Alexandria Journal of Veterinary Sciences
www.alexjvs.com

AJVS. Vol. 55 (2): 36-49. Oct. 2017
DOI: 10.5455/ajvs.276295

Effect of Dietary Vitamin C and B-Glucan to Alleviate the Toxic Effect of Copper Sulphate in Tilapia Fish

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Key words:
Nile tilapia – Copper toxicity – Vitamin C – β-glucan–Growth performance – Histopathological changes.

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ABSTRACT

This study was designed to investigate the possibility of copper (Cu) to induce oxidative stress and biochemical perturbations in Nile tilapia liver, spleen, gills, kidney, intestine and muscle and the role of Vitamin C (Vit. C) and/or β-glucan in alleviating its toxic effects. Nile tilapia fish were randomly allotted into five groups of twenty-five each, group one served as control without any treatment, group two exposed to Cu (100µg/liter water), group three to five exposed to Cu plus Vit. C (500 mg/Kg diet), β-glucan (100mg/Kg diet) or combination of Vit. C and β-glucan respectively. Fish exposed to copper significantly (P≤0.05) reduced final weight, total gain, weight gain%, RGR and significantly deteriorate FCR and PER compared to control, while, Vitamin C and/or β-glucan supplementation markedly improved the mentioned parameters. Copper exposure reduce blood serum total protein, triglycerides and total cholesterol concentrations, while significantly increased serum uric acid, urea and concentrations compared with control. Moreover, copper exposure increased serum AST, ALT, CAT and SOD enzymes activities and significantly reduced phagocytic index and activity compared to control. Vitamin C and/or β-glucan supplementation with copper exposure maintained the previous parameters near the normal and modulated antioxidant enzymes. Also, Vit. C or β-glucan reduce Cu residue in Nile tilapia muscle. Cu exposure leading to various pathological changes in liver (necrosis and mild vacuolation), kidney (tubular vacuolation), spleen (lymphoid depletion), gills (lamella fusion), intestine (enteritis) and muscles (Zenker's necrosis), while dietary Vit.C and/or β-glucan supplementation reduce the pathological lesions induced by copper exposure. The present study could concluded that dietary supplementation of 500 mg/Kg coated vitamin C plus 100 mg/Kg β-glucan reduced the harmful effects of Cu on Nile tilapia.

1. INTRODUCTION

Among all types of pollution, aquatic pollution is of greater concern as each and every kind of the life depends on water. Among all types of aquatic pollutants, heavy metals are of greatest concern. Heavy metals when reach the aquatic bodies deteriorate the life sustaining quality of water and cause damages to both flora and fauna (Girish and Bijoy, 2014).Even though most of the heavy metals are micronutrients, they exert a prominent role in environmental deterioration. The heavy metal and pesticide contamination of aquatic ecosystems has increased manifold in the last few decades due to their extensive use in agricultural, chemical and industrial processes and is a real threat to the aquatic fauna.

Among metals, copper is used in industries manufacturing organic chemicals, fertilizers, iron and steel works, electrical works, antifouling paints, pulp and paper industries, pesticides, fungicides and automobile accessories. Even though copper is an

essential trace element required in low concentrations, it is discharged into freshwater environments in large concentrations as an industrial effluent and severely affect the freshwater fauna, especially fishes. Copper sulphate is widely used as an algaecide for controlling phytoplankton in fish ponds and lakes as well as a herbicide used in aquatic weed control since 1882 (Carbonell and Tarazona, 1993).

Fishes acquire copper by the gills from the surrounding water, as well as from the diet by the digestive tract (Kamunde *et al.,* 2002). It is necessary for the synthesis of Hb and act as a component of many enzymes such as cytochrome oxidase. Elevated aquatic copper levels cause a range of negative effects on fish such as reduced growth, interference with whole body iono-regulation and endocrine disruption (Schjolden *et al*., 2007).The toxic effect of Cu is related to its capacity for catalyzing oxidative reactions, leading to the production of reactive oxygen species**.** These highly reactive compounds may also induce tissue alterations and physiological derangement in fish (Varanka *et al*., 2001).

Ascorbic Acid (AA) is an essential vitamin for normal growth and physiological functions in animals including fish. Most teleosts are unable to synthesize AA due to the lack of L-gulonolactone oxidase (Fracalossi *et al*., 2001). Therefore, an exogenous source of AA is required in fish diets. It functions as a general water-soluble redox reagent, on collagen formation, iron metabolism and the response to stress (Vijayavel *et al*., 2006). Collagen is a principal constituent of skin, scales, mucous, cartilaginous tissues, bones and in conjunctive tissue formation, which involves all organs of the body. Ascorbic acid was also reported to have curative effects due to its properties, by many authors. (Ghazaly, 1994) reported that ascorbic acid reduced the mortality, lowered metal content of tissue and prevented the inhibition of blood AST and LDH activities of Tilapia zillii after exposure to mercury.

β-glucans represent part of a group of physiologically active compounds generally called "biological response modifiers." They are highly conserved carbohydrates forming structural components of cell walls of some plants, fungi, yeast, seaweed and bacteria. The first studies showed that glucan application significantly stimulated the phagocytic system and enhanced general defense and resistance to experimental tumors. During subsequent decades of intensive research by laboratories around the world, glucans were found to significantly stimulate defense reactions against infections and cancer (Novak and Vetvicka, 2009). In addition, several additional effects were later shown. These included reduction of stress (Vetvicka and Vetvickova, 2011). Hypoglycemic effects, lowering cholesterol (Raharet *et al.,* 2011). Reduction of cytotoxic effects (Vetvicka and Vetvickova, 2011) and improving treatment of diseases such as ulcerative colitis (Lavi *et al.,* 2012). Another advantage of using glucan as a stimulator of immune reactions is the fact that it has been shown to act in all species tested so far, starting with earthworms and ending with humans (Vetvicka and Sima, 2004).

The present study was planned to evaluate the hazard efficacy of water pollution by copper on growth performance and health status as well as the ameliorating capacity of dietary vitamin C and/or β-glucan in the Nile tilapia fish.

2. MATERIALS AND METHODS

2.1. Experimental Fish:

A total of 125 apparently healthy monosex *O.niloticus* with average body weight of 50±10g/fish were obtained from a private fish farm at Kafer El-Sheikh governorate. Fish were transported alive to the laboratory of Animal health research institute at Kafr El-Sheikh. Fish were kept in glass aquaria. These aquaria supplied with chlorine free tap water**.** The aquaria were continuously aerated by electric pump and held at 28±2^{o}C and half of the water was changed daily for ten weeks. Fish were acclimated for two weeks during the acclimation fish fed on the basal diet only.

2.2. Feeding diets and experimental design:

The diets were formulated to meet nutrient requirements of Nile tilapia fish according to (NRC, 2011) Table (1). Fish were randomly allotted into five equal groups (25 fish per group) received the prepared pelleted experimental diet according to the experimental design Table (2). All dietary ingredients were finally ground, well mixed and pelleted through 3.0 mm die. Three diet samples have been collected for proximate analysis**.** One was taken at the beginning, one in the middle and one at the end of the experimental as grab sample from the feed stocks. Feed samples were stored at -4^{o}C for later analysis.

2.3. Experimental procedure:

The 5 aquaria were randomly assigned to one of the five treatments. The fish were fed by hand twice times a day at 9:00 and 14:00 h. Fish were fed to apparent visual satiation and utmost care was taken to assure that all feed supplied was consumed. All fish

in each aquarium were weighed at the beginning (W0) and biweekly for a continuous 10 weeks (70 days). Weight gain, was calculated as: Weight gain = (Final body weight- Initial body weight). Gain% = (Total gain/Initial Wt.) X100. Feed Conversion Ratio (FCR) was calculated by dividing total feed intake per aquarium by the total body weight gain per the same aquarium, Protein Efficiency Ratio (PER) was calculated.

2.4. Analytical procedure:

Collected feed samples were analyzed for Dry Matter (DM), moisture and ash contents according to (AOAC,1985),crude protein using Kjeldahl method according to (Randhir and Pradhan, 1981) and ether extract was determined according to (Bligh and Dyer, 1959) technique as modified by(Hanson and Olly, 1963).

2.5.Serum biochemical parameters:

Biochemical examinations of the Nile tilapia were performed on surviving fish. The body surfaces were cleaned and blotted dry with adsorbent paper. Blood samples, collected 2ml/fish from three fish of each group at the end of the experiment from caudal vessel using disposable 3-cc syringes and 21-gauge needles, were transferred into Eppendorf tubes without anticoagulants for serum separation, as described for the assessments of serum total protein, albumin, triglycerides, total cholesterol, HDL, LDL, VLDL, urea, uric acid, creatinine levels, and AST, ALT, CAT, SOD activities, which were spectrophotometrically (LABOMED Co., Lab. American Inc., USA) estimated according to the manufacturer's instructions. Another blood sample was collected with anticoagulant for estimation of phagocytic activity and index according to (Kawahara *et al.*, 1991).

Table (1): Ingredients and proximate analysis of the basal diet:

Physical composition		Chemical composition	
Ingredients	**%**	**Items**	**%**
Yellow corn7%	31.0	Dry matter (DM)	90.04
Soybean meal 42.88%	40.5	Moisture	9.96
Fish Meal 58.28%	5.0	Crude protein (CP)	32.75
wheat bran 14.1%	2.0	Ether extract (EE)	5.64
Corn gluten 60%	16.5	Crude fiber (CF)	4.14
Soybean oil	3.5	Ash	5.61
Di-calcium phosphate	1.0	NFE[2]	41.9
Salt	0.2	Methionine[3]	0.64
Vitamins and minerals mixture[1]	0.3	Lysine[3]	1.65
		Calcium	0.73
		Total phosphorus	0.82
		Digestible energy (DE)[4]	3091.32 Kcal/Kg

[1]The used Vitamins and minerals mixture (Multivita Co.) composed of vitamin A 12000000 IU, vitamin D3 2200000 IU, vitamin E 10g, vitamin K3 2g, vitamin B1 1g, vitamin B2 5g, vitamin B6 1.5g, vitamin B12 0.01g, Niacin 30g, Biotin 0.050g, Folic acid 1g, Pantothenic acid 10g, Iron 30g, Manganese 60g, Copper 4 g, Zinc 50g, Iodine 1g, Cobalt 0.1g, Selenium 0.1g, calcium carbonate (CaCO3) carrier to 3000g. [2]NFE = Nitrogen free extract and calculated by difference {100 – (moisture% + CP% + EE% + CF% + Ash%)}
[3]methioine, lysine, calcium and phosphorus were calculated based on chemical composition of feed stuffs nutrients (NRC, 2011).
[4]Digestible energy(DE) was calculated (kcal/kg) using formula based on chemical composition of feed stuffs nutrients (NRC, 2011).

Table (2): Outline of the experimental design:

Groups No.	Copper water exposure	Dietary supplementation	
	(100µg/L)*	Vit. C (500mg/Kg)**	B-glucan (100mg/Kg)***
1 (Negative control)	--	--	--
2 (positive control)	+	--	--
3	+	+	--
4	+	--	+
5	+	+	+

*Copper used at sublethal concentration (100µg/L water) according to (Hamilton *et al.*, 1977) and was calculated from copper sulphate "$CuSO_45H_2O$" (Analar grade). Manufactured by Fine-CHEM Limited, India and added subsequently to the water in experimental tanks to obtain desired test concentration for 70 days to all groups except control negative.
**Coated vitamin C 97%, produced by Nutristar Co. and added 500mg/Kg diet according to NRC (2001). ***Produced by Allteck Co. and used according to manufacture recommendation.

2.6.Copper Residue:

For measuring Cu residue in fish dorsal musculatures, three samples from each group were collected and oven-dried at 85^0C until constant weight. Afterwards, one gram of dry sample was ashed in muffle furnace at 550^0C for 6 hours and was digested with concentrated HNO_3, and diluted with 2N HCl to a constant volume. Copper concentrations were measured using an atomic absorption spectrophotometer (Thermo 6600, Thermo Electron Corporation, Cambridge, UK) according to (Iwegbue, 2008).

2.6. Clinical symptoms and postmortem examination of fish:

Through all the experimental period clinical signs and post mortem examination of monosex *Oreochromis niloticus* was performed according to (Austin and Austin, 1987).

2.7. Histopathological examination:

At the end of the experiment specimens from different parts of ovaries, livers, kidneys, spleen, intestine and gills were immediately fixed in 10% neutral buffered formalin, processed to obtain 4µ paraffin section. Sections were stained with Hematoxylin and Eosin stain for microscopic examination according to (Bancroft *et al*., 2013).

2.8. Statistical analysis:

Statistical analysis was made using Analysis of Variance (ANOVA) one-way analysis of variance for study the effect of different treatment groups on the different studied variables studied that includes (growth performance parameters, hematological and biochemical) variables using (SAS, 2004).

3. RESULTS

Our results concerned with determining the sub-sub-chronic (lethal) impact of copper exposure (100µg/L) and the possible protective effect of vitamin C (500 mg/kg diet) and/or β-glucan supplementation on some biochemical and immune parameters including; liver and kidney functions, and antioxidant status and serum lipid profile and histopathological changes of gills and liver in Nile tilapia fish.

3.1. Growth performance parameters:

Table (3), showed that fish exposure to copper significantly ($P \leq 0.05$) reduced final weight, total gain, weight gain%, RGR and significantly deteriorate FCR and PER compared to control. Vitamin C or β-glucan supplementation markedly improved growth performance and feed efficiency parameters compared with fish group exposed to copper without feed supplement, however that improvement still lower than control.

3.2. Blood serum units:

In relation to control copper exposure non-significantly ($P \geq 0.05$) reduced serum total protein and increased albumin levels (table, 4). Vitamin C and/or β-glucan supplementation increased the serum protein level almost closer to control value. However, copper exposure significantly reduced blood serum globulin concentration compared with control while exposure and vitamin C and/β-glucan supplementation non-significantly improved serum globulin level compared with control.

Table (3): Variation of growth performance parameters of Nile tilapia on exposure to copper without or with dietary vitamin C and/or β-glucan supplementation

Items	Copper exposure and feed supplement				
	Control	**Exposed to CuSO4 (5H2O)**	**Exposed + Vit. C**	**Exposed + β-glucan**	**Exposed + (Vit. C & β-glucan)**
Initial weight (g/fish)	44.5±0.29^a	42.5±0.29^b	42.5±0.29^b	43.0±0.58^b	42.5±0.29^b
Final weight (g/fish)	88.5±0.29^a	67.5±0.29^c	75.0±1.16^b	73.0±1.73^b	74.0±1.73^b
Total weight gain (g/fish)	44.0±0.58^a	25.0±0.58^c	32.5±1.04^b	30.0±1.16^b	31.5±1.44^b
Weight gain%	98.0±1.94^a	58.85±1.76^c	76.47±2.36^b	69.72±1.75^b	74.08±2.89^b
RGR	65.16±0.05^a	45.45±1.04^c	54.94±1.13^b	51.68±0.97^b	54.06±1.54^b
Feed intake (g/fish)	65.86±4.22^a	62.36±1.44^a	65.12±3.91^a	66.27±6.01^a	56.37±1.05^a
FCR values	1.5±0.12^c	2.5±0.12^a	2.0±0.06^{bc}	2.2±0.12^{ab}	1.8±0.12^c
PER values	2.06±0.16^a	1.23±0.06^c	1.53±0.04^{bc}	1.40±0.07^{bc}	1.71±0.11^b
Mortality rate %	-	32	-	-	-

Values are means ± standard error. Means within the same row of different litters are significantly different at ($P \leq 0.05$).

Table (4): Variation of some blood serum parameters of Nile tilapia on exposure to copper without or with dietary vitamin C and/or β-glucan supplementation

Items	Copper exposure and feed supplement				
	Control	**Exposed to $CuSO_4$ ($5H_2O$)**	**Exposed + Vit. C**	**Exposed + β-glucan**	**Exposed + (Vit. C & β-glucan)**
Total protein (g/dl)	6.30 ±0.25[a]	5.96 ±0.03[a]	6.11 ±0.05[a]	6.05 ±0.03[a]	6.09 ±0.01[a]
Albumin (g/dl)	5.11 ±0.02[a]	5.17 ±0.07[a]	5.08 ±0.06[a]	5.09 ±0.06[a]	5.02 ±0.02[a]
Globulin (g/dl)	0.95 ±0.02[a]	0.79 ±0.02[b]	1.15 ±0.03[a]	0.97 ±0.01[a]	1.07 ±0.01[a]
A/G ratio	5.37 ±0.01[b]	6.54 ±0.02[a]	4.42 ±0.02[e]	5.25 ±0.01[c]	4.69 ±0.01[d]

Values are means ± standard error. Means within the same row of different litters are significantly different at (P ≤0.05).

3.3.Phagocytosis:

Copper exposure to Nile tilapia fish significantly decreased phagocytic index and activity (table, 5) compared to control. On the other hand, Cu-Vitamin C or β-glucan and its combination significantly improved phagocytic index and activity compared with copper exposed fish however that improvement still lower than control.

3.4. Kidney and liver function related parameters:

In relation to control copper exposure significantly (P≤0.05) increased blood serum AST, ALT, uric acid and urea blood serum concentrations while, had no significant effect on blood serum creatinine level (table, 6). Vit. C or β-glucan alone and their combination has no effect on the measured hepatorenal biochemical parameters, fortunately it returned the increased levels of AST, ALT, urea, uric acid and creatinine to their normal values in Cu + additives treated fish.

Table (5): Variation of phagocytosis of Nile tilapia on exposure to copper without or with dietary vitamin C and/or β-glucan supplementation

Items	Copper exposure and feed supplement				
	Control	**Exposed to $CuSO_4$ ($5H_2O$)**	**Exposed + Vit. C**	**Exposed + β-glucan**	**Exposed + (Vit. C & β-glucan)**
Phagocytic index	2.67±0.01[a]	1.68±0.01[e]	2.07±0.01[b]	1.82±0.01[d]	1.92±0.01[c]
Phagocytic activity	42.48±0.08[b]	34.75±0.04[e]	44.79±0.02[a]	42.11±0.02[c]	38.24±0.01[d]

Values are means ± standard error. Means within the same row of different litters are significantly different at (P ≤0.05).

Table (6): Variation of kidney and liver functions related parameters of Nile tilapia on exposure to copper without or with dietary vitamin C and/or β-glucan supplementation

Items	Copper exposure and feed supplementation				
	Control	**Exposed to $CuSO_4$ ($5H_2O$)**	**Exposed + Vit. C**	**Exposed + β-glucan**	**Exposed + (Vit. C & β-glucan)**
Creatinine(mg/dl)	1.91 ±0.01[ab]	1.96 ±0.01[a]	1.89 ±0.02[b]	1.93 [ab] ±0.03	1.93 ±0.01[ab]
Urea (mg/dl)	54.4 ±0.17[e]	74.9 ±0.17[a]	63.8 ±0.12[d]	71.7 [b] ±0.12	65.9 ±0.17[c]
Uric acid (mg/dl)	5.16 ±0.09[c]	5.42 ±0.03[ab]	5.69 ±0.01a	52.0 ±0.13[ab]	5.69 [a] ±0.20
AST(u/ml)	27.0 ±1.2[c]	36.0 ±0.6[a]	23.0 ±0.6[d]	22.0 ±1.7[d]	31.0 ±0.6[b]
ALT (u/ml)	89.0 ±0.57[c]	94.0 ±0.58[a]	91.0 [b] ±0.57	85.0 ±1.15[b]	90.0 ±0.58[b]

Values are means ± standard error. Means within the same row of different litters are significantly different at (P ≤0.05).

3.4. Lipid profile:

Fish with copper exposure showed significantly (P≤0.05) lower blood serum total cholesterol, triglyceride, HDL, LDL while increased VLDL concentration (table, 7) compared with control. Vitamin C or β-glucan and its combination supplementation with copper exposure increased blood serum total cholesterol and HDL concentration compared with fish group exposed to copper without supplement.

3.5.Antioxidants enzyme activities:

In relation to control copper exposure significantly increased CAT serum activity compared with control. Dietary supplementation of vitamin C with copper exposure significantly increased blood serum CAT activity compared with copper exposed group without supplement, in contrast β-glucan reduced CAT activity and both vitamin C and β-glucan had no effect. Moreover, both vitamin C and/or β-glucan significantly increased blood serum SOD activity.

3.6. Copper residue:

Fish with copper exposure showed higher copper residue in dorsal muscle (table, 9) compared by negative control, however vitamin C, β-glucan or combination of both Vit. C and β-glucan reduced copper residue by about 40.9%, 56.2% and 59.9% respectively compared with fish group exposed to copper without any supplement.

3.7. Fish health:

No negative control fish died during the experimental period. Mortality occurred only in the exposed fish (table, 3) and before death, exposed fish showed unstable swimming with unbalanced movements, exhaustion, suspended in vertical position with the mouth up near the water surface and finally submerged in the bottom of water with no motion. Compared to the control, the exposed fish showed pale gills, some damaged gills, flabby intestine, and yellowish discoloration of liver, distended gall bladder, eye protrusion and skin darkness (fig. 1 – 3).

Table (7): Variation of blood serum lipid of Nile tilapia on exposure to copper without or with dietary vitamin C and/or β-glucan supplementation

Items	Copper exposure and feed supplement				
	Control	**Exposed to $CuSO_4$ ($5H_2O$)**	**Exposed + Vit. C**	**Exposed + β-glucan**	**Exposed + (Vit. C & β-glucan)**
Total cholesterol (mg/dl)	203.4±0.06^{b}	179.6±0.06^{e}	194.1±0.06^{d}	204.4±0.06^{a}	201.7±0.06^{c}
Triglyceride (mg/dl)	204.7±0.06^{b}	193.2±0.12^{e}	206.8±0.06^{a}	203.4±0.06^{d}	204.2±0.06^{c}
HDL (mg/dl)	52.5±0.06^{c}	50.4±0.06^{e}	51.9±0.06^{d}	54.8±0.06^{a}	53.7±0.12^{b}
LDL (mg/dl)	112.26±0.01^{a}	88.22±0.01^{e}	100.84±0.01^{d}	108.92±0.01^{b}	107.17±0.01^{c}
VLDL (mg/dl)	38.64±0.01^{e}	40.98±0.01^{b}	41.36±0.01^{a}	40.68±0.01^{d}	40.84±0.01^{c}
T-CHO/HDL ratio	3.87±0.01^{a}	3.56±0.01^{d}	3.74±0.01^{c}	3.73±0.01^{c}	3.76±0.01^{b}

Values are means ± standard error. Means within the same row of different litters are significantly different at (P ≤0.05).

Table (8): Variation of blood serum antioxidant enzyme activity of Nile tilapia on exposure to copper without or with dietary vitamin C and/or β-glucan supplementation

Items	Copper exposure and feed supplementation				
	Control	**Exposed to $CuSO_4$ ($5H_2O$)**	**Exposed + Vit. C**	**Exposed + β-glucan**	**Exposed + (Vit. C & β-glucan)**
Catalase(CAT) (u/ml)	14.9 ±0.06^{d}	18.2 ±0.06^{b}	21.7 ±0.06^{a}	16.6 ±0.06^{c}	18.3 ±0.06^{b}
Superoxide dismutase (SOD) (u/ml)	137.6 ±0.2^{c}	138.2 ±0.12^{c}	172.9 ±0.057^{a}	146.1 ±0.057^{b}	159.2 ±0.057^{b}

Values are means ± standard error. Means within the same row of different litters are significantly different at (P ≤0.05).

Table (9): Variation of copper concentration in Nile tilapia muscle on exposure to copper without or with dietary vitamin C and/or β-glucan supplementation.

Items	Copper exposure and feed supplementation				
	Control	**Exposed to $CuSO_4$ ($5H_2O$)**	**Exposed + Vit. C**	**Exposed + β-glucan**	**Exposed + (Vit. C & β-glucan)**
Copper (µg/Kg)	0.05	1.37	0.81	0.60	0.55

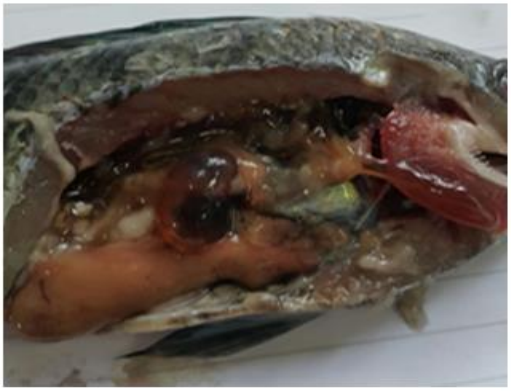

Fig. (1): Experimentally $Cuso_4$ intoxicated *O.niloticus* showed, yellowish discoloration of liver and distended abnormal colored gall bladder

Fig. (2): Experimentally $Cuso_4$ intoxicated *O.niloticus* showed, skin darkness, abnormal coloration of operculum and eye protrusion

Fig. (3): Experimentally $Cuso_4$ intoxicated *O. niloticus* showed erosions of dorsal, anal fins and tail

3.8. Histopathological findings:

Histopathological examination of different organs (liver, spleen and gills) and (kidney, intestine and muscle) of fish from different groups were illustrated in Fig. 4 and 5 respectively. The examined sections from normal fish revealed normal liver consisting from both normal hepatic and pancreatic tissues, normal spleen which showed normal red and white pulp and normal melanomacrophage center, normal gills revealed normal primary and secondary gill lamellae, kidneys consisted from normal renal tubular and glomerular structures, normal intestinal villi and normal striated muscle fibers.

While the fish treated with copper showed different organs-associated Cu toxicity. The liver showed necrobiotic changes within the hepatocytes including vacuolar degeneration and necrosis. Interestingly, hepatopancreas was severely affected and showed marked necrosis accompanied with leukocytic infiltration with peripancreatic haemorrhages. The spleen demonstrated multifocal necrotic areas, severe lymphoid depletion associated with reticular cells proliferation. The gills showed complete loss of their lamellae, with complete fusion of some lamellae and mostly associated with goblet cells proliferation. The kidney showed congestion of glomerular tufts and diffuse vacuolation of tubular renal cells. The intestine showed features of enteritis associated with intestinal metaplasia. The striated muscle fibers were greatly atrophied with marked leukocytic cells infiltration in addition there were multifocal areas showed Zenker's necrosis.

The β-glucan-treated fish revealed decrease the histological alteration induced by cupper toxicities. The liver showed moderate degree of pancreatic cells degeneration. The spleen showed mild degree of lymphoid depletion. Gills showed moderate degree of lamellar fusion. Kidneys revealed mild renal tubular degeneration. Intestine showed moderate degree of necrotic enteritis. The skeletal muscle showed mild degree of Zenker's necrosis.

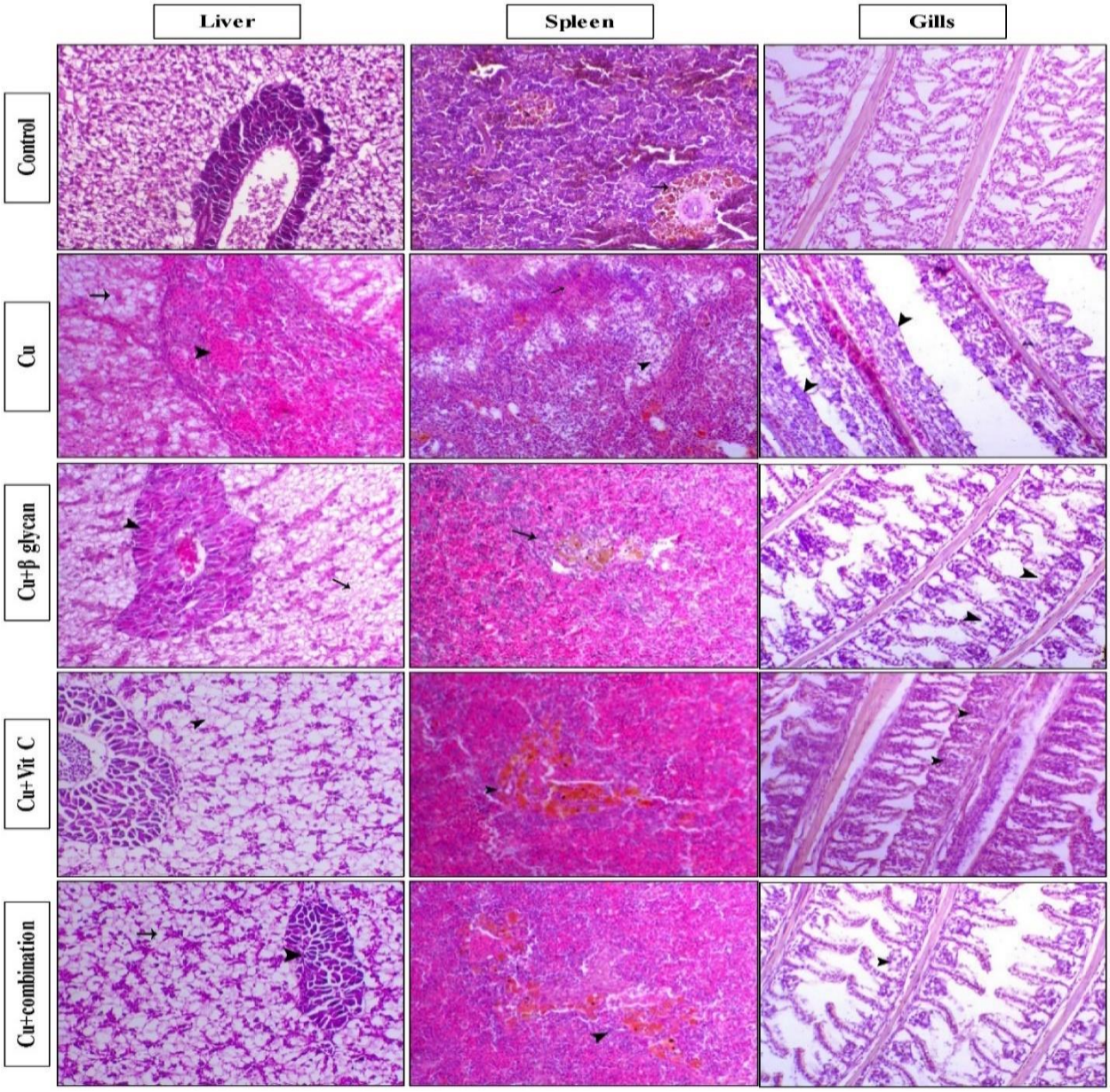

Figure (4): Variation of histopathological lesions of liver, gills and spleen in Nile tilapia muscle on exposure to copper without or with dietary vitamin C and/or β-glucan supplementation

Vitamin C treated fish also reduce the pathological lesions induced by cupper exposure. The liver of this group showed mild degree of hepatic and pancreatic tissues degeneration. The spleen showed mild degree of lymphoid depletion with normal melano macrophage center. The gills showed moderate degree of fusion of gill lamellae. The kidney revealed mild renal tubular degeneration. Intestine showed mild enteritis. The muscle demonstrated focal areas of Zenker's necrosis.

Interestingly administration of both β-glucan and Vit. C alleviate the multi-systematic intoxication with cupper. The liver showed mild degree of hepatic vacuolation. The spleen was apparently increase the lymphocytes within the while pulp with increase the melano macrophage cells. The gills showed mild degree of secondary lamellae fusion. The kidney revealed mild renal tubular vacuolation. The intestine and muscle was mildly affected and were within normal limits.

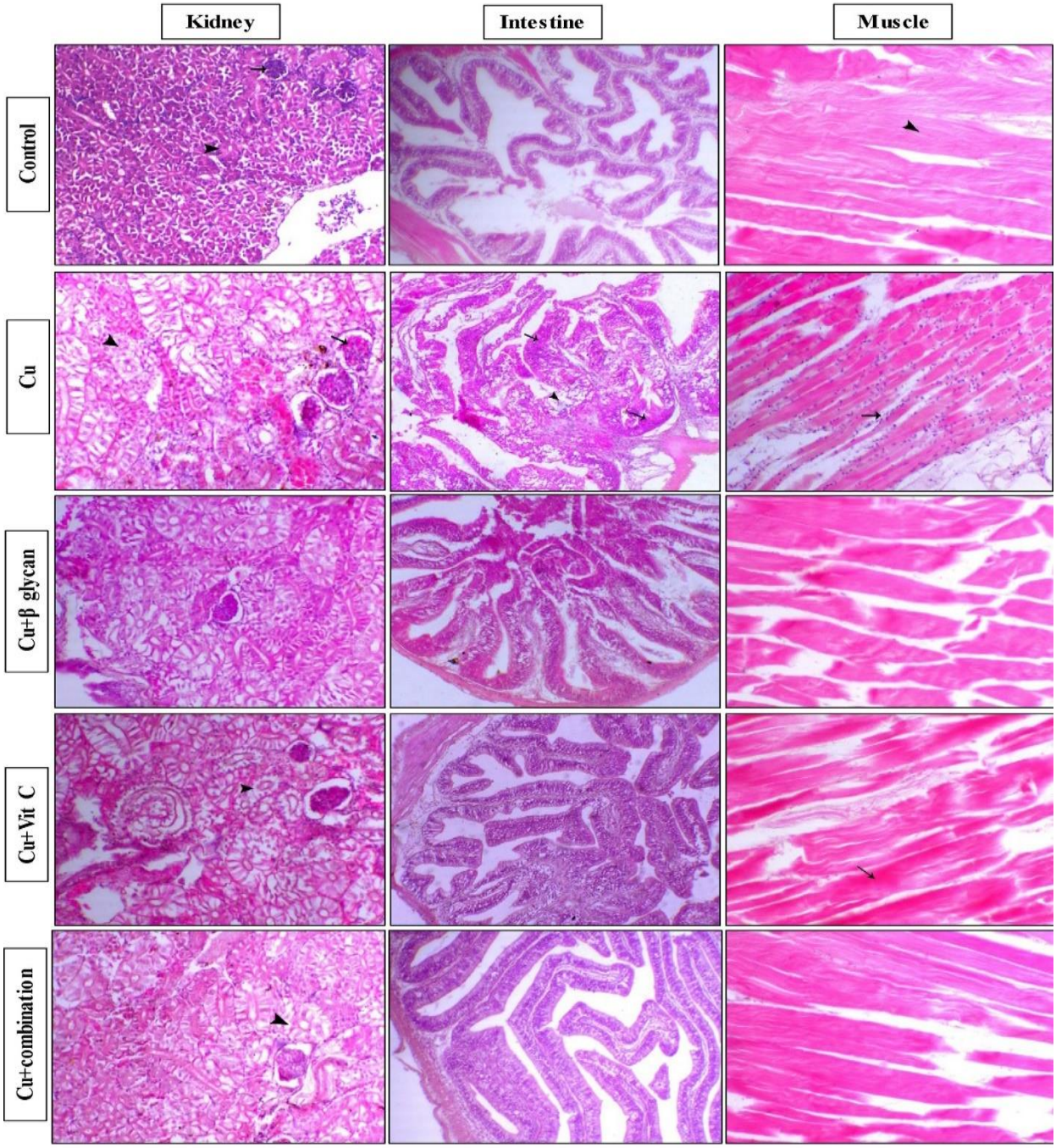

Figure (5): Variation of histopathological lesions of kidney, intestine and muscle in Nile tilapia muscle on exposure to copper without or with dietary vitamin C and/or β-glucan supplementation

4. DISCUSSION

Copper sulphate exposure at 100µg/L water for 10 weeks caused significant decrease in growth rate and deteriorate exposed feed conversion ratio & protein efficiency ratio which is consistent with other studies(Ali *et al.,* 2003) reported that significant decreases were observed in the total weight gain and specific growth rate of the *O. niloticus* reared in water containing different concentrations of copper; thus, these decreases were linearly correlated with the increase of the copper level in water. However, these data are in contrast with those obtained by (Mutlu *et*

al., 2015) indicated that the differences of growth rates between control and copper sulfate-exposed groups were not significant (P>0.05) at the end of 35, 65 and 95 days. Moreover, vitamin C or β-glucan supplementation markedly improved growth performance and feed efficiency parameters which agree with (Baccarin and Pezzato, 2001). When fed lower content of protein (28.0% DP/3000 kcal DE/kg), but with addition of vitamin C (400 and 600 mg kg-1) and ß-glucan (0.10.8%), that species presented a satisfactory performance even with shorter periods of time (six weeks) than the ones observed in the present study (Falcon, 2007). The increase in the values of weight gain (WG) in fishes might be related to a probable glucan degradation by the glucanase, promoting the transference of more proteins (protein saver effect) for the growth (Lopéz *et al.*,2003).

The plasma protein decreased non-significantly at exposure of copper in the present study The observed decrease of serum proteins could result from the breakdown of protein in to amino acids first to be fed in to the Kreb's cycle to cope up the energy crisis manifested by the metal intoxication (Hosetti *et al.,* 2011).The depletion in serum protein in test organisms might be due to impaired protein synthesis or the functional deterioration of the liver or excessive loss of protein caused by nephrosis (Kori-Siakpere and Ake, 1995) corroborates the present study. Similar decrease in serum and liver protein on exposure to copper has been reported in Labeorohita (Meenakumari *et al.,* 2010). I n the present study, vitamin C or β-glucan supplementation were found to have an ameliorating effect on copper toxicity enhancing the protein content near to the control. Similar decrease in plasma protein has been documented in Labeorohita exposed to chromium and *Oreochromis niloticus* exposed to copper (Barad and Kulkarni, 2010). Copper exposure reduced Nile tilapia fish immune response through lower blood serum globulin concentration and lowering phagocytosis. while vitamin C and/or β-glucan supplementation improve fish immunity. Beyond phagocytosis mechanism, ascorbic acid (vitamin C) is involved in leukocyte migration and retarded hyper sensibility. It also participates in the mutagenic proliferation of lymphocytes, in the increase of the level of serum complements and in the production of interferon; therefore, it is considered as anti-infection vitamin (Soliman, *et al.,* 1994). Also, these data are in harmony with those obtained by (Lirancoe*t al.*, 2013) reported that tilapia that had received the diet supplemented with β-glucan in a period of 90 days showed a favorable condition of the immune system.

The high creatinine, urea and uric acid might be expected in the serum of the exposed fish, as has been reported with exposures of copper (Chen *et al.*, 2004). The increases in serum urea and creatinine concentrations have frequently been used in fish as an indicator of gill and kidney dysfunction (Adham *et al.*, 2002; Yang and Chen, 2003), because there is a relationship between exposure to heavy metals and kidney disease. This refers to a kidney failure and increased muscular tissue catabolism. This finding is in parallel with the fact that the most prominent rise among serum nitrogenous compounds in these fish was reported for uric acid. It could be suggested that the branchial excretion of uric acid and other nitrogenous compounds was inhibited leading to an accumulation of uric acid in blood. This leads to confusion about the role of uric acid and urea levels in assessing fish health. The results obtained in the present study revealed that the metal intoxicated fish recovered at a faster rate on supplementation of vitamin C and/or β-glucan. Supplementation of vitamin C, β-glucan or their combination in copper exposed fishes significantly reduced the serum AST and ALT activity compared to fishes without supplement clearly reveals the prophylactic role of vitamin C as a typical antioxidant protecting fishes from oxidative stress and tissue injury to a great extent. These results agree with earlier findings (Kumar *et al.,* 2014). Vitamin C is one among the most important biological antioxidants. Majority of animals synthesize vitamin C from D-glucose. However most of the fishes are incapable of self-synthesis of vitamin C. Therefore, it could be concluded that vitamin C is efficient for reducing copper toxicity in fish. Vitamin C is closely related to the immunological system performance and has antioxidant properties favoring the integrity and fluidity of membranes and capable of controlling the oxidizing reactions of fatty acids, thus keeping cellular respiration and avoiding cell death (Brake, 1997).On the other hand, ascorbic acid (vitamin C) and β-glucan has been shown to enhance also the urinary elimination of metal to reduce hepatic and renal burden of metal (Ismail *et al.,* 2014).Generally, as showed from the results (table, 9) dietary β-glucan

supplementation alone or combined with vitamin C more efficient in copper elimination outside fish body.

Lipid is an important source of energy in fish (Haggag *et al.,* 1993). The present study indicated a significant decrease in blood serum total cholesterol and triglycerides concentrations in Nile tilapia exposed to copper toxicity compared to control or fish exposed to copper with dietary vitamin C, β-glucan and its combination supplementation. This is agreement with (Abdel-Khalek *et al.,* 2015) stated that copper toxicity reduced blood serum triglycerides in Nile tilapia. This may also be due to the decrease in insulin levels because insulin has a greater effect on protogenic and lipogenic pathways (El-Naggar *et al.,* 1998).

Catalase a primary antioxidant defense component protects fish from oxidative stress by converting the hydrogen peroxide to oxygen and water (Atli and Canli, 2007). In the present study there were CAT activity increased in Cu exposed fish. Also, (Basha and Rani, 2003) demonstrated that there were simultaneous increases in the levels of CAT activity in the liver following Cd exposure of *O. niloticus*. They indicated that there was a possible shift toward a detoxification mechanism under long term metal exposure. Also, at higher concentrations, chemicals may directly inhibit the activity of enzymes, or indirectly reduce the concentration of the enzymes by damaging cell organs (Jemec *et al.*, 2007). The reduction of the CAT activity may also result from the accumulation of H_2O_2 and other oxy-radicals (Choi *et al.,* 2010). Increased H_2O_2 levels resulting from CAT inhibition could ultimately further inhibit the SOD activity (Kono and Fridovich, 1982). It was observed that vitamin C supplementation increase blood serum CAT or SOD activities, these results indicated that vitamin C has a positive effect on antioxidant capacity of Nile tilapia. These data are in harmony with those obtained by (Asaikkutti *et al.*, 2016).

The histology showed that copper caused some alterations of the gill lamella, liver parenchyma, spleen, kidney, muscle and intestinal membrane of Nile tilapia fish. These alterations are often associated with a degenerative-necrotic condition (Myers *et al.*, 1987). 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 (Varanka *et al.*, 2001). Alterations in liver hepatocytes associated with stress have been well studied and reported the formation of vacuoles in hepatocytes (Metelev *et al.*, 1971). Vacuolar degeneration and disrupted hepatocytes detected in exposed fishes substantiates the potency of copper in causing liver damage (Fig. 4). Vacuolar degeneration and focal necrosis in hepatocytes in the present study coincides with similar observations in Etroplus maculatus exposed to lindane (Bijoy *et al.,* 2011). Hypoxia due to gill degeneration is attributed to be the reason for cellular degeneration in the liver (Eder and Gedigk, 1983). In the present study, the gills showed obvious degenerative changes like proliferation of epithelial lining with reduction in respiratory surface area lowering diffusion of oxygen. In this context it is imperative to study the histological deviations of liver along with biochemical changes in exposed fishes as a reliable biomarker of metal toxicity. All these reports strongly support the conviction that the liver, kidney, spleen and gill of the heavy metal exposed fish is severely damaged. The biochemical and histopathological results of the present study revealed that the metal intoxicated fish recovered at a faster rate on supplementation of vitamin C and/or β-glucan.

Moreover, dietary supplementation of both vitamin C and β-glucan markedly improve intestinal villi and recover necrotic tissues of all organs. Improved intestinal health may increase the ability to absorb digested nutrients, thereby providing the potential to enhance growth performance in fish. These data are in harmony with (Abid *et al.*, 2013) reported that supplementation with a synbiotic that included P. acidilactici and FOS improved the microvilli in rainbow trout.

5. CONCLUSION

It could be concluded that dietary vitamin C and/or β-glucan supplementation played a role in reducing the harmful effect of water born Cu on Nile tilapia fish, which in turn improved the growth, feed utilization, liver and kidney health and antioxidant capacity. Also, vitamin C and/or β-glucan supplementation decreased Cu residue found in fish dorsal muscle, thereby mitigating potential hazards to human health. Combination of both vitamin C and β-glucan more effective to recover of intoxicated Nile tilapia fish.

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