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Effect of Various Dietary Concentrations of Copper Nanoparticles And Copper Sulfate on Growth Performance, Body Composition, Cu Retention, Antioxidant Capacity, Metabolic and Digestive Enzymes Activity in Blue Tilapia, Oreochromis Aureus

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Abstract

The use of mineral supplementation to enhance the efficacy of aquafeeds is already established. Therefore, the present study was designed to evaluate the effects of various concentrations of dietary copper nanoparticles (CuNPs) on growth performance, Cu retention, antioxidant capacity, and metabolic enzyme status of Nile *tilapia* (*Oreochromis* niloticus) by supplementing its diets with CuNPs. Four isoproteic (crude protein; 317 g/kg) and isolipidic (crude lipid; 118 g/kg) dietary treatments were supplemented with CuNPs, viz: D I was set as control (0 mg CuNPs/kg) whereas, D II, D III, and D IV were supplemented with 2, 4 and 6 mg CuNPs/kg, respectively. Three replicates of fish having an initial mean weight of 5.64 ± 0.16 g were hand-fed daily. Significant improved growth performance, feed efficiency ratio (FER), and protein efficiency ratio (PER) was recorded in the group fed with D III (4 mg CuNPs/kg) as compared to the control (D I) as well as other treatment groups, D II and D IV ($p < 0.05$). The survival rate remained unaffected among all the dietary treatments. Whereas, a significant difference in hepatosomatic and viscerosomatic indices was observed in the group fed with D IV. The significantly improved Cu retention and serum glucose level however, reduced serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity was recorded with dietary treatment D III. Additionally, improved oxidative stability and protease activity (PA) were found in the same nutritional treatment. Based on the results of the present study, supplementation of the fish diet up to 4 mg/kg of CuNPs is suggested to improve growth performance and overall health of *O. niloticus* by reducing oxidative stress.

Keywords: Antioxidant Enzymes; Body Indices; Copper Nanoparticles; Feed Efficiency; Growth Performance; Liver Functioning Enzymes; *Oreochromis* Aureus

Abbreviations

CuNPs: Concentrations of Dietary Copper Nanoparticles; FER: Feed Efficiency Ratio; PER: Protein Efficiency Ratio; AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase; PA: Protease Activity; SOD: Superoxide Dismutase; ACP: Acid Phosphatases; AKP: Alkaline Phosphatases; CuNPs: Cu Nanoparticles; MFTRC: Manawa Fisheries Training and

Research Complex; GCUL: Government College University, Lahore; POX: Peroxidase; Glu: Glucose; LA: Lipase Activity; PA: Protease; SEM: Standard Error of Mean; THS: Tukey's Honestly Significant; SGR: Specific Growth Rate; HSI: Hepatosomatic Index; VSI: Viscerosomatic Index; NPs: Nanoparticles; SGPT: Serum Glutamic-Pyruvic Transaminase; SGOT: Serum Glutamic-Oxaloacetic Transaminase; MDA: Malondialdehyde; GIT: Gastrointestinal Tract; TBARS: Thiobarbituric Acid Reactive Substances.

Introduction

Minerals are inorganic elements required to regulate physiological processes in all animals, including fish [1]. These nutrients maintain acid-base equilibrium and colloidal systems in the fish body [2]. Among various elements, copper is an essential trace mineral and is used as a co-factor of some antioxidant enzymes [3]. Various biological processes, including scavenging of radical species, hemoglobin and collagen syntheses, and transportation of electrons are enabled by these copper-containing enzymes. Additionally, along with other minerals, Cu serves as a vital part of soft tissues, such as proteins, and hard tissues including bones, fins, rays, teeth scales, etc [4]. Hence, diets of cultured terrestrial animals (including pigs, poultry, and livestock) and various aquatic species (including fish, mollusks, and crustaceans) are supplemented with Cu [3-34].

In aquaculture, synthetic/formulated diets must be supplemented with copper for enhanced production because aqua feed ingredients including fish and plant meals are not adequate sources of copper [10]. Reasonably sufficient data have already been generated indicating the importance of Cu supplementation in the diet for growth and other health parameters of animals including cultured fish species [11,12]. Dietary Cu requirement varies from species to species and has been reported for some terrestrial as well as aquatic animals, such as pigs 200-250 mg/kg diet, channel catfish 5 mg/kg diet, Penaeus vannamei < 34 mg/kg diet, Penaeus orientalis 53 mg/kg diet, Epinephelus malabaricus 2-3 mg/kg diet, and Acipenser gueldenstaedtii 8 mg/kg diet [3,14-18]. Also, deleterious effects of Cu deficiency in several fish species, such as decreased growth in grouper Epinephelus malabaricus, reduced hepatic cytochrome C oxidase and Cu-Zn superoxide dismutase (SOD) activities in channel catfish Ictalurus punctatus, decreased activities of acid phosphatases (ACP) and alkaline phosphatases (AKP) in blunt snout bream Megalobrama amblycephala have been reported [14,17]. In this context, it is imperative to determine the optimum Cu supplementation content for attaining maximum productivity while avoiding undesirable effects [12,19].

Additionally, the physical/chemical form of supplemented minerals is one of the most important factors, that influences mineral absorption, assimilation, and distribution in the animal body [20]. In this context, nano-scale forms (nanoparticles) are potential candidates due to their enhanced magnitude and their improved interaction with other dietary components. Also, the large surface area of nanoparticles is conducive to high rates of nutrient absorption and endocytosis in cells [21]. At present, there is a dearth of information in the scientific literature regarding the availability and use of Cu nanoparticles as dietary supplements. A previous study conducted with piglets had shown better absorption and other biological effects with Cu nanoparticles (CuNPs) as compared to bulk form [7]. Moreover, few published reports suggested an enhancement in the absorption of various drugs and nutrients in Pagrus major as well [1,22]. However, there is a dire need to test the use of vital fish dietary supplements, such as CuNPs in commercially valuable fish species. The species of *Tilapia* such as blue *tilapia* (*Oreochromis aureus*) belong to the cichlid group of fishes that are native to Northern and Western Africa and the Middle East and are the second most important farmed fish species worldwide [23]. Currently, more than 85 countries are culturing *tilapia* species in various culturing facilities including paddy fields, raceways, cages, earthen ponds, and concrete tanks [24]. Additionally, the availability of a wide range of culturing techniques, including monosex, mixed sex, intensive, semi-intensive, and extensive is a favorable factor for the expansion of *tilapia* culture around the world [25]. Although a reasonable amount of data concerning macro- and micronutrient requirements of *tilapia* have been generated, the use of Cu nanoparticles as feed supplements is yet to be explored at large. Therefore, the present study was undertaken to evaluate the effect of copper nanoparticles (CuNPs) in comparison to its conventional form on the growth performance, body composition, Cu retention, antioxidant capacity, and metabolic and digestive enzyme activity in blue tilapia, *Oreochromis aureus*.

Materials and Methods

Fish Transportation and Acclimation

The experimental fish (at the fingerling stage) were sourced from the Manawa Fisheries Training and Research Complex (MFTRC), Government of the Punjab Lahore, Pakistan, and were transported and acclimatized by keeping in outdoor concrete-lined ponds at the Animal House Facility of Department of Zoology, Government College University, Lahore (GCUL). The subsequent feeding trial was conducted at the same department in an indoor facility. Before initiating the experiment, the experimental fish were starved for 24 hours and then weighed. The fish were acclimatized for two weeks to experimental regimes and maintained on commercially available fish feed (Oryza Organics ®: crude protein 327 g/kg). A prophylactic dip with 5-gram sodium

chloride (NaCl) in 1000 mL distilled water was provided to the experimental fish to reduce the risk of disease or infections [26].

Characterization of CuNPs

The commercially available copper sulfate ($CuSO_4$) (Sigma Aldrich: Product No. Reagent Plus® 7758-98-7) and copper nanoparticles (CuNPs) (Sigma Aldrich® Product No. 774103; Particle size: 60-80 nm, Boiling point: 2567 ºC (lit.), Melting point: 1083.4 ºC(lit.) - manufacturer's details) were used as a source of copper for the experimental diets. The different stock solutions were prepared and their maximum absorbance was recorded. The calibration curve was formed and the maximum absorbance peak was obtained at 585 nm (Figure 1).

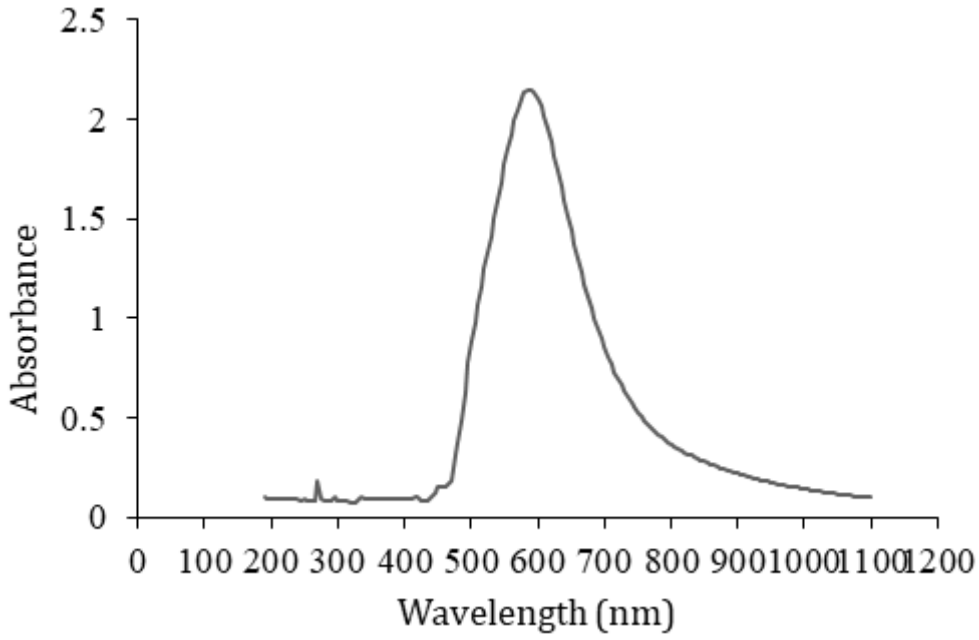

Figure 1: The graphical representation of ultra-visible (UV)-spectrophotometer of commercially prepared copper nanoparticles (CuNPs). The maximum absorbance peak for CuNPs was observed at 585 nm.

Diets and Experimental Design

The raw ingredients of experimental diets containing fish meal, soybean meal, corn gluten meal (60 %), rice polish, wheat flour, cod liver oil, minerals, and vitamins mixture purchased from a local fish feed company; KHUSI FEED COMPANY® in Lahore, Pakistan. These ingredients were ground into fine powder form (0.5 mm) using an electric grinder (Cambridge CG 502, China). The experiment contained overall five dietary treatments; one diet was used without any copper supplementation and one diet was supplemented with CuSO4 (at 4 mg/kg) whereas, the remaining three diets were supplemented with CuNPs (at three different levels viz: 2, 4 and 6 mg/kg) designated as D I (control), D II (4 mg/kg CuSO4), D III (2 mg/kg CuNPs), D IV (4 mg/kg CuNPs) and D V (6 mg/kg CuNPs), respectively. After a thorough mixing of dry ingredients, 200 mL of distilled water was added. Thereafter, $CuSO_4$ and CuNPs were added to the diets, and pellets (3.0 mm) were made with the help of an electric fish feed extruder (Jinan Saibainuo Machinery Co., Ltd., Model no. SYSLG30-IV Experimental Extruder). The cod liver oil was sprayed afterward, pellets were air dried to remove extra moisture, packed into air-tight plastic bags, and stored at -20°C until use (feeding). The proximate composition of experimental diets (g/kg) was determined according to the guidelines of the Association of Official Analytical Chemists (AOAC, 2005) and presented in Table 1. The randomly collected fish specimens (initial mean weight of 5.64 ± 0.16 g/fish) were stocked in 15 glass tanks (80 L water capacity; L 60 cm × H 45 cm × W 30 cm) with each tank containing 15 fish (45 fish per dietary treatment) and each dietary treatment was randomly assigned to three identical replicates.

Ingredient (g/kg)	Experimental diets			
	D I	D II	D III	D IV
Soybean meal	600	600	600	600
Fish meal	100	100	100	100
Corn gluten (60 %)	90	90	90	90
Rice polish	90	90	90	90
Cod liver oil	90	90	90	90
Mineral mixture	10	10	10	10
Cu free[a]				
Vitamin premix[b]	10	10	10	10
Toxin binder	10	10	10	10
CuNPs (mg/kg)	0	2	4	6
Analyzed proximate composition of diets (g/kg on a dry basis)				
Crude protein	314.95	318.1	320.43	317.67
Lipids	129.6	118.27	110.7	115.6
Dry matter	876.1	890.67	893.59	895.51
Energy (kcal/g)	19.41	18.92	19.29	19.83
Ash				

[a]Obtained from Oryza Organics® Limited Pakistan (Fish Feed Company). Each kg of mineral mixture contains; Calcium (Ca; 155 gm), phosphorous (P; 135 gm), magnesium (Mg; 55 gm), sodium (Na; 45 mg), zinc (Zn; 3000 gm), manganese (Mn; 2000 gm), iron (Fe; 1000 gm), cobalt (Co; 40 mg), iodine (I; 40 mg), selenium (Se; 3 mg).

[b]Obtained from Oryza Organics® Limited Pakistan (Fish Feed Company). Each kg of vitamin premix contains; Vitamin A (5.0 mg), Vitamin B1 (0.5 mg), Vitamin B2 (3.0 mg), Vitamin B3 (5.0 mg), Vitamin B6 (1.0 mg), Vitamin B7 (0.05 mg), Vitamin B9 (0.18 mg), Vitamin B12 (0.002 mg), Vitamin C (5.0 mg), Vitamin D3 (0.002 mg), Cellulose 815.26 mg, Choline 100 mg.

Table 1: Ingredient and proximate composition (g/kg) of experimental diets with various concentrations of CuNPs fed to *O. niloticus.*

Fish Feeding Regimes

The fish were fed twice a day (in the morning session at 8:00 and the evening session at 16:00) at 2 % of live wet body weight. During the 8 weeks of feeding, water variables, such as dissolved oxygen (mean value of DO: 5.6 ± 0.14 mg/L), pH (mean value of pH: 7.9 ± 0.17), and water temperature (mean value of temp.: 28.5 ± 0.4°C) were monitored daily using digital meters. Aeration was provided through the capillary system and stone aerators. After the feeding session of three hours, the leftover diet was collected daily from each tank individually, dried, and stored for subsequent analysis. The glass tanks were refilled with clean fresh water after washing and a natural photoperiod (L:D; 12 hr.:12 hr.) was also provided throughout the feeding experiment.

Determination of Growth Performance, Feed Consumption and Body Indices

The mean initial weight was kept similar for all replicated tanks and an increase in weight was calculated by weighing individual tanks after every two weeks. For this purpose, fish were not fed on the day of weighing to avoid the addition of eaten feed weight in the calculation of fish weight. At the termination of the feeding experiment, growth performance and feed consumption were determined by applying the following equations. Additionally, viscera and liver were dissected out of randomly selected three fish (n = 3) from each tank (a total of 9 fish per dietary treatment) and weighed individually to calculate body indices.

Percentage Weight Gain

$$\text{PWG} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

Specific growth rate:

$$\text{SGR}\left(\% / \text{day}\right) = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

Survival rate:

$$SR(\%) = \frac{\text{Final number of fish}}{\text{Initial number of fish}} \times 100$$

Feed conversion ratio:

$$FCR = \frac{\text{Dry feed consumed (g)}}{\text{Wet weight gain (g)}}$$

Protein efficiency ratio:

$$PER = \frac{\text{Dry feed consumed (g)}}{\text{Wet weight gain (g)}}$$

Viscerosomatic index:

$$VSI = \frac{\text{Weight of viscera (g)}}{\text{Total body weight (g)}} \times 100$$

Hepatosomatic index:

$$HSI = \frac{\text{Weight of liver (g)}}{\text{Total body weight (g)}} \times 100$$

Sampling

At the end of the feeding experiment, fish were starved for 24 and euthanized using 2-phenoxy-ethanol (2% solution). Five specimens (n = 5) were randomly taken from each tank (a total of 15 fish per dietary treatment) and samples of scales, muscles, and whole body were collected and stored at -20°C to analyze the whole-body proximate composition and Cu retention. Another batch of five (n = 5) fish were collected from each replicated tank and blood was drawn through cardiac puncture. The non-heparinized tubes (containing 3-6 mL blood) were centrifuged at 3000 g for 10 minutes at 4°C and serum was separated and frozen at -80°C to determine the serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), superoxide dismutase (SOD), peroxidase (POX) activity, glucose (Glu) and thiobarbituric acid reactive substances (TBARS) content. The intestine samples of two fish (n = 2) from each tank (a total of 6 fish per dietary treatment) were excised out, enzyme extract was prepared by adding phosphate buffer in samples (intestine tissue: phosphate buffer; 1:3), centrifuged at 12,000 RMP for 30 minutes at 4°C [26] and stored at -20°C to evaluate protease (PA) and lipase activity (LA).

Chemical Analyses

The proximate composition of experimental diets and fish whole-body was determined by following the standard procedures of AOAC [25]. The percentage of nitrogen (% N_2) was calculated through the Kjeldhal method (Hanon K1100F micro- Kjeldhal auto-analyzer) and crude protein content was determined by multiplying nitrogen (%) with a coefficient of 6.25 [AOAC-960.52]. Ether extraction technique [AOAC- 2003.05] was used to determine crude lipid content (Soxtec system HT2 1045). Dry matter was measured by constant oven (Electric oven: Memmert UN30) drying at 105°C for 6 hours [AOAC-2005-925.10]. The ash content was determined by burning the dried samples in an electric ash furnace (CARBOLITE GERO AFF 11/18) at 550-600°C till the residue was obtained and gross energy was calculated through the oxygen bomb calorimeter. The Cu content in whole-body, scales, and muscle tissues of experimental fish was determined through a wet digestion process (nitric acid and perchloric; 3:1, respectively) by atomic absorption spectrophotometer (AAS) method (AA 7000 F, Shimadzu, Japan). The serum AST and ALT were determined spectrophotometrically (UV/Vis -spectrophotometer AE-S70-10 UK) at 405 nm and 340 nm respectively, using commercially available kits (Randox AST: AS101 and ALT: AL7930 assay kits®). The serum SOD and POX activity and TBARS content were measured as described by Sabatini [26]. Serum glucose content was measured by applying a drop of serum to a chemically treated, disposable 'test strip', which was then inserted into an electronic serum glucose meter. The reaction between the test strip and the serum was detected by the meter and displayed as unit mg/dL. The activity of PA and LA was determined spectrophotometrically (UV/Vis-spectrophotometer AE-S70-10 UK) at 450 nm and 550 nm, respectively. The specific activity of PA (casein as substrate) and LA (palmitic acid as substrate) was presented as units per milligram of protein.

Calculations and Statistical Analysis

The SPSS software Version 22.0 IBM for Windows was used for all the statistical calculations. Data related to the growth performance, feed consumption, body indices, whole-body proximate composition, Cu retention, glucose content, AST, ALT, SOD, POX, PA and LA activity, and TBARS content of *O. aureus* fed with various concentrations of CuNPs and $CuSO_4$ were collected as a mean of three replicates (n = 3) ± standard error of mean (SEM) and subjected to one-way analysis of variance (ANOVA). The homogeneity of variances and normality of data were analyzed by Welch and Brown-Forsythe tests, respectively. Tukey's honestly significant (THS) test was applied as a post-hoc test to compare the

difference among the means of different dietary treatments by adjusting the level of significance at $P < 0.05$.

Results

Effect of CuNPs and CuSO4 on Growth Performance and Survival Rate

The various dietary concentrations of copper nanoparticles (CuNPs) and copper sulfate (CuSO4) had a significant effect on the growth performance of blue tilapia, *Oreochromis aureus* (Table 1). The experimental groups fed with CuNPs supplemented diets such as D III, D IV, and D V showed significantly ($P < 0.05$) higher growth performance in terms of final weight (FW), percentage weight gain (PWG), and specific growth rate (SGR) as compared to the groups received D I (control) and D II ($CuSO_4$ supplemented). However, the maximum increase in growth performance was observed in the experimental group fed with D IV (4 mg/kg CuNPs) in comparison to other CuNPs-supplemented groups. The significantly lowest growth performance was exhibited by the experimental group fed with D I (control). Furthermore, no mortality was experienced during the experiment among different experimental groups of *O. aureus* fed with various dietary concentrations of CuNPs and $CuSO_4$ ($P > 0.05$) (Table 1).

Effect of CuNPs and CuSO4 on Feed Consumption and Body Indices

The feed intake of O. aureus fed with various dietary concentrations of CuNPs and CuSO4 was not significantly ($P > 0.05$) different among the experimental groups however, feed conversion ratio (FCR) and feed efficiency ratio (FER) was significantly affected by the various dietary concentrations of CuNPs and $CuSO_4$ in *O. aureus* (Table 2). The significantly ($P < 0.05$) lowest FCR was found in the experimental group fed with D IV whereas, the highest FCR was displayed by the experimental group received D I (control), which was statistically similar to the FCR of the group fed with D II (containing CuSO4). An inverse relationship was observed regarding the protein efficiency ratio (PER); significantly improved PER was exhibited by the fish group that received D IV while, the lowest value of PER was obtained with the experimental group fed with D I (control) that was similar to the D II when compared to other experimental groups ($P < 0.05$).

Parameters	Experimental diets				*p*-value
	D I	D II	D III	D IV	
IW (g)	5.72±0.01	5.72±0.01	5.74±0.00	5.73±0.00	NS
FW (g)	11.76±0.14^{c}	13.16±0.10^{b}	18.32±0.02^{a}	11.64±0.07^{d}	0.0001***
LWG (g)	6.04±0.13^{c}	7.44±0.08^{b}	12.58±0.02^{a}	5.905±0.06^{d}	0.0001***
PWG	105.67±2.0^{c}	129.95±1.0^{b}	219.25±0.3^{a}	102.96±0.9^{d}	0.0001***
SGR (%/day)	2.30±0.02^{c}	2.51±0.01^{b}	3.04±0.00^{a}	2.28±0.0^{d}	0.0001***
SR (%)	87.49±5.89^{c}	95.83±5.89^{b}	100±0.00^{a}	83.33±0.00^{d}	0.0001***
FER	1.59±0.08^{b}	1.23±0.01^{d}	1.68±0.00^{a}	1.56±0.01^{c}	0.0001***
PER	0.17±0.00^{c}	0.21±0.00^{b}	0.36±0.00^{a}	0.13±0.04^{d}	0.0001***
VSI	7.476±0.09^{a}	7.108±0.07^{b}	6.050±0.05^{d}	6.436±0.20^{c}	0.000***
HSI	1.754±0.05^{a}	1.629±0.03^{b}	1.150±0.02^{d}	1.369±0.02^{c}	0.000***

Table 2: Growth performance, feed consumption, and body indices of *O. niloticus* fed with various concentrations of CuNPs.

In the present study, body indices including hepatosomatic index (HSI) and viscerosomatic index (VSI) of *O. niloticus* fed with various concentrations of CuNPs were significantly lowest ($p < 0.05$) in the fish group fed with dietary treatment D III however, the highest values of VSI and HSI was observed in the control group. (Table 2).

Effect of CuNPs and CuSO4 on Whole-Body Proximate Composition

Effect of CuNPs and $CuSO_4$ on Cu retention in tissues. Figure 2 shows the effect of various concentrations of dietary CuNP supplementation on Cu retention in the whole body, scales, and muscle tissues of *O. niloticus*. A direct relationship was observed between the dietary supplementation of CuNPs and Cu retention. A significant increase in the whole body, scales, and muscle Cu content was observed in the fish group fed with D IV, followed by D III, D II, and D I ($p < 0.05$).

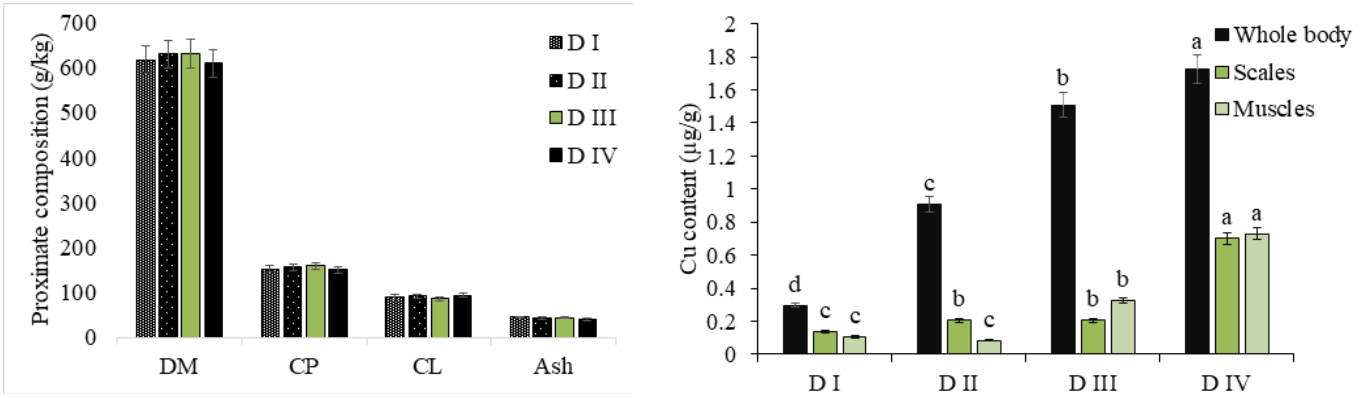

Figure 2: Shows the Cu content (µg/g on a dry matter basis) in the whole body, scales, and muscle tissue of *O. niloticus* fed with various concentrations of CuNPs. The graph bars show the mean values of three replicates (n =3) ± standard error of the mean (SEM). Different superscripts show significant differences at $p < 0.05$ among the means of different dietary treatments. D I was the control (without CuNPs) whereas, D II, D III, and D IV were supplemented with 2, 4, and 6 mg/kg CuNPs, respectively.

Effect of CuNPs and $CuSO_4$ on Liver Functioning Enzymes Activity

The activity of liver functioning enzymes including, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity in the serum of *O. niloticus* was significantly influenced by various concentrations of dietary CuNPs (Figure 3). The significantly elevated serum AST activity (U/L) was found in fish fed with dietary treatment D IV; however, the highest ALT activity (U/L) was exhibited by the fish-fed control diet ($p < 0.05$). The lowest AST and ALT activity (U/L) was measured in the fish group fed with dietary treatment D III as compared to the control group and other CuNPs-supplemented groups ($p < 0.05$).

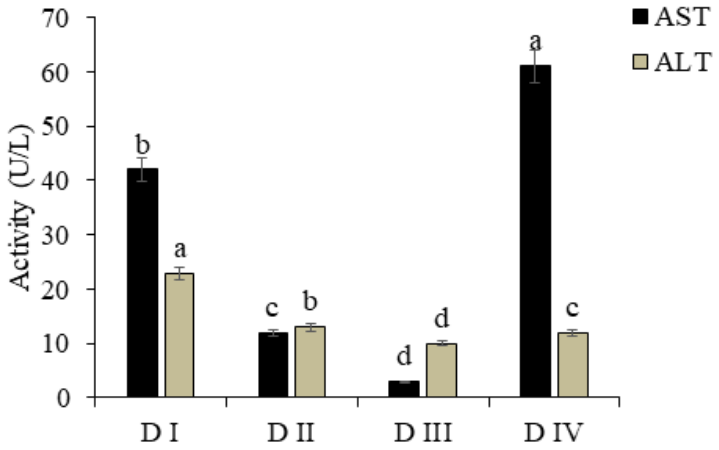

Figure 3: Represents the serum aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) activity of *O. niloticus* fed with various concentrations of CuNPs. The graph bars show the mean values of three replicates (n =3) ± standard error of the mean (SEM). Different superscripts show significant differences at $p < 0.05$ among the means of different dietary treatments. D I was the control (without CuNPs) whereas, D II, D III, and D IV were supplemented with 2, 4, and 6 mg/kg CuNPs, respectively.

Effect of CuNPs and CuSO4 on Antioxidant Enzymes Activities

The superoxide dismutase (SOD) and peroxidase (POX) activity (U/L) was significantly influenced by various concentrations of dietary (Figure 4). SOD activity was found to be significantly higher in fish groups fed with dietary treatments D I (control) and D IV whereas, the lowest SOD activity was measured in the group receiving D III ($p < 0.05$). Moreover, significantly increased POX activity was observed

in fish fed with dietary treatment D I (control) while the lowest POX activity was exhibited by the group fed with D III ($p < 0.05$).

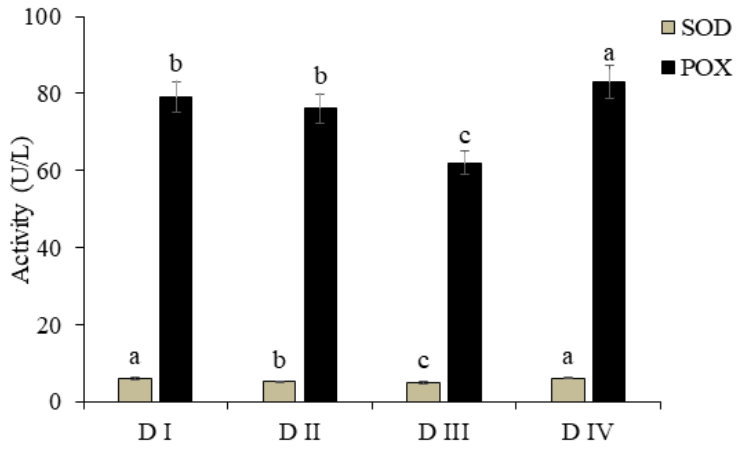

Figure 4: Illustrates the serum superoxide dismutase (SOD) and peroxidase (POX) activity (U/L) in *O. niloticus* fed with various concentrations of CuNPs. The graph bars show the mean values of three replicates (n =3) ± standard error of the mean (SEM). Different superscripts show significant differences at $p < 0.05$ among the means of different dietary treatments. D I was the control (without CuNPs) whereas, D II, D III, and D IV were supplemented with 2, 4, and 6 mg/kg CuNPs, respectively.

Effect of CuNPs and $CuSO_4$ on Digestive Enzymes Activity

Figure 5 illustrates a significant difference in the protease (PA) activity in the intestine of *O. niloticus* fed with various concentrations of CuNPs. The significantly higher PA activity (U/mg protein) was observed in fish fed with dietary treatment D III whereas, lower PA activity (U/mg protein) was measured in the control group ($p < 0.05$) (Figure 5).

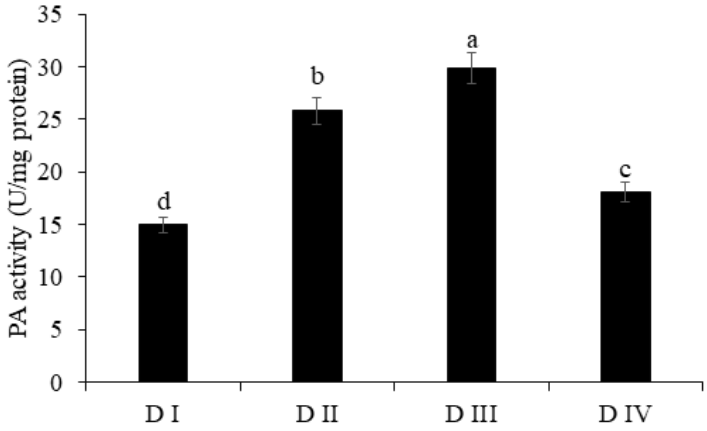

Figure 5: Indicates the protease (PA) activity (U/mg protein) in the intestine of *O. niloticus* fed with various concentrations of CuNPs. The graph bars show the mean values of three replicates (n =3) ± standard error of the mean (SEM). Different superscripts show significant differences at $p < 0.05$ among the means of different dietary treatments. D I was the control (without CuNPs), whereas D II, D III, and D IV were supplemented with 2, 4, and 6 mg/kg CuNPs, respectively.

Effect of CuNPs and $CuSO_4$ on Serum Glucose Level

The serum glucose level of *O. niloticus* fed with various concentrations of CuNPs was significantly enhanced in the fish group fed with dietary treatment D III ($p < 0.05$) compared to other dietary treatments whereas, this increase was considered to be within the normal ranges of healthy fish (Figure 6).

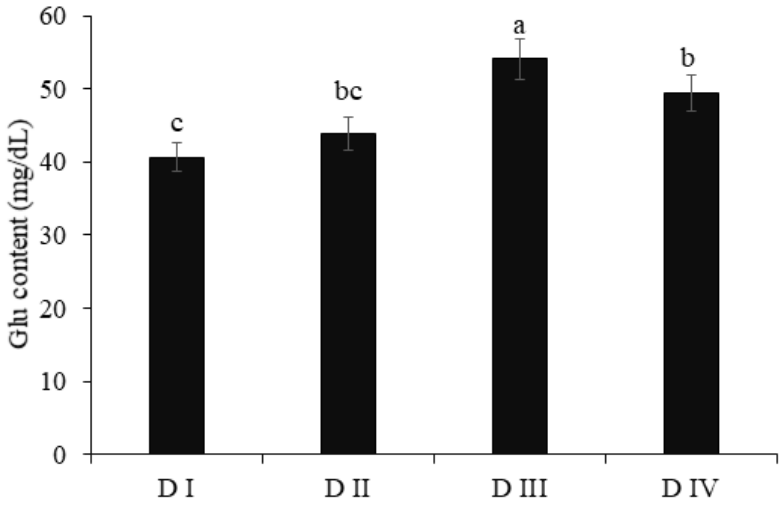

Figure 6: Shows the serum glucose content (mg/dL) of *O. niloticus* fed with various concentrations of CuNPs. The graph bars show the mean values of three replicates (n =3) ± standard error of the mean (SEM). Different superscripts show significant differences at $p < 0.05$ among the means of different dietary treatments. D I was the control (without CuNPs), whereas D II, D III, and D IV were supplemented with 2, 4, and 6 mg/kg CuNPs, respectively.

Discussion

In the present study, observation of improved growth performance of *O. niloticus* fed with a diet supplemented with CuNPs is comparable to the previous studies suggesting enhanced growth and immune response in several fish species, including grass carp (*Ctenopharyngodon idella*) [33], beluga huso (*Huso huso*) [29], orange-spotted grouper (*Epinephelus coioides*) [34], Nile *tilapia* (*Oreochromis niloticus*) [10] and red seabream (Pagrus major) [32]. Additionally, poor performance was reported in some crustacean species such as Malabar grouper (*Epinephelus malabaricus*) [18] and giant freshwater prawn (*Macrobrachium rosenbergii*) [5]. In pursuit of discovering more efficient diets for fish, supplementation of diet with vital dietary components, such as copper in the form of nanoparticles (NPs), has been found to play an important role [28,29]. Therefore, a plausible explanation for this improved growth performance would be the enhanced absorption and bioavailability of nutrients due to the dietary supplementation of NPs [30,31]. Since the trace elements in NPs show higher absorption rates and better interaction with other nutritional components due to their increased surface area [32]. As previous data suggested Cu plays a vital role in absorption and metabolism of other micronutrients, such as iron which is subsequently involved in the formation of haemoglobin molecules [30,35,36]. Moreover, Cu is imperative for the enhancement of immune capacity in the animal body [8]. However, some past observations made with grass carp and beluga had suggested growth retardation and suppression in the immune system due to Cu deficiency [29,33].

Likewise, the survival rate is also known as an important index in aquaculture practices. These findings of survival rate are in line with the observation of Mohseni, et al. [27] suggested a maximum survival rate up to a certain level of supplementation in juvenile H. huso and thereafter a decreasing trend was found in the survival rate. Previous studies conducted with red sea bream and freshwater prawn suggested improved survival rates with CuNPs at lower dietary concentrations of CuNPs [1,5]. The reduced survival rate at high dietary CuNP levels might be due to the initiation of a Fenton-type reaction which produces radical species and increases oxidative stress in fish [22]. In addition, exposure to excessive CuNPs disrupts the mitochondrial bioenergetics and osmoregulatory processes which consequently causes high mortality rates in fish [6].

These results of feed consumption are in agreement with some previous reports that suggested improved feed utilization rates in P. major [1], E. malabaracus [18], piglets [7], and hybrid *tilapia* (*O. niloticus* (L.) x O. aureus) [37-41] in response to dietary CuNPs administration. This could be explained by the fact that dietary CuNP supplementation improves the feed intake which might be helpful for protein retention [42]. Furthermore, the penetration and absorption of nutrients through small capillaries by the nano-sized Cu is more efficient as compared to its conventional form [7] therefore, CuNPs bind with the proteins to form Cu-protein complexes, such as ceruloplasmin which shows antioxidant

activity in blood plasma [2]. Thus, high FER and PER might be proportionate to the various factors; such as enhanced feed intake and metabolic and intestinal absorption by CuNP supplementation [43].

Similar findings for body indices have already been reported by El-Basuini, et al. [1] in P. major. However, another study indicated the detrimental effects of high concentrations of Cu on intestinal proliferation in Atlantic salmon, Salmo salar (L.) [41] and a study involving broiler concluded that high Cu intake results in Cu accumulation in the liver causing liver enlargement [9]. The toxicity of Cu and its effects on the growth or the enlargement of organs, especially the liver, and heart, and metallothionine response have been extensively studied but to date, their mechanism is not fully understood [44-52].

Whole-Body Proximate Composition

A similar trend was observed for Cu retention in previous studies conducted with rainbow trout (Salmo salar), *E. malabaricus*, pig, cow, and ewe [18,52-55]. In the current study, it was observed that the accumulation of CuNPs in the muscle and carcass of juvenile *O. niloticus* is a dose-dependent function. The retention of Cu in the animal body is directly associated with several factors such as form (nano or conventional), size, dietary Cu content, Cu concentration in rearing water, and the rate of Cu absorption through the intestine [56]. By contrast, El-Basuini, et al. [30] found no significant effect on Cu retention in red sea bream when fed with CuNPs. This difference in response might be due to the difference in particle size used in both studies because, particle size is the key factor that greatly influences efficiency whereas, the chemical form of Cu is another significant factor regarding its bioavailability and accumulation in the body [57].

Similarly, increased activities of serum glutamic-oxaloacetic transaminase (SGOT) and serum glutamic-pyruvic transaminase (SGPT) were measured with high doses of CuNPs in Chinese mitten crab [58], sea anemone [58], freshwater prawn [5] in previous experiments. The increased AST and ALT activity indicates the pro-oxidant effect of Cu by enhancing the production rate of radical species (ROS and RNS) in fish groups fed with high concentrations of CuNPs. The possible explanation for this condition might be that the high doses of CuNPs may cause damage to various organs including the liver, heart, kidney, or muscle, and therefore, liberate the transaminase enzymes in the blood circulation [59]. However, Cu deficiency may cause stress by suppressing the immune system in fish bodies therefore, excess or deficiency of CuNPs, both conditions result in the form of increased activity of serum AST and ALT.

A similar result was observed for antioxidant enzyme activity in juvenile grouper by Lin, et al. [17]. It is a proven fact that optimum growth and good health; both vital physiological processes are greatly influenced by the proper functioning of antioxidant enzymes in fish. The enhanced SOD and POX activity with the deficient and excess supplementation of CuNPs might be attributed to the increased production of oxyradicals and other secondary products of the oxidation process including malondialdehyde (MDA), aldehydes, and ketone bodies [29]. Hence, a plausible reason behind the improved oxidative stability might be the improved profile of antioxidant enzymes, such as Cu-Zn superoxide dismutase, lysyl oxidase, and cytochrome oxidase due to the appropriate level of CuNPs supplementation [35].

The result of digestive enzyme (protease and lipase) activity is in line with the finding of Muralisankar, et al. [35] in *M. rosenbergii*. Undeniably, the appropriate regulation of nutritional physiology is entirely associated with the digestive machinery (digestive enzymes and gut microbiota) in the fish body. The dietary supplementation of CuNPs forms various complexes along with various types of proteins inside the gastrointestinal tract (GIT) which enhances the activity of protein digestion enzymes and consequently improves the bioavailable of proteins in the gut. Furthermore, the nano-sized particles of Cu facilitate the transport of proteins into the small capillaries of the intestine [3] hence, supplementation of aquafeeds with CuNPs is of great significance. On the other side, CuNPs in excess could modulate the enterocyte surface and thus, may cause inhibition in the digestive enzyme activity in the intestine [35].

Glucose Thus, it reflects that fish were not facing any stress conditions related to the experimental diets. Earlier studies conducted with red sea bream, *P. major*, and *pirarucu, Arapiama gigas* showed similar results [50,51]. The studies including Cu requirements, Cu deficiency, and water-borne toxicity of Cu salts have been abundantly documented but research investigations on positive effects and diet-borne toxicity of Cu in the nano form are limited. Nevertheless, guidelines laid down by the present study should be of value in feed formulation to avoid diet-borne toxicity in fish. In future studies, histological analyses of organs in response to CuNP supplementation should help understand the mode of action of CuNPs as a dietary additive.

Conclusion

The findings of the present study suggested that supplementation of *O. niloticus* diets with CuNPs (at 4 mg/kg diet) resulted in positive effects on growth performance, feed consumption, body indices, Cu retention, and overall health status by reducing the oxidative stress in juvenile *O. niloticus*.

Additionally, the use of more efficient forms of minerals in aquafeeds could reduce the overall production cost.

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Author's contribution

The project was designed and supervised by Atif Yaqub. Nadia Nazir, Maria Basit, and Ayesha Tariq performed practical work. Muhammad Ayub helped in analytical work. Data compilation and manuscript writing was done by Atif Yaqub and Maryam Iqbal. All authors read and revised the manuscript.

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