.E)

These results are similar to those reported by, Kim et al. (2003) who mentioned that Nile tilapia fed diet supplemented with 150 mg vit.E had significantly ( $P < 0.05$ ) higher growth performance than those fed the control diet. Also, Galaz et al. (2010) noted that dietary inclusion of vit.E significantly influenced growth performance and feed utilization of the juvenile parrot fish (*Oplegnathus fasciatus*). Up to 38 mg vit.E/kg diet



**Fig. 2** Gill longitudinal section of *O. niloticus* fish stained with H&E. **a** Primary lamellae (Pr) and secondary lamellae (Sc) (normal). **b** Primary lamellae (Pr) (1 g vit.E). **c** Degeneration (D) in secondary lamellae (2 g vit.E). **d** Hemorrhage (Hr) in primary lamellae and separation (S) in epithelial cells of secondary lamellae (copperoxychloride). **e** Necrosis (N) and curling (Cr) in epithelial cells of secondary lamellae (1 g vit.E and copperoxychloride). **f** Separation (S) in epithelial cells of secondary lamellae (2 g vit.E and copperoxychloride)

and beyond that level no further increase was observed. The same pattern was also noticed for protein efficiency ratio (PER). Lower feed conversion ratio (FCR) was observed in fish fed the 38 mg vit.E/kg diet. Similarly, Sau et al. (2004) reported that, in rohu fry fed five semi-purified diets supplemented with 0, 50, 100,



150, 200 mg vit.E/kg diet as DL- $\alpha$ -tocopheryl acetate, fish fed diets containing less than 100 mg supplemental vit.E/kg diet had significantly ( $P < 0.05$ ) reduced WG, FER and other nutritional indices compared to those fed diets supplemented with vit.E at 100–200 mg/kg diet. Values of PER were significantly higher in 115.70 mg/kg diet vs other groups and lowest in control group (without supplementation of vit.E), at the last, FCR was observed in fish fed the diet supplemented with 100, 150 and 200 mg/kg diets. Furones et al. (1992) observed that in rainbow trout (*Oncorhynchus mykiss*) fed diets containing 7, 86 or 806 mg vit.E/kg diet for 22 weeks and exposed to virulent *Yersinia ruckeri* by bath or injection, mortalities were always least among those fed the highest concentration of vit.E. Similar trend was observed by Abdel-Tawab (1999) and Sweilum (2000) with *Mugil cephalus* and *O. niloticus*, respectively.

The present study indicated slight differences between fish untreated with fungicide and fed normal level of vit.E and fish exposed to 0.25 LC<sub>50</sub> copperoxychloride and fed 1 or 2 g vit.E. This indicates that 1 or 2 mg vit.E/kg diet of fish minimized the toxic effect of copperoxychloride. The outcomes of the present study came in agreement with those of Mourente et al. (2002) that recorded that growth and survival of sea bream were relatively unaffected by heavy metals in a vit.E-supplemented diet. Also, Steven (2002) recorded growth depression in fish fed a Pb-supplemented diet was prevented with the supplementation of antioxidants.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes are liver enzymes and a kind of transferase. They have the function of transferring amino group from alpha-amino acids to alpha-keto acids. Large amount of ALT and AST is released into blood mostly during liver cell damage. Thus, detection of serum level of ALT and AST tells monitored liver cell damage (Soltan et al. 2008). The results of the present study indicated that ALT and AST activities were increased significantly ( $P < 0.05$ ) with exposure to sublethal dose of copperoxychloride fungicide. The increase of ALT and AST activities in fish exposed to copperoxychloride level might be due to dysfunction and hepatocellular damage of liver. These results are in accordance with those reported by many investigators. Hashim and Zaki (2005) reported that catfish exposed to fungicide Dithane in dose of 0.5 ppm/l for 30 days showed an increase ( $P < 0.05$ ) in serum ALT and AST activities compared with the control. Jiraungkoorskul et al. (2003) indicated that *O. niloticus* exposed for 3 months to 5 and 15 ppm of the commercial herbicide Roundup showed significant ( $P < 0.05$ ) increase in plasma ALT and AST activities. Also, Ramzy (2009) indicated that ALT and AST enzyme activities of Nile tilapia were significantly ( $P < 0.05$ ) increased throughout the whole experimental period of acute exposure and chronic exposure (1/10 LC<sub>50</sub>) to seletoron insecticide. The AST activity of fish exposed to 0.25 LC<sub>50</sub> of copperoxychloride increased in the present study; however, amelioration in the AST activity of fish exposed to fungicide and fed high level of vit.E could be the consequence of vit.E increasing the anti-stress ability. These results confirmed those of Metwally et al. (2002) who reported that in *O. niloticus* receiving 500 mg CuSO<sub>4</sub>/kg diet and fed diets supplemented with (0, 250, 500, 750 and 1,000 mg vit.E/kg diet), the activity of liver function enzymes AST and ALT increased significantly in the blood serum of specimens fed the diet containing 500 mg Cu/kg diet compared to the control diet.

The present study indicated a significant decrease in Ht and Hb after exposure to a sublethal dose of copperoxychloride. These results may be due to the inhibition of erythropoiesis, hemosynthesis, osmoregulatory dysfunction or due to increased rate of erythrocyte destruction in the hematopoietic organ, as came in *Catla catla* exposed to deltamethrin as recorded by Vani et al. (2011). Also, Svobodova et al. (2003) reported significantly lower values of red blood cells and Hb in *Cyprinus carpio* after acute exposure to deltamethrin. The reduction in hemoglobin might be due to the inhibition of erythropoiesis, hemosynthesis, osmoregulatory dysfunction or due to increased rate of erythrocyte destruction in the hematopoietic organ, as reported in *Cyprinus carpio* exposed to endosulfan (Jenkins et al. 2003). Whereas fish exposed to 0.50 and fed diet supplemented with 1 or 2 g vit.E/kg diet showed slightly increased Ht and Hb compared to the fish not exposed to fungicide and fed diet with normal level of vit.E, this indicates that supplemented diet with 1 or 2 g vit.E decreased the toxic effect of copperoxychloride and stimulated immune response to cope up the copperoxychloride stress. In this respect, Wise et al. (1993) reported that susceptibility of red blood cells of channel catfish fingerlings to oxidative hemolysis decreased with increasing levels of dietary  $\alpha$ -tocopherol acetate.

Serum uric acid and creatinine can be used as a rough index of the glomerular filtration rate (Maita et al. 1984). Low values of creatinine and uric acid have no significant, but increased values cause several disturbances in the kidney (Maxine and Benjamin 1985). The increased uric acid and creatinine activities were shown by fish exposed to copperoxychloride. The obtained results are in agreement with those obtained by Abdel-Tawwab et al. (2007) who reported that *O. niloticus* exposed to copper concentrations 0.503 or 1.25 mg



$\text{Cu}^{2+}/\text{l}$  showed significant ( $P < 0.05$ ) increase in creatinine and uric acid. Also, Hashim and Zaki (2005) reported that catfish exposed to fungicide Dithane at dose of 0.5 ppm/l for 30 days revealed an increase ( $P < 0.05$ ) in creatinine and uric acid values compared with the control. However, supplemented diet with 1 or 2 g vit.E/kg diet decreased serum uric acid and creatinine activity for fish exposed to 0.25  $\text{LC}_{50}$  of copperoxychloride, this might be due to the protective function of vit.E. In partial agreement with our results, Metwally et al. (2002) reported non-significant changes in serum creatinine of *O. niloticus* fed diet containing to vit.E, on the other hand uric acid was significantly increased ( $P < 0.01$ ) in blood serum of *O. niloticus* fed diet containing 500 mg  $\text{CuSO}_4/\text{kg}$  diet compared to group treated with vit.E. Also, Senug et al. (2007) recorded that vit.E appears to provide protection against oxidative stress caused by Pb toxicity.

The present study showed a significant decrease in WBC counts in fish exhausted by copperoxychloride fungicide; this result may be due to the inhibition of the hemopoietic tissue activity. In agreement with the results in this study, Svoboda et al. (2003) concluded that the changes in leukocyte differential count gave an evidence for decreased level of non-specific immunity in fish after exposure to deltamethrin. *O. niloticus* exposed to low levels of the organophosphate pesticide chlorpyrifos have significantly lower total pronephros cell counts and depressed macrophage phagocytic ability compared to untreated controls (Holladay et al. 1996). Also, Khoshbavar-Rostami et al. (2006) indicated that great sturgeon (*Huso huso*) exposed to a long-term sublethal concentration of diazinon showed significantly ( $P < 0.05$ ) lower WBCs and lymphocytes values compared with the control fish group. However, the decrease in WBCs differential count of fish exposed to sublethal levels of the copperoxychloride may be due to the toxicant effect on the fish cellular immunity. Similarly, Wise et al. (1993) reported that the ability of macrophages to phagocytize virulent *Edwardsiella ictaluri* was enhanced by vit.E, but agglutinating antibody titers were not affected by dietary vit.E levels for channel catfish fingerlings fed with purified diets containing  $\alpha$ -tocopherol acetate to provide 0, 60 and 2,500 mg vit.E/kg for 180 days. Verlhac et al. (1991) also concluded that vit.E deficiency impaired antibody response and lymphoproliferation. Conversely, Vani et al. (2011) reported that the total leukocyte count was significantly less ( $P < 0.05$ ) in fish exposed to deltamethrin concentration of 1.61  $\mu\text{g}/\text{l}$  and fed normal diet. The WBCs was significantly increased ( $P < 0.05$ ) in fish exposed to 0.25 copperoxychloride level and fed diet supplemented with 1 and 2 g vit.E/kg and recorded the value cope up treatment of zero fungicide.

Serum immunoglobulins are major components of the humoral immune system and IgM is the main immunoglobulin present in fish (Wilson et al. 1995). Our study revealed the significant decrease in IgM values in *O. niloticus* exposed to copperoxychloride fungicide chronic exposure (90 days). In full agreement with results of the effect of copperoxychloride level on IgM values, Hashim and Zaki (2005) reported that, in catfish exposed to fungicide Dithane in dose of 0.5 ppm/l (equivalent to 1/10 of  $\text{LD}_{50}$ ) for 30 days, IgM in serum decreased significantly ( $P < 0.05$ ) compared with control. Similarly, Giron et al. (2009) reported that the IgM levels in plasma from *Oreochromis niloticus* exposed to 1/20  $\text{LC}_{50}$  (0.39) and 1/10  $\text{LC}_{50}$  (0.78) mg/l diazinon were not affected, but at 1.96 mg/l diazinon increased IgM concentrations for 96 h (short exposure) and also observed that fish could be exposed to concentrations of diazinon for long periods (chronic exposure) or in combination with another pesticide. Similarly, Martha et al. (2010) revealed that *O. niloticus* exposed to short sublethal (7 ppb, 96 h) of insecticide endosulfan increased significantly ( $P < 0.05$ ) secretion of IgM. The obtained results revealed that, IgM values were significantly increased ( $P < 0.05$ ) in fish exposed to 0.25  $\text{LC}_{50}$  copperoxychloride level and fed diet supplemented with 1 and 2 g vit.E/kg and recorded the value cope up treatment of zero fungicide. Ndoye et al. (1989) reported that only an overdose of vit.E improved the antibody production of rainbow trout. Waagbø et al. (1993) showed higher total antibody level in Atlantic salmon fed vit.E-supplemented diets, an opposite effect to the differences found as regards to specific antibodies. In this respect, Verlhac et al. (1991) observed that antibody titre against red mouth disease at 40 days after vaccination was higher for fish fed 450 mg vitamin E kg/diet as compared to those fed at 45 mg/kg. However, the beneficial effect on stimulation was time dependent, and after 120 days of feeding no difference was observed. Also, Sahoo and Mukherjee (2002) reported that vitamins E and C increased specific immunity and reduced mortality only in immunocompromised fish and failed in healthy fish. Lall and Olivier (1991) recommended that higher dose of vitamin E (>2,000 mg/kg) should not be used in studies on immune response of fish because of problems due to hypervitaminosis.

DNA damage has been proposed as useful parameter for assessing the genotoxic properties of environmental pollutants (Kohn 1983). Our results revealed that fish exposed to sublethal doses of copperoxychloride fungicide had significantly ( $P < 0.05$ ) increased DNA damage. The present investigation is in agreement with



the results obtained by Mahmoud (2006) who revealed that both agricultural and industrial water pollution caused an increase in the percentage of DNA fragmentation in *Tilapia Zillii* but the percentage of industrial pollution was higher than that of agriculture. Also, Vittoria et al. (2004) found that at the middle part of Gota harbor, which is chronically subjected to heavy chemical pollution, the viviparous eelpout (*Zoarces viviparus*) had a high level of DNA damage. Arabi (2004) studied the effect of metal ion contamination on the functional capacity of carp gill cells with antioxidant interactions in an in vitro study. The results obtained showed that copper and mercury could cause a high rate of DNA breaks (single stranded) in carp gill cell suspensions as a comet appearance and DNA fragmentation is frequent in metal ion contamination. Similarly, Chang et al. (2009) reported that, DNA damage in hemocytes and hepatopancreas cells of shrimp *Litopenaeus vannamei* increase after treatment with cadmium chloride ( $\text{CdCl}_2$ ) compared with controls. They also indicated that Cd can induce oxidative stress and DNA damage in the shrimp *L. vannamei*. Moreover, the results also demonstrated that this parameter can be used as sensitive indicators of exposure to this genotoxicant.

Histology has been used as a test for evaluating toxic effects of water pollutants (Eifac 1983; Murthy 1986). Liver is responsible for the digestion, filtration and storage of glucose. It is considered as the principle organ of detoxification and good indicator of aquatic environmental pollution (Ahmed 2012). The liver was examined because it plays a primary role in the metabolism and excretion of xenobiotic compounds with morphological alterations occurring in some toxic conditions (Rocha and Monteiro 1999). Metals can either increase or decrease hepatic enzyme activities and can lead to histopathological hepatic changes, depending on the metal type and concentration, fish species, period of exposure and other factors (Paris-Palacios et al. 2000). The histological changes in fish liver are a highly sensitive and accurate way to assess the effect of xenobiotic compounds in field and experimental studies (Figueiredo-Fernandes 2007). In a previous study, the normal morphology of the liver Nile tilapia was uniform in appearance and dark red in color. Moreover, liver in normal histology was made up of hepatocytes which were polygonal cells with a central spherical nucleus and a densely stained nucleolus. These cells were arranged in branches separated by sinusoids (Figueiredo-Fernandes et al. 2006). The present study also demonstrates that the liver of control fish exhibits a normal appearance and structure with no pathological abnormalities. The hepatocytes present a homogenous cytoplasm and a large central or subcentral spherical nucleus, except some samples only showed fatty degeneration. However, fish exposed to copperoxychloride cleared severe hemorrhage in hepatic tissue; this alteration is in agreement with the results obtained by Figueiredo-Fernandes et al. (2007) for the same species exposed to waterborne copper. Several studies had shown a variety of changes in the liver of *O. niloticus*, resulting from exposure to different toxic chemicals (Visoottiviseth et al. 1999; Figueiredo-Fernandes et al. 2006). Moreover, it was also reported by several studies that chronic copper accumulation in the liver of fish causes hepatocyte lysis, cirrhosis and ultimately death (Pourahamad and O'Brien, 2000; Varanka et al. 2001; Păunescu et al. 2010). Osman et al. (2009) observed similar liver changes following the administration of copper sulfate by *O. niloticus*. On the other hand, fish exposed to copperoxychloride and fed diet supplemented with vit.E in present study showed decrease in the appearance of several liver alterations. Also, fish fed diet supplemented with vit.E and not treated with copperoxychloride showed no lesions of liver. These results are in agreement with those of Mahmoud (2009). Vitamin E deficiency could cause hepatic necrosis in fish (Idris and Hassan, 2002). Wahli et al. (1998) studied the effect of combinations of vitamin C and E on immune response and disease resistance and found increasing in macrophage oxidative burst activity in fish receiving both vitamins at high levels.

Gills participate in many important functions in fish, such as respiration, osmoregulation and excretion. They remain in close contact with the external environment, and particularly sensitive to changes in the water quality. Also they are considered the primary target of the contaminants (Mazon et al. 2002). In the present study, histological examination of *O. niloticus* gills shows a typical structural organization of the lamellae in fish fed normal level, 1 and 2 g vit.E/kg diet and not exposed to copperoxychloride; some samples showed cell degeneration in secondary lamellae. However, fish exposed to copperoxychloride show several histological alterations, namely hemorrhage in primary lamellae and separation in secondary lamellae. These observations are in agreement with those obtained by Figueiredo-Fernandes et al. (2007) on the same species of fish with exposure of copper metals. These histological alterations of gill have been observed also by several authors in fish and shellfish submitted to copper (Karan et al. 1998; Chen and Lin 2001; De Boeck et al. 2001). Fish exposed to copperoxychloride and 1 g vit.E showed necrosis and curling in secondary lamellae, while those exposed to copperoxychloride and 2 g vit.E cleared separation in secondary lamellae. In the present study, the observed histological alterations of gill and liver have been related to copperoxychloride exposure.



## Conclusion

The exposure of *O. niloticus* to sublethal concentration of copperoxychloride caused alterations in growth, blood parameters, DNA fragmentation and histology. These alterations were reversed to a great extent with supplement of higher dose of vitamin E in diets. We recommended that cultured fish will be protected against any possible pesticide or fungicide stress using natural antioxidants such as vitamin E by supplemented it with higher level than it is normally required in the fish diet.

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**Authors' contributions** MH carried out the molecular genetic studies, participated in the sequence alignment, and drafted the manuscript, carried out the immunoassays and design the study and performed the statistical analysis. AG conceived of the study and participated in its design and coordination. SM and ST carried out the histology study. All authors read and approved the final manuscript.

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