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Zinc Oxide Nanoparticles Boost the Immune Responses in *Oreochromis niloticus* and Improve Disease Resistance to *Aeromonas hydrophila* Infection

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Abstract

Zinc is an essential element affecting immune responses in aquatic organisms. In the present research, the immunomodulating effect of zinc oxide nanoparticles (ZnO NPs) was studied in Nile tilapia (*Oreochromis niloticus*). The minimum inhibitory concentration of zinc oxide nanoparticles (ZnO NPs) for *Aeromonas hydrophila* was estimated at 60 µg/mL. To evaluate the efficacy of ZnO NPs for improving disease resistance against *A. hydrophila*, three hundred fish were divided into 5 groups. Fish in the group T1 maintained on the control feed, T2 and T3 feed on ZnO at 60 and 30 µg/g, while T4 and T5 received ZnO NPs at 60 and 30 µg/g, respectively for 8 weeks. Immune responses were evaluated by determining the phagocytic activity, serum antibacterial activity, lysozymes, respiratory burst activity, and also gene expression of immunoglobin M-2, tumor necrosis factor-α, interleukin (IL)-1β, heat shock proteins, IL-10, insulin growth factor 1, transforming growth factor-β2, superoxide dismutase enzyme, and catalase enzyme genes. Results indicated that groups that received ZnO NPs have exaggerated immune response and upregulation in the most of expressed immune-related genes. After the feeding trial, all groups were experimentally infected with *A. hydrophila*, and the mortality rate was monitored. Among all the treated groups, a higher survival rate and disease resistance were observed for fish that received ZnO NPs at 30 and 60 µg/g. The inclusion of ZnO NPs in *O. niloticus* feed improves both fish immune response and disease resistance against *A. hydrophila*.

Keywords Zinc oxide · Nanoparticles · Gene expression · Cytokines · Antioxidants

Introduction

Aquaculture is an important sector in the agriculture economy, as it participated in 42.2% of the global fish production in 2016. Aquatic animals and their products are the keys to meeting the nutritional protein needs of humans in Egypt [1]. The nutritional requirements of farmed fish should be fulfilled in their feed to positively affect reproductive performance and optimize production in the aquatic sector [2–4].

Zinc (Zn) is an essential micronutrient for fish, as it plays a vital role in biological activities [5, 6]. As a microelement, Zn participates in several enzymatic processes of animal metabolism such as growth, immune response, and enzyme function [7, 8]. Zn restrains the generation of reactive oxygen species (ROS) such as superoxide anion radical, hydroxyl radical, and hydrogen peroxide [9]. Zinc cannot be stored in the body, necessitating regular dietary intake [10, 11]. There are several Zn sources, including Zn carbonate, chloride, oxide, and sulfate; however, the primary mineral element content is higher in Zn than in other sources and represents 803.4 mg/g [6]. The bioavailability of Zn in fish feed is greatly affected by the tricalcium phosphate content [12]. Higher levels and continuous Zn supplementation should be included in feeds to compensate for the reduced bioavailability, so the continued search for additional techniques to enhance Zn bioavailability in fish is important.

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In 2015, about 30,000 metric tons of zinc oxide nanoparticles (ZnO NPs) was produced and used that reflect the importance of this mineral form. Different suggestions have been offered to explain the effect of ZnO NPs on bacterial cells, for example, ZnO NPs can alter the function of the bacterial cell wall by establishing hydrogen bonds and ligands, changing its protein structure [13]. In addition, by binding to the cytoplasmic bacterial membrane disrupting its integrity, ZnO NPs subsequently interrupt the fundamental role of electron transport phosphorylation and energy transduction processes [14].

This work aimed to investigate the protective role of ZnO NPs against bacterial infection in *Oreochromis niloticus* by studying its influence on fish immunity and antioxidant activity.

Materials and Methods

Preparation of Zn NPs

Zinc sulfate ($ZnSO_4 \cdot 7H_2O$) and zinc oxide (ZnO), El-Nasr Co., Giza, Egypt, were purchased from the local market. Nanocrystalline ZnO was prepared using the precipitation method of Daneshvar et al. [15]. Briefly, zinc sulfate was mixed with sodium hydroxide, then the mixture was continuously stirred for 12 h and then the produced zinc hydroxide precipitate was washed with deionized water. The precipitant was dried then calcined in air at a specific temperature to produce nanosized ZnO. The particle size of ZnO NPs was determined by a transmission electron microscope.

Fish Feed Preparation

ZnO NPs were ultrasonically distributed in water according to the procedure developed by Sherif et al. [16]. Briefly, 30 and 60 mg of ZnO NPs were sonicated in 100 mL of Milli-Q water and the same procedure was done for ZnO. One kg of pelleted fish feed was sprayed with 100 mL of the prepared ZnO NP solution to achieve a concentration of 30 and 60 µg/g. The prepared ration was dried at 50 °C for 1 h to remove the excess moisture. The physical and chemical composition of the experimental basal diet is presented in Table 1.

Table 1 Ingredient composition and chemical analysis of the experimental diet

Ingredients	%	Chemical analysis	%
Corn	15	**Moisture**	11.09
Soya (44%)	30	**CP**	42.05
Fish meal (60%)	25	**Ether extract**	5.71
Wheat flour	7	**Ash**	7.23
DDGs	5	**Crude fiber**	2.63
Corn gluten	15	**NFE**	35.29
Soya oil	1.5	**DE (kcal/kg)**[3]	2954
MCP	1		
Salt	0.2		
Methionine	0.05		
Choline chloride	0.05		
Mineral premix[1]	0.1		
Vitamin premix[2]	0.1		

[1]Mineral premix: each 1 kg contains manganese 60 g, copper 4 g, zinc 50 g, iodine 1 g, iron 80 g, cobalt 0.1 g, selenium 0.1 g, calcium carbonate (CaCO3) carrier to 1000 g

[2]Vitamin premix: each 1 kg contains vitamin A 12,000,000 IU, vitamin D3 2,200,000 IU, vitamin E 10 g, vitamin K3 2 g, vitamin B1 1 g, vitamin B2 5 g, vitamin B6 1.5 g, vitamin B12 0.01 g, vitamin C 250 g, niacin 30 g, biotin 0.050 g, folic acid 1 g, and pantothenic acid 10 g and carrier to 1000 g

[3]DE kcal/kg = digestible energy (DE) was calculated using formula based on chemical composition of feed stuffs nutrients according to NRC [6]

DDGs dried distilled grains

NFE nitrogen-free extract

Determination of Zn Concentration in Fish Tissues

Zn concentrations in the fish tissues were analyzed following the method designated by Li et al. [17]. Briefly, the whole body of three randomly selected fish from each group was killed by an overdose of MS222 anesthetic, and 2 g of the dorsal musculature of each fish was taken and labeled. Each fish sample was minced then dried and finely ground and each muscle sample was ground in a clean mortar after that whole fish tissue and muscle samples were dried in a drying oven at 90 °C for 1 day. One hundred and fifty milligrams of dried sample was added to 15 mL of 65% nitric acid and 2 mL of 70% perchloric acid in Kjeldahl flasks after complete digestion, and 33 mL of deionized water was added. The sample was assayed in atomic absorption spectrophotometer equipped with a graphite furnace (Model AA-240Z; Varian, Australia).

The Minimum Inhibitory Concentration of ZnO NPs for Aeromonas hydrophila

The minimum inhibitory concentration (MIC) was calculated following the recommendations described by Ravikumar et al. [18]. Briefly, the overnight *Aeromonas hydrophila* broth culture was adjusted to 1×10^6 CFU, then diluted to 1:200 with sterile broth. One hundred and ninety microliters from the diluted broth was added to each well of a 96-well microtiter plate. Ten microliters of ZnO NP solution was added to each well to achieve a final concentration of 100, 90, 80, 70, 60, 50, 40, 30, 20, and 10 µg/mL then the plate surface was sealed with cello tape to avoid dehydration. The

whole setup was performed in triplicates and culture microplates were incubated at 37 °C for 48 h. MIC is determined as the lowest concentration of ZnO NPs that completely inhibited bacterial growth.

Experimental Design and Fish Accommodations

A total number of 320 apparently healthy *O. niloticus* (28 ± 3 g) were collected from a private fish farm in Kafrelsheikh, and fish were stocked in the wet laboratory of the Animal Health Research Institute, Egypt. Fish were acclimated in a 3-m^3 fiberglass tank for 2 weeks, after that 20 fish were examined for clinical signs and postmortem following recommendations of Austin and Austin [19] to ensure that the experimental fish is free from any disease. Experimental fish were randomly divided into 5 groups (T1–T5) in triplicates (20 fish/replicate). Fish in the first group (T1) were maintained as a control and received a basal diet without any additives. The second and third treatment groups (T2 and T3) received ZnO at 60 and 30 µg/g feed while the fourth and fifth treatment groups (T4 and T5) were received ZnO NPs at 60 and 30 µg/g feed, respectively. Each replicate was maintained in 250-L glass aquaria (110 × 50 × 50 cm). During the experimental work, the water temperature was adjusted at 28 °C ± 1.5 °C, dissolved oxygen more than 5.5 ± 0.5 mg/L, pH 7.8, and salinity around 0.3 g/L. In addition, one-third of the water was exchanged daily with the removal of fish excreta.

Innate Immunity Responses

Blood Sampling

Blood samples were collected from 5 fishes in each group on the last day of the feeding trial, about 2 mL of blood was collected from the caudal vessels of each fish. Serum samples were separated by centrifugation at 2000 rpm for 10 min, and leukocytes were isolated from each blood sample as described by Faulmann et al. [20].

Phagocytic Activity

Phagocytic activity (PA) and the phagocytic index (PI) were calculated according to Kawahara et al. [21]. Twenty-four-hour *Candida albicans* culture was counted with hemocytometer then adjusted to 1×10^6 cells/mL, and the leukocytes of each sample were adjusted to 2.5×10^6 cells/mL. One millliter of adjusted leukocytes was added to 1 mL of *C. albicans* suspension, and the mixture was incubated in a CO_2 incubator at 27 °C for 1 h. One smear was prepared from each sample then stained with Giemsa then one-hundred leukocytes were counted under the oil immersion lens, and the number of engulfed yeast cells was also determined. The PA and PI were calculated according to the following equations:

$$\mathbf{PA} = \frac{\text{No. of ingesting phagocytes}}{\text{Total no. of phagocytes}} \times 100.$$

$$\mathbf{PI} = \frac{\text{No. of ingested } C.albicans \text{ cells}}{\text{No. of ingesting phagocytes}}.$$

Serum Bactericidal Activity Percentage

Serum bactericidal activity (SBA) was determined by mixing equal volume (100 µL) of fish serum and *A. hydrophila* bacterial suspension containing 1×10^6 CFU/mL. The mixture was incubated for 1 h at 25 °C, and the blank sample was prepared by replacing fish serum with sterile phosphate-buffered saline (PBS). A series of tenfold dilutions were prepared from each mixture and streaked on blood agar, and plates were incubated for 24 h at 37 °C then the number of viable bacterial colonies was counted [22].

Analysis of Lysozyme Activity

Lysozyme (LYZ) activity was assayed as described by Helal and Melzig [23]. Initially, 10 µL of fish serum was added to 100 µL of *Micrococcus lysodeikticus* suspended in PBS (360 µg/mL, pH:6.24). The absorbance of samples was measured at 450 nm after incubation at 37 °C for 5 min.

Respiratory Burst Activity

Respiratory burst activity (RBA) was estimated for isolated leukocytes using nitro blue tetrazolium (NBT) assay via the method reported by Jang et al. [24]. Fifty from previously prepared leukocyte suspension was loaded in 96-well plate and incubated at 30°C for 1 h. The wells were rinsed with PBS (pH 7.2) and set aside for 1 h at 30°C after adding NBT (50 µL). Methanol (30%) was added to each well to fix the cells and left undisturbed for 5 min. The air-dried wells were filled with 60 µL of 2 mM potassium hydroxide and 70 µL of dimethyl sulfoxide. Absorbance was ultimately measured on a plate reader at 540 nm.

Gene Expression of Cytokines and Antioxidant Enzyme Genes in O. niloticus

Total RNA was extracted from the head kidney tissues that were collected from three fish in each group on the last day of the feeding trial using the Trizol reagent (iNtRON Biotechnology Inc., Korea) following the manufacturer's procedure. The quantity and quality of extracted RNA were assessed by Nanodrop D-1000 spectrophotometer

(NanoDrop Technologies Inc., USA). The complementary DNA (cDNA) was synthesized by the reverse transcription-polymerase chain reaction using SensiFAST cDNA synthesis kit (Bioline, USA) following the manufacturer's protocol. For studying the gene expression, the resulting cDNA was used as a template in the quantitative real-time PCR using Nile tilapia-specific primer sets (Table 2) for immunoglobin (Ig) M-2, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, heat shock protein (IL-10), insulin growth factor (IGF) 1, and transforming growth factor (TGF)-β2, as well as superoxide dismutase enzyme (SOD) and catalase (CAT) enzyme, while the gene encoding β-actin was used as the housekeeping gene, owing to its constitutive expression. After the efficiency of PCR was confirmed to be around 100%, the data of gene expression were calculated using the Eq. $2^{-\Delta\Delta CT}$ method according to the procedure of Livak and Schmittgen [25].

Table 2 Sequences of the primers used in the expression of the studied genes

Gene	Sequence 5–3	GenBank accession No
IgM-2	F: CCACTTCAACTGCACCCACT R: TGGTCCACGAGAAAGTCACC	KC677037.1
TNF-α	F: AGGGTGATCTGCGGGAATACT R: GgCCCAGGTAAATGGCGTTGT	NM_001279533.1
IL-1β	F: TCTTCTACAAACGCGACACC R: TCTGGAGCTGGATGTTGAAG	KF747686.1
IL-10	F: ACCCCGTTCGCTTGCCA R: CATCTGGTGACATCACTC	[26]
IGF1	F: TCTTCAAGAGTGCGATGTGC R: GGCCATAGCCTGTTGGTTTA	XM_003448059
TGF-β2	F: GCTCACGATCTTCCGTCTTC R: CACTCCCCCTCTGTTTGTGT	NM_001311314
SOD	F: CATGCCTTCGGAGACAACAC R: ACCTTCTCGTGGATCACCAT	AY491056.1
CAT	F: AGCTCTTCATCCAGAAACGC R: GACGTCAGGCGTCACATCTT	JF801726.1
β-actin	F: CCACACAGTGCCCATCTACGA R: CCACGCTCTGTCAGGATCTTCA	EU887951.1

F forward, *R* reverse, *IgM-2* immunoglobin M-2, *TNF-α* tumor necrosis factor-alpha, *IL-1β* interleukin-1 beta, *IL-10* interleukin-10, *IGF1* insulin growth factor 1, *TGF-2* transforming growth factor-beta 2, *SOD* superoxide dismutase enzyme, *CAT* catalase enzyme, *β-actin* beta-actin

Bacterial Challenge

After the end of the feeding trial, two replicates in each group (40 fish/group) were experimentally infected with *A. hydrophila* (AHRAS22) strain accession number in NCBI (MW092007), in which one replicate was injected intraperitoneally (I/P) with the LD_{50} that equals 2.4×10^5 CFU/fish and the other replicate infected through the addition of the infective agent in aquarium water (cohabitation) to simulate the natural infection conditions in fish farms.

Daily mortality was recorded for 14 successive days and the cumulative mortality rate (CMR) was calculated using the following equation:

$$\mathrm{CMR}(\%) = \frac{\mathrm{Number\,of\,deaths\,in\,a\,specific\,period}}{\mathrm{Total\,population\,size\,during\,that\,period}} \times 100.$$

The relative level of protection (RLP) was verified among the challenged fish according to Ruangpan et al. [27] as follows:

$$\mathrm{RLP}(\%) = \left[1 - \frac{\mathrm{mortality\%\,in\,the\,treated\,group}}{\mathrm{mortality\%\,in\,the\,control\,group}}\right] \times 100.$$

A. hydrophila were re-isolated from dead and moribund fish according to the methods described by Austin and Austin [19]. For bacteriological examination, a loopful from the kidney, liver, spleen, and heart was added to brain heart infusion broth tube, then tubes were incubated at 28 °C for 24 h [28]. A loopful from the broth was streaked on Aeromonas selective agar base with ampicillin supplement then plates were incubated at 28 °C for 24 h, and after that, single pure colony from each plate was identified biochemically using API 20E strips. Phenotypic characterization of the bacterial isolates was confirmed according to the published instructions of Madigan and Martinko [29].

Biosafety Considerations

This study followed the biosafety measures outlined on the pathogen safety data sheets entitled infectious substances — *A. hydrophila*, Pathogen Regulation Directorate [30].

Statistical Analyses

Data were statistically analyzed for variance (ANOVA) with SPSS software, SPSS Inc., Chicago, IL, USA. Duncan's multiple range test [31] was used to determine differences among treatments at a significance level of 0.05.

Results

Characterization of Zn NPs

Synthesized ZnO NPs used in this work were approximately 45 nm (99.7% purity). Scanning electron microscopy results show the different sizes of produced ZnO NPs as represented in Fig. 1.

Zn Residue in Fish Tissues

High Zn level was significantly retained in the whole body of *O. niloticus* that received 60 µg/g of ZnO NPs compared to other treatments while there was a non-significant difference between groups received 30 µg/g (T3 = 26.17 and T5 = 28.6 µg/g) regardless of the form of ZnO. The residue of Zn in fish muscles followed the same whole-body pattern, except in groups that received ZnO NPs (T4 = 22.2 and T5 = 17.4 µg/g) in which zinc levels were higher than other groups received ZnO as represented in Table 3.

The Minimum Inhibitory Concentration of ZnO NPs for A. hydrophila

The estimated MIC of the synthesized ZnO NPs for *A. hydrophila* was 60 µg/mL.

Effect of In-feed Supplementation with ZnO NPs on the Innate Immunity

The incorporation of ZnO NPs in the fish diet has a significant booster effect on innate immunity as represented in Table 4. In addition, the higher concentration of ZnO regardless of its form has improved innate immunity for groups T2 and T4 than other groups.

Level of Cytokine Gene Expression

Feeding dietary ZnO modulated the gene expression of IgM-2, TNF-α, IL-1β, IL-10, IGF 1, and TGF-β2 in the head kidney tissue of *O. niloticus* as shown in Figs. 2 and 3. IgM-2 and anti-inflammatory cytokines (IL-10 and TGF-β2) were significantly increased in fish that received

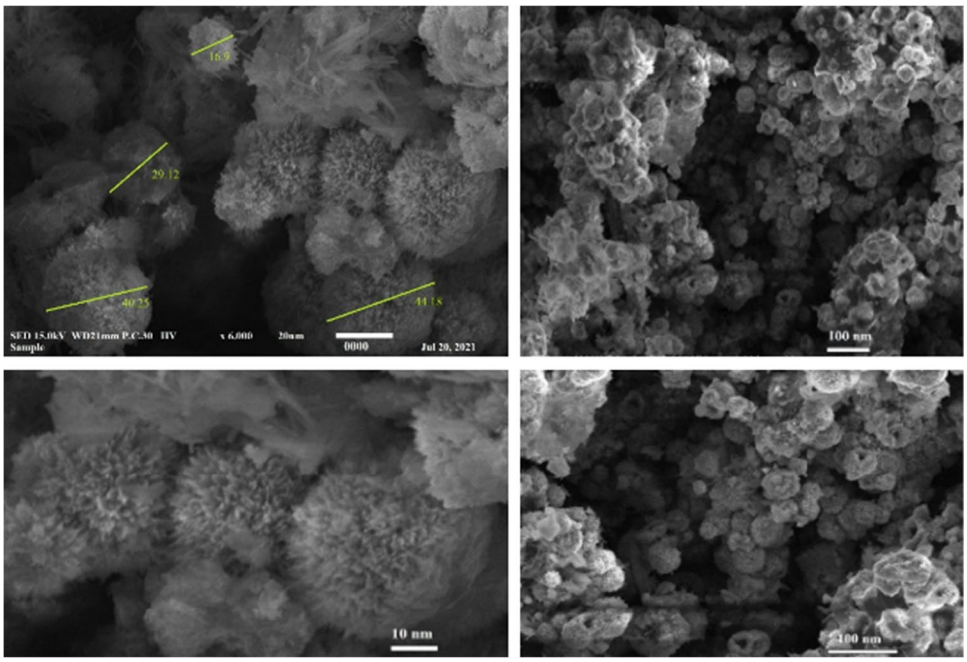

Fig. 1 Characterization of ZnO NPs using a transmission electron microscope

Table 3 Zn concentration in the body and muscles of *O. niloticus* based on dry body weight

Item	T1	T2	T3	T4	T5
Whole body	$22.4^{a} \pm 0.34$	$32.1^{b} \pm 1.04$	$26.17^{c} \pm 1.35$	$37.6^{a} \pm 1.3$	$28.6^{c} \pm 0.6$
Muscles	$9^{d} \pm 0.5$	$17.8^{b} \pm 1.2$	$12.8^{c} \pm 1.2$	$22.2^{a} \pm 0.8$	$17.4^{b} \pm 0.3$

Values are mean zinc concentration in µg/g ± SE; *T1* control, *T2* ZnO in feed at 60 µg/g, *T3* ZnO in feed at 30 µg/g, *T4* ZnO NPs in feed at 60 µg/g, *T5* ZnO NPs in feed at 30 µg/g. The same letter indicates nonsignificant differences at $P \leq 0.05$

Table 4 Immune responses in *O. niloticus* that received different concentrations of ZnO

Item	T1	T2	T3	T4	T5
PA %	$42.33^{c} \pm 1.45$	$52.7^{b} \pm 1.4$	$42^{c} \pm 1.2$	$59.3^{a} \pm 1.3$	$53.67^{ab} \pm 3.18$
PI	$0.67^{a} \pm 0.7$	$2.7^{a} \pm 0.3$	$1.33^{a} \pm 0.88$	$3^{a} \pm 0.58$	$2.67^{a} \pm 0.9$
SBA %	$24.3^{c} \pm 2.85$	$49.3^{a} \pm 1.8$	$33.7^{b} \pm 3.2$	$53.7^{a} \pm 3.5$	$49.3^{a} \pm 1.86$
LYZ	$0.14^{d} \pm 0.03$	$0.84^{b} \pm 0.04$	$0.29^{cd} \pm 0.01$	$1.17^{a} \pm 0.1$	$0.36^{c} \pm 0.04$
RBA	$6.67^{c} \pm 0.9$	$8.3^{c} \pm 0.7$	$7.7^{c} \pm 0.9$	$14.7^{a} \pm 1.2$	$11.7^{b} \pm 0.3$

Values are mean ± SE; *T1* control, *T2* ZnO in feed at 60 µg/g, *T3* ZnO in feed at 30 µg/g, *T4* ZnO NPs in feed at 60 µg/g, *T5* ZnO NPs in feed at 30 µg/g, *PA* phagocytic activity, *PI* phagocytic index (No. of ingested cells), *SBA* serum bactericidal activity, *LYZ* lysozyme (U/mL), *RBA* respiratory burst activity. The same letter indicates nonsignificant differences at $P \leq 0.05$

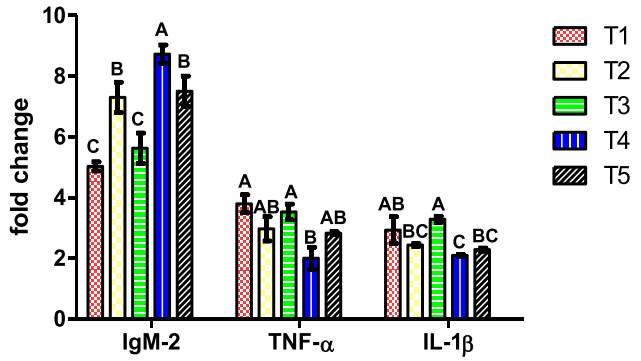

Fig. 2 The level of gene expression in *O. niloticus* fed different forms of ZnO. IgM-2, immunoglobin M-2; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1 beta. The same letter indicates nonsignificant differences at $P \leq 0.05$

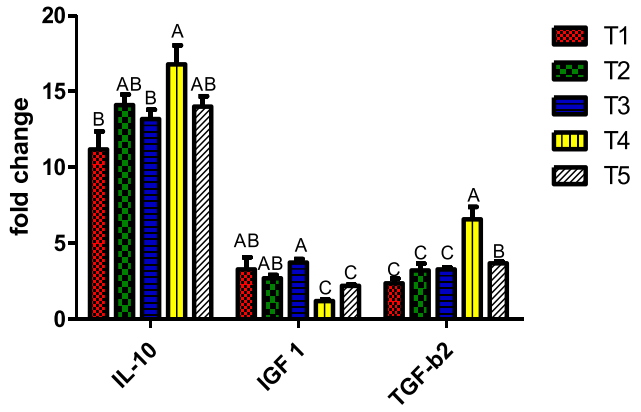

Fig. 3 The level of gene expression of different cytokines. IL-10, interleukin10; IGF1, insulin growth factor 1; TGF-β2, transforming growth factor-beta 2. The same letter indicates nonsignificant differences at $P \leq 0.05$

ZnO NPs at 60 µg/g in their feed in comparison with the control group and representing 8.73-, 16.8-, and 6.6-fold, respectively; this finding indicates the enhancement of fish immune status.

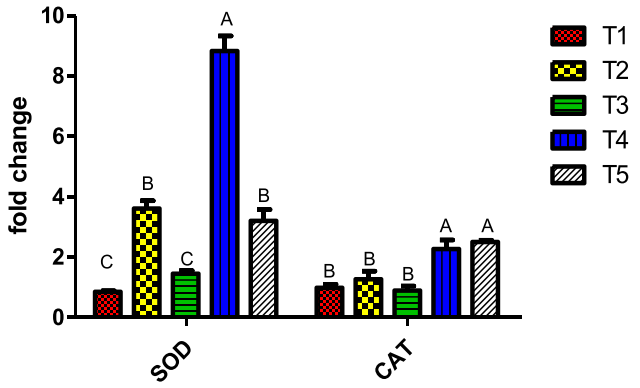

Fig. 4 Gene expression level of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT). The same letter indicates nonsignificant differences at $P \leq 0.05$

Gene Expression of Antioxidant Enzymes

The gene expression levels of SOD and CAT had a significant upsurge in the head kidney of *O. niloticus* that received dietary ZnO NPs at both 30 and 60 µg/g. There was 8.83- and 3.2-fold increase for SOD also there was 2.27- and 2.5-fold increase for CAT in comparison with the control group, as represented in Fig. 4. Group received ZnO also showed 3.8-fold increase in SOD expression compared with the control group.

Results of Challenge Test with A. hydrophila Bacteria

In Table 5, fish that received dietary ZnO NPs in T4 and T5 showed a lower cumulative mortality rate (40%), and this was lower than the cumulative mortality in the other groups. Fish that received ZnO NPs at 30 and 60 µg/g also showed a higher relative level of protection estimated by 33.33% and 60%, respectively. While ZnO could not protect challenged fish against bacterial infection, it decreased the mortality in cohabitant fish compared to I/P-infected fish.

Table 5 Cumulative mortality and relative protective effect of ZnO on *O. niloticus* challenged with *A. hydrophila*

Item	T1 –ve	T1 + ve		T2		T3		T4		T5	
MOF	-	**I/P**	**W**	**I/P**	**W**	**I/P**	**W**	**I/P**	**W**	**I/P**	**W**
MR %	0	60	50	60	40	60	40	40	20	40	20
SR	100	40	50	40	60	40	60	60	80	60	80
RLP	-	-	-	0	20	0	20	33.33	60	33.33	60

T1 -ve control feed non-infected, *T1 + ve* control feed infected, *I/P* infected by intraperitoneal inoculation, *W* infected through cohabitation with the pathogen, *T2* ZnO in feed at 60 µg/g, *T3* ZnO in feed at 30 µg/g, *T4* ZnO NPs in feed at 60 µg/g, *T5* ZnO NPs in feed at 30 µg/g, *MR* mortality rate, *SR* survival rate, *RLP* relative level of protection

Discussion

Farmed fish are mainly fed diets of plant origin in which Zn absorption and bioavailability are low due to the presence of dietary phytate and tricalcium phosphate [32, 33].

In this study, whole body and muscle tissues of *O. niloticus* which received dietary ZnO NPs retained higher Zn levels than fish in the control group and both groups that received dietary ZnO. In all treatments, Zn concentration in the muscle showed higher values than the controls but was still within the range of the permissible limit (40 ppm) set by the Food and Agriculture Organization [34]. Accordingly, *Cyprinus carpio* fed ZnO NPs (30 and 100 µg/g) had a higher Zn level in their tissues than those provided similar concentrations of $ZnSO_4$ and Zn-proteinate. These findings are due to the ability of ZnO NPs to pass through the intestine epithelium and accumulate in the tissues [35]. In addition, Zn concentrations in the whole body of *O. niloticus* were insignificantly affected ($P > 0.05$) by incorporation of a ZnO mix of zeolite and ZnO or $ZnSO_4$ in their diets [36]. Supporting our results, inorganic Zn forms (ZnO, $ZnSO_4$, and zinc carbonate [$ZnCO_3$]) possess a low rate of absorption via fish intestine [37]. Different findings obtained with rainbow trout larvae [38] found that feeding the inorganic form of Zn ($ZnSO_4$) resulted in a higher accumulation in fish tissues than ZnO NPs and organic Zn.

Based on our results, the MIC of ZnO NPs was determined to be 60 µg/mL for *A. hydrophila*. Accordingly, when the potential bacterial inhibition property of *Murraya koenigii* berry extract-based ZnO NPs was assessed by Yazhiniprabha et al. [39], a concentration of 25 µg/mL was the least quantity required to inhibit 1×10^6 cells/mL of *A. hydrophila*, whereas a concentration of 50 µg/mL could diminish approximately 70–80% of biofilm activity. The antibacterial properties of ZnO NPs could be due to their ability to penetrate the bacterial cell membrane by releasing ions to disrupt cellular metabolism [40] and/or to generate ROS within bacterial cells, causing damage to the cell envelope, cell membrane, cell structure, and biomolecules [41].

Respiratory burst activities are initiated when the immune system identified any microbial pathogen as a part of the innate immune response [42, 43]. The LYZ is a part of the innate immunity of fish that protects against diseases [44]. Therefore, the significant increase of PA, PI, SBA, LYZ, and RBA in the experimental fish fed dietary ZnO NPs showed that ZnO NPs could boost the innate immunity of *O. niloticus*. In a similar study, Gharaei et al. [45] found that the activity of LYZ is significantly increased in beluga (*Huso huso*) after feeding dietary chitosan–ZnO NP. In addition, the response of *Oreochromis mossambicus* resembled that of our study as the LYZ and myeloperoxidase activities were increased in the serum after ZnO NP-based β-glucan binding protein was added to the feed [46]. Many enzymes and proteins in freshwater fish could restrict the invasion and replication of pathogenic bacteria [47]. In this study, dietary ZnO NPs activated SBA in *O. niloticus*. Similarly, Yazhiniprabha et al. [39] found that ZnO NPs promote the innate immunity of *O. mossambicus* by stimulating serum antiprotease activity and natural complement activity, which in turn restricted the invasion and replication of pathogens in the serum.

The innate and adaptive immune response could be promoted by NPs [48] through activating toll-like receptors [49] and downregulating the pro-inflammatory cytokines [7, 43, 50]. Our results showed that dietary ZnO NPs (60 µg/g) could enhance the immunity of *O. niloticus* as the expression of some genes involved in immune responses had a significant upsurge, such as IgM-2 and anti-inflammatory cytokines (IL-10 and TGF-β2). High gene expression of cytokines TNF-α and IL6 is considered signs of an inflammatory response [51–53], so low levels of mRNA gene expression of IL6 and TNF-α indicated an enhanced health status of blunt snout bream (*Megalobrama amblycephala*) fed zinc-bearing palygorskite (Zn-Pal) [54, 55]. Accordingly, Song et al. [52] mentioned that pro-inflammatory cytokines are downregulated in young grass carp (*Ctenopharyngodon idella*) that received dietary Zn.

Similarly, the gene expression of IgM was upregulated in response to dietary ZnO NPs (30 mg/kg) in *O. niloticus* [56]. The sera globulin in *C. carpio* was also significantly upregulated [57]. Zn bioavailability affects the potential immune role. Soaudy et al. [58] reported that the gene expression of IgM-2 is enhanced by supporting Zn with K. This upregulation may be due to the continuous and slow release of Zn from the ZnO-K form compared with other Zn forms.

It is well known that Zn and ZnO NPs can stimulate antioxidant responses via activation of enzymatic antioxidants, glutathione peroxidase [59], and CAT [60, 61], as well as non-enzymatic cellular responses, eliminating the generation of ROS and protecting tissues from oxidation stress-based damages. In this study, regardless of the concentration, the gene expression of SOD and CAT was upregulated in *O. niloticus* fed dietary ZnO NPs. Similarly, with freshwater fish, the antioxidant status of *O. niloticus* fed dietary ZnO NPs was enhanced through improving the activities of SOD and CAT [56]. In addition, Dekani et al. [35] recorded a significant increase in the activity of SOD and minimum activity of CAT in *C. carpio* fed a diet containing 500 mg/kg of ZnO NPs compared with those that received the same concentration of $ZnSO_4$. Gene expression of mRNA of CAT and glutathione S-transferase mu was increased and attained the peak in abalone (*Haliotis discus hannai*) fed a diet containing 33.8 mg/Zn/kg [62], supporting our findings. Different findings were obtained by Soaudy et al. [58], who noticed that enhancing the Zn availability of the ZnO-K form resulted in an increase in the expression of CAT and SOD genes in fish compared with those fed dietary ZnO NPs.

From our results, *O. niloticus* fed dietary ZnO NPs and experimentally infected I/P with *A. hydrophila* and cohabitants achieved RPL of 33.33% and 60%, respectively. These results may be because ZnO NPs could enhance the innate immunity (cellular and humoral) and oxidative status of *O. niloticus* that could compete for the bacterial infection. Zhang et al. [63] stated that the intestinal digesta of blunt snout bream fed dietary Zn-Pal shows a decrease in *Escherichia coli* and *A. hydrophila*. Jiao et al. [64] claimed that a low bacterial number could be due to the released Zn ions from Zn-Pal. Moreover, it is well documented that Zn deficiency reduces immune responses and disease resistance in humans and animals [65]. The high SBA of fish is mediated by molecules capable of inhibiting bacterial growth [66]. LYZ and phagocytosis are non-specific humoral immune defenses against gram-positive and gram-negative bacteria [67], activated in response to dietary ZnO NPs. Similarly, Yazhiniprabha et al. [39] found that *O. mossambicus* fed with Mb-ZnO NP-supplemented diet at different concentrations (0.5, 1, and 2 mg/kg) has improved immune responses and the ability to resist *A. hydrophila* infection. In freshwater fish *Pangasius hypophthalmus*, ZnO NPs enhanced immunological parameters such as total protein, albumin, globulin, and A/G ratio, as well as stress biomarkers such as blood glucose, cortisol, and HSP 70 that allow the fish to resist biotic (*Aeromonas veronii biovar sobria*) and abiotic multi-stressors as lead toxicity and water temperature of 34 °C [68]. Survival of freshwater prawn (*Macrobrachium rosenbergii*) and rainbow trout (*Oncorhynchus mykiss*) was significantly increased after dietary supplementation with the same concentration of ZnO NPs at 60 mg/kg [38, 69].

Conclusion

The study results indicate that ZnO can boost the immune status of *O. niloticus* and protect fish against bacterial infection. ZnO nanoparticles have a superior effect over the inorganic ZnO form in stimulating the immune system of fish through increasing the innate immune response together with upregulating the expression of immune-related, cytokines and antioxidant enzyme genes. ZnO nanoparticles also have a protective effect for *O. niloticus* against *A. hydrophila* infection when administrated infeed at 60 ppm.

Data Availability Data are available on request from the authors.

Declarations

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

Conflict of Interest The authors declare no competing interests.

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