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Transcriptomic profile change, immunological response and disease resistance of *Oreochromis niloticus* fed with conventional and Nano-Zinc oxide dietary supplements

Ashraf Awad[a], Asmaa W. Zaglool[a], Shaimaa A.A. Ahmed[b], Samah R. Khalil[c,*]

[a] Animal Wealth Development Department, Faculty of Veterinary Medicine, Zagazig University, Egypt
[b] Fish Disease and Management Department, Faculty of Veterinary Medicine, Zagazig University, Egypt
[c] Forensic Medicine and Toxicology Department, Faculty of Veterinary Medicine, Zagazig University, Egypt

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ABSTRACT

The present investigation was performed to evaluate the efficiency of Zinc oxide (ZnO) as a fish feed additive in immunomodulation of *Oreochromis niloticus*. Fish were fed on ZnO nano-particles (nZnO) and conventional (ZnO) in two concentrations (30 and 60 mg/kg diet), in addition to the control fish which was fed on Zn free diet. After 6° days, the highest survival rate was recorded in the $nZnO_{30}$ –supplemented group. The total antioxidant capacity (TAC) and antioxidant enzymes were improved in different dietary Zn supplementation, obviously in the $nZnO_{30}$ –supplemented group, while the lowest antioxidant status was noticed $nZnO_{60}$ supplemented fish. The lipid peroxides (MDA) level was diminished upon Zn supplementation, particularly in $nZnO_{30}$-supplemented group but showed a significant elevation in the $nZnO_{60}$-supplemented group. Furthermore, the immune parameters examined, lysozyme activity, bactericidal activity, and IgM were significantly higher in ZnO_{60}, and $nZnO_{30}$ supplemented groups. The C-reactive protein (CRP) level showed no significant increase in response to Zn supplementation in the both forms at level of 30 mg/kg diet, but showed marked elevation in $nZnO_{60}$-supplemented group. The mRNA expression profile of both interleukin 8 (*IL-8*), interleukin 1, beta (*IL-1β*) encoding genes showed an up-regulation that was found in all Zn- supplemented groups, but more pronounced in $nZnO_{60}$-supplemented group. On the other hand, the expression pattern of myxovirus resistance (*Mx*)-encoding gene showed no remarkable difference between the Zn- supplemented and control fish. The expression level of CXC-chemokine, toll-like receptor 7 (*TLR-7*), immunoglobulin M heavy chain (*IgM* heavy chain) and interferon gamma (*IFN-γ*) gene was upregulated in Zn-supplemented groups particularly in the $nZnO_{30}$- supplemented group. While, the lowest expression was found in $nZnO_{60}$- and ZnO_{30}-supplemented groups. Here, Zn supplementation promoted the immune and antioxidant strength in fish mainly in nano form at the level of 30 mg/kg diet but not at 60 mg/kg diet that disrupt the immune and antioxidant status and promote inflammatory response.

1. Introduction

Feed supplements have been an increasingly a well-known management technique in fish cultivating systems where numerous supplements have demonstrated an efficiency to improve the fish immune response or controlling the severity of infections [1,2]. The ideal feed ingredients used in fish feed should ensure growth, immunity and health promoting factors to achieve a great effect on the farm net gain. Minerals are essential nutrients for normal body processes; mineral requirements vary depending on forms, interactions with other elements, water quality and fish itself (age, size and species). Minerals are required in fewer amounts than other ration required nutrients e.g. protein, carbohydrates and fat. As they are essential, they have other side of being toxic [3].

Zinc (Zn) is an essential trace mineral that is required for growth and metabolism of all vertebrates including fish. It is required for structural, catalytic and regulatory proteins, which are important for growth, development and physiology of animals [4,5]. It is well documented that normal Zn levels in freshwater [6] and seawater [7] are insufficient to meet the requirement of growing aquatic species. Therefore, Zn is considered as an essential nutrient in finfish feed [8]. Dietary Zn requirements have been established for a number of

* Corresponding author. Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Zagazig University, 44511, Zagazig, Egypt.
E-mail addresses: resamah@zu.edu.eg, dr_samahkhalil@yahoo.com (S.R. Khalil).

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different fish species by using zinc sulfate (ZnSO4) as a dietary source and found in a range between 15 and 35 mg/kg diet [9]. Eid and Ghonim [10] demonstrated the suitable zinc level required for maximum weight gain of the Nile tilapia is 30 mg/kg diet.

High dietary levels of Zn may negatively affect the status of other elements such as iron, copper and cadmium [11], increase the cost of feed and contribute in minerals load of aquatic environment [12]. Therefore, the use of the trace minerals at nanometer dimension exhibit novel properties, which led to their use in less amount to improve the growth and immunity through antioxidant effect. [13,14], higher bioavailability, greater specific surface area, higher surface activity, and stronger adsorbing ability than its normal sized particles [15]. Moreover, enhanced bactericidal and catalytic efficiency [16,17].

Nano zinc oxide (nZnO) is third most noteworthy globally delivered nano metal after nano SiO2 and nano TiO2 [18]. The abrupt increase in the interest of nZnO is mostly owing to its efficient antibacterial properties over the conventional Zinc oxide (ZnO) [19]. Previous studies have already stated the dose dependent efficacy of nZnO supplementation on growth performance traits in animal and poultry [20–25]. Faiz et al. [26] stated that the nZnO supplementation has improved growth and immune status in grass carp (*Ctenopharyngodon idella*), In addition, a considerable increase in antioxidant enzymes activity, total protein content, and enhanced weight in freshwater prawn (*Macrobrachium rosenbergii*) [27].

For the assessment of dietary efficiencies of feed additives in animals, some of critical markers for the health status can be considered as hematological, serum biochemical and immune response-linked parameters [28]. Lately, an evaluation of immune-related gene expressions regulation profile is considered an important research area to monitor the effect on immune status. Therefore, genes related to immune systems were appointed to assess the effect of numerous immunomodulatory agents on tilapia fish. It is notable that the interferon gamma (*IFN-γ*), CXC-chemokine, interleukin 8 (*IL-8*), interleukin 1-beta (*IL-1β*), immunoglobulin M heavy chain (*IgM* heavy chain), myxovirus resistance (*Mx*) and toll-like receptor 7 (*TLR-7*) are utilized for the evaluation of innate, acquired and adaptive immune responses in teleost fish species [29–32].

Nile tilapia, *Oreochromis niloticus* (L.), is a popular fish category among fish farmers throughout the world attributable to its good development and high marketing value [33]. The tilapia fish is an economically significant fish species cultured chiefly in China, Indonesia and Egypt, with a global creation around 5.9 million tones [34].

The current study targeted to evaluate the influence of the supplementation of ZnO as a feed additive to *O. niloticus* in two forms; the conventional and the nano one. This was carried out by evaluating the physiological and immunological endpoints such as lysozyme activity, serum bactericidal activity and, protein profile. Besides, the expression of pattern of immune-encoding genes, and the susceptibility to infection such as that mediated by *Aeromonas sobria* were also assessed.

2. Materials and methods

2.1. Oreochromis niloticus fish

An apparently healthy *O. niloticus* fingerlings (N = 150, 35 ± 0.40 g weight) was purchased from El-Abbassa Fish Hatchery, Al-Sharkia Province, Egypt. Fish were acclimated for two weeks before the beginning of the experiment in glass aquaria (80 × 40 × 30 cm) filled with 60 L of dechlorinated tap water and fed on a basal diet formulated without addition of feed additives. All the aquaria were kept under the constant conditions of temperature (25 ± 1.02 °C), pH (6.9 ± 0.1), dissolved oxygen (7.4 ± 0.34 mg/L), and ammonia (0.035 ± 0.01 mg/L) with a controlled photoperiod (10 h light: 14 h dark) in the laboratory. Feed was allowed three times daily at a rate of 5% of their biomass. The bottom water was changed (one third of the water) daily to remove excretory wastes.

Table 1
Composition and proximate analysis of the basal diet.

Ingredients	Percentage of diet (g/kg)
Ground Yellow corn	26.50
Soybean meal	22.00
Fish oil	5.00
Meat meal	20.00
Fish meal	25.50
Vitamin and mineral mixture[a]	1.50
Calculated chemical analysis	
Crude protein	39.9%
Crude lipid	10.89%
Crude fiber	3.68%

[a] Vitamin and Mineral mixture (free from Zinc): Each 1 kg contains: Vit. A 580000 I.U, Vit. D3 8600 I.U, Vit. E. 720 mg, Vit. K_3 142 mg, Vit C 0.1 mg, Vit B_1 58 mg, Vit B_2 34 mg, Vit. B_6 34 mg, Vit.B_{12} 58 mg, Folic acid 86 mg, Pantothenic acid 8 mg, Manganese sulfate 65 mg, Iron sulfate 2000 mg, Copper sulfate 3400 mg, Cobalt sulfate 572 mg.

2.2. Zinc oxide nanoparticles (nZnO)

The tested Zinc oxide nano particles (nZnO), was a white powder obtained from Sigma-Aldrich Chemical Company., St. Louis, MO, USA. The nZnO average particle size was < 65 nm with particle size distribution of < 100 nm using dynamic light scattering technique, pH 7 ± 0.1, and density 1.7 ± 0.1 g/ml at 25 °C.

2.3. Experimental design

The proximate analysis of used basal diet showed a 39.9% crude protein, 10.89% crude lipid, and 3.68% fiber, based on the Association of Analytical Communities, (AOAC) [35]. Zn treated diets were prepared by mixing Zn separately at level of 30 mg and 60 mg ZnO/kg diet for ZnO_{30} and ZnO_{60} groups, respectively and at the same levels of nZnO for $nZnO_{30}$ and $nZnO_{60}$ groups. The ingredients were blended followed by formation of pellets by using 1.5 mm diameter pellet machine. The pellets were air-dried at room temperature at 27 °C for 24 h and kept in a refrigerator at 4 °C until used (Table 1).

The experiment lasted for 60 days where *O, niloticus* were equally divided into five groups, each group containing three replicates (10 fish/replicate). The first group served as the control group (CT) fed on a Zn free basal diet. The second and third groups (ZnO_{30} and ZnO_{60}) were fed on a basal diet supplemented with conventional ZnO (30 mg/kg and 60 mg/kg respectively). The fourth and fifth groups ($nZnO_{30}$ and $nZnO_{60}$) were fed on a basal diet supplemented with nZnO (30 mg/kg and 60 mg/kg respectively). Throughout the exposure period, the daily mortality was recorded to evaluate the survivability percentage at the end of experiment. The Ethics of Animal Use in Research Committee (EAURC) of Zagazig University has approved the experimental procedures which were followed the NIH general guidelines for the Care and Use of Laboratory Animals in scientific investigations.

2.4. Blood sampling

At the end of the experimental period, blood samples were obtained by puncturing the caudal vein without using anticoagulant to collect the serum by centrifugation at 3000 rpm/15 min for the estimation of biochemical biomarkers.

2.5. Serum biochemical parameters

2.5.1. Physiological biomarkers and protein profile

Serum total protein and creatinine were estimated using a commercial diagnostic kits supplied by Spinreact Co., Spain. The levels of serum alanine transaminase (ALT) and aspartate transaminase (AST)

were assessed using kits supplied by Diamond Diagnostics, Egypt.

2.5.2. Immunological response biomarkers

The serum bactericidal activity against *A. sobria* suspension was determined as mentioned by Kajita et al. [36] and the results were expressed as the percentage of serum killing. The serum lysozyme activity was measured by turbidimetric assay [37]. Commercial fish ELISA kits for C- Reactive Protein (CRP) and Immunoglobulin M (IgM) were provided by *MyBioSource,* San Diego, CA, USA (CAT. No. MBS072783 and MBS042385, respectively).

2.5.3. Evaluation of oxidative stress and antioxidant indices

The superoxide dismutase (SOD (and catalase (CAT) activity were stated following the methods described previously by Misra and Fridovich [38] and Sinha [39]. Total antioxidant capacity (TAC) was estimated using kit from Biodiagnostic Co., Egypt (CAT. No. TA 25 13). The level of lipid peroxides (MDA) was determined by colorimetric assay as described previously by Ohkawa et al., [40].

2.6. Transcriptional analysis of inflammatory cytokines and immune-related genes in the spleen tissue

The spleen samples were collected from six fish per group and quickly-frozen in liquid nitrogen and stored at −80 °C till isolation of total RNA. The total RNA was extracted by TRIzol reagent (easy-REDTM, iNtRON Biotechnology, Korea), as per the manufacturer's protocol. The quantity and quality of RNA of samples were measured at 260/280 nm using GeneQuant spectrophotometer (Pharmacia Biotech, Freiberg, Germany). The RNA was loaded on 1.5% Agarose gel to verify the integrity of 18S and 28S rRNA bands. Then, the first-strand cDNA synthesis was performed by a Quantitect® Reverse Transcription kit (Qiagen, Germany) based on the manufacturer's directions.

All the sequences of gene specific primers including interleukin1 beta (*IL-1β*), interleukin 8 (*IL-8*), immunoglobulin M heavy chain (*IgM* heavy chain), CXC-chemokine, interferon (*IFN*-γ) and myxovirus resistance (*Mx*), Toll-like receptors (*TLR-7*) and β-actin (as a house keeping gene) are described in Table (2). The qPCR performed in a Rotor-Gene Q instrument (Qiagen, Germany) with a QuantiTect® SYBR® Green PCR kit (Qiagen, Germany) under the following condition: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15s and 60 °C for 15s and 72 °C for 15s. Melt-curve analysis performed to verify the specificity of PCR. The relative mRNA expression calculated using the comparative $2^{-\Delta\Delta Ct}$ method [46].

2.7. Challenge test

After 60 days, 14 fish from each group were injected intraperitoneally with 0.2 ml of 24 h old culture (9×10^8 CFU) of pathogenic *Aeromonas sobria* previously isolated from moribund fish and identified by the VITEK® C15 automated system for bacterial identification (BioMerieux Inc., France) following the manufacturer's directions. After inoculation, fish were kept under observation for 14 days to record clinical signs of infection and mortality rates. The average mortality among all replicates in the groups was determined for calculating relative percent survival (RPS) [47].

2.8. Statistical analysis

Statistical analysis was done by one-way analysis of variance (one-way ANOVA) by using the SPSS 16.0 computer program. Tukey's multiple comparisons *post hoc* test was conducted to compare means among the experimental groups where the value of $p < 0.05$ was considered as statistically significant. Data were presented as a mean ± SE.

3. Results

3.1. Survival percentage in response to ZnO and nZnO supplementation in O. niloticus

After 60 days of Zn supplementation, the highest survival rate was recorded in the groups that supplemented with 30 mg/kg diet nZnO ($nZnO_{30}$) (90%) and in that supplemented with 60 mg/kg diet conventional ZnO (ZnO_{60}) (86.6%). Feeding *O. niloticus* fish in the conventional ZnO supplemented diet at level of 30 mg/kg diet, revealed 83.33% survivability. On the other hand, the lowest survival % was noticed in the control group fed on Zn free-basal diet (73.33%) and $nZnO_{60}$ (53.33%) (Table 3).

3.2. Physiological indices in response to ZnO and nZnO supplementation in O. niloticus

Total protein level was increased in response to ZnO supplementation; such increase was significantly recorded in the $nZnO_{30}$ and ZnO_{60} supplemented groups. No significant difference in the creatinine level was observed in the groups supplemented with different Zn dietary levels except in the $nZnO_{60}$ group showed a non-significant increase, compared with the control values. The levels of liver function indices, ALT showed a non-significant decrease while AST level showed a significant decrease in response to supplementation with Zn relative to control fish. Meanwhile, fish supplemented with $nZnO_{60}$ showed a significant elevation in their levels compared with control value (Table 3).

3.3. Immunological indices in response to ZnO and nZnO supplementation in O. niloticus

The serum-bactericidal activity of *O. niloticus* exhibited a significant higher activity in ZnO_{60}, and $nZnO_{30}$ supplemented groups as compared to the control group. While, there was no significant difference recorded in ZnO_{30} and $nZnO_{60}$-supplemented groups compared with the control one. The highest lysozyme activity was observed in $nZnO_{30}$-supplemented groups compared with control value, followed by the both groups that supplemented with conventional Zn (ZnO_{30} and ZnO_{60}) where there was no significant difference between their activities. In contrast, $nZnO_{60}$ -supplemented group showed a lower activity

Table 2
Primer sequences used for real-time qPCR analysis.

Genes	Forward (5′-3′)	Reverse (5′-3′)	Accession no. / References
IL1β	TGC ACT GTC ACT GAC AGC CAA	ATG TTC AGG TGC ACT ATG CGG	DQ061114.1 [41]
IL-8	GCA CTG CCG CTG CAT TAA G	GCA GTG GGA GTT GGG AAG AA	NM_001279704.1 [42]
CXC-Chemokine	CTA TCC ATG GAG CCT CAG GT	CTT CTT GAG CGT GGC AAT AA	XM_003452201 [43]
IFN-γ	AGC ACA ACG TAG CTT TCC CT	TAA ACA GGG CAA ACA GGT CA	XM_003460533.2 [43]
MX	GGA TCC TGA TGG AGA GAG GA	GCA TTT GAC CAC CAT GTA GC	XM_003460517.2 [43]
TLR-7	TCA GCA GGG TGA GAG CAT AC	ACA TAT CCC AGC CGT AGA GG	XM_005477981.1 [43]
IgM	AGG AGA CAG GAC TGG AAT GCA CAA	GGA GGC AGT ATA GGT ATC ATC CTC	KJ676389.1 [44]
β-actin	CAG CAA GCA GGA GTA CGA TGA G	TGT GTG GTG TGT GGT TGT TTT G	XM_003455949.2 [45]

Table 3
Effect of dietary ZnO and nZnO supplementation for 60 days on survivability and physiological variables in *O. niloticus*.

Parameters	Zn, g/kg of diet					*p*-value
	Control	ZnO_{30}	ZnO_{60}	$nZnO_{30}$	$nZnO_{60}$	
Survivability (%)	73.33	83.33	86.66	90	53.33	-
Total Protein (gm/l)	$2.25^{d} \pm 0.14$	$2.88^{c} \pm 0.07$	$3.61^{ab} \pm 0.22$	$4.18^{a} \pm 0.006$	$3.37^{bc} \pm 0.23$	< 0.001
ALT(U/l)	$18.80^{b} \pm 0.94$	$16.89^{b} \pm 0.58$	$16.73^{b} \pm 0.63$	$18.38^{b} \pm 0.27$	$22.54^{a} \pm 0.28$	< 0.001
AST(U/l)	16.20b ± 0.11	14.03c ± 0.30	14.68c ± 0.40	14.45c ± 0.14	20.54a ± 0.12	< 0.001
Creatinine (mg/dl)	$0.43^{ab} \pm 0.016$	$0.41^{b} \pm 0.003$	$0.40^{b} \pm 0.005$	$0.42^{b} \pm 0.013$	$0.47^{a} \pm 0.013$	0.007

Means bearing different superscripts (a,b,c and d) within the same row are significantly different ($P < 0.05$). Values are mean ± SEM.

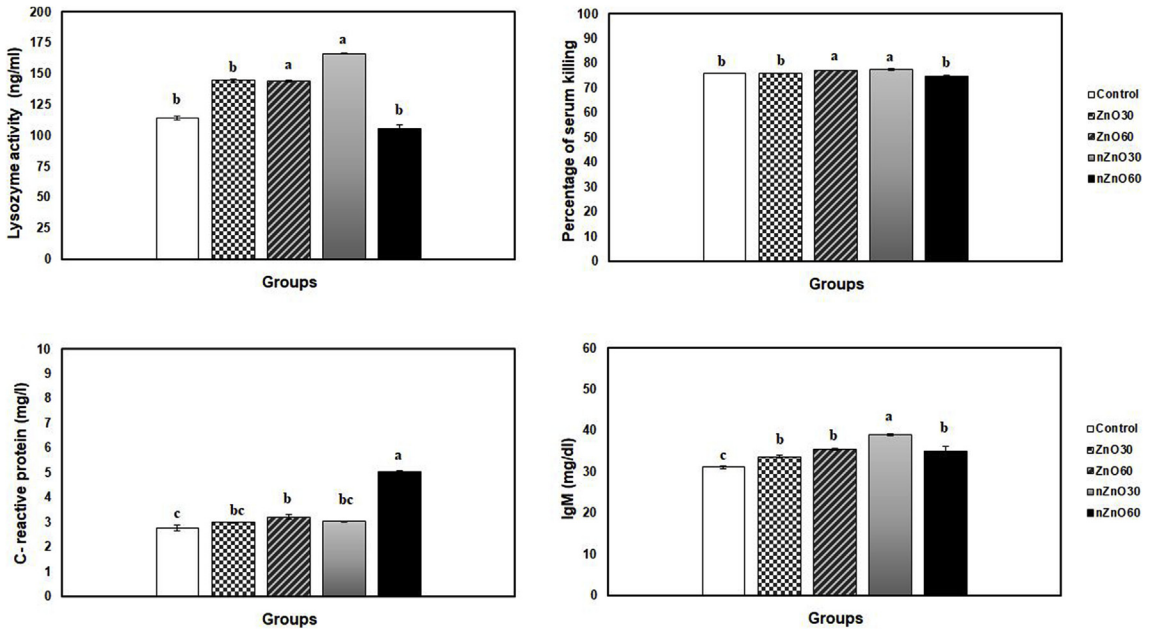

Fig. 1. Effect of dietary ZnO and nZnO supplementation for 60 days on lysozyme activity, serum killing percentage, C-reactive protein and IgM level in *O. niloticus*. Values are mean ± SE. Bars with different letters are significantly different (at $P < 0.05$).

than control fish. IgM was significantly high in the $nZnO_{30}$ as compared to the control group. Besides, there was no significant difference between ZnO_{30}, ZnO_{60} and $nZnO_{60}$- supplemented groups but the level was higher than that recorded in control group. The groups those supplemented with Zn at level of 30 mg/kg diet either in nano or conventional forms showed no significant difference in CRP level compared with control group. Meanwhile, the level showed marked elevation in $nZnO_{60}$- supplemented group, followed by ZnO_{60}- supplemented group (Fig. 1).

3.4. Antioxidant and oxidative stress indices in response to ZnO and nZnO supplementation in O. niloticus

The TAC level of *O. niloticus* that were fed on different dietary Zn supplementation was improved, such improvement was significant in the $nZnO_{30}$ –supplemented group, compared with the control level. On the other hand, TAC level displayed non-significant improvement in the other Zn- supplemented groups. The highest activity of antioxidant enzymes (SOD and CAT) was demonstrated in $nZnO_{30}$-supplemented group, followed by groups that supplemented with conventional Zn. The lowest antioxidant enzymes activity was noticed by supplementation with nZnO at the level of 60 mg/kg diet (Fig. 2). On contrary, The MDA level was significantly high in the $nZnO_{60}$-supplemented group, but modulated and diminished in the other ZnO supplemented groups, particularly in fish of $nZnO_{30}$-supplemented group (Table 4).

3.5. Immune encoding gene expression pattern in response to ZnO and nZnO supplementation in O. niloticus

The mRNA expression profile of both *IL-8* and *IL-1β* -encoding genes showed an up-regulation that was found in all ZnO- supplemented groups, compared to the control fish, significantly in ZnO_{60} and $nZnO_{30}$-supplemented group and non-significantly in ZnO_{30} group. This up regulation was more pronounced and markedly in $nZnO_{60}$-supplemented group. On the other hand, the expression pattern of *Mx*-encoding gene showed no remarkable difference between the Zn- supplemented and control fish. A significant upregulation of the expression levels of CXC-chemokine and *IFN*-γ gene was found in all Zn-supplemented group except ZnO_{30} group that was non-significantly upregulated, compared with control group. The more pronounced up regulation of *TLR-7* and *IgM* -encoding genes was significantly recorded in the $nZnO_{30}$- supplemented group when compared to the control group. Besides, the lowest expression was found in $nZnO_{60}$- and ZnO_{30}-supplemented groups. In $nZnO_{60}$- supplemented group, the *TLR-7* expression pattern showed a non-significant difference compared with the control group (Fig. 2).

3.6. Challenge test with Aeromonas sobria

Clinically infected control and ZnO_{30}- administered fish displayed severe erythema of skin, ascites and loss of scales (Fig. 3A and B, respectively). Severe hemorrhage at caudal peduncle region with erythematous skin and fin rot in $nZnO_{60}$-supplemented fish (Fig. 3E). Fish

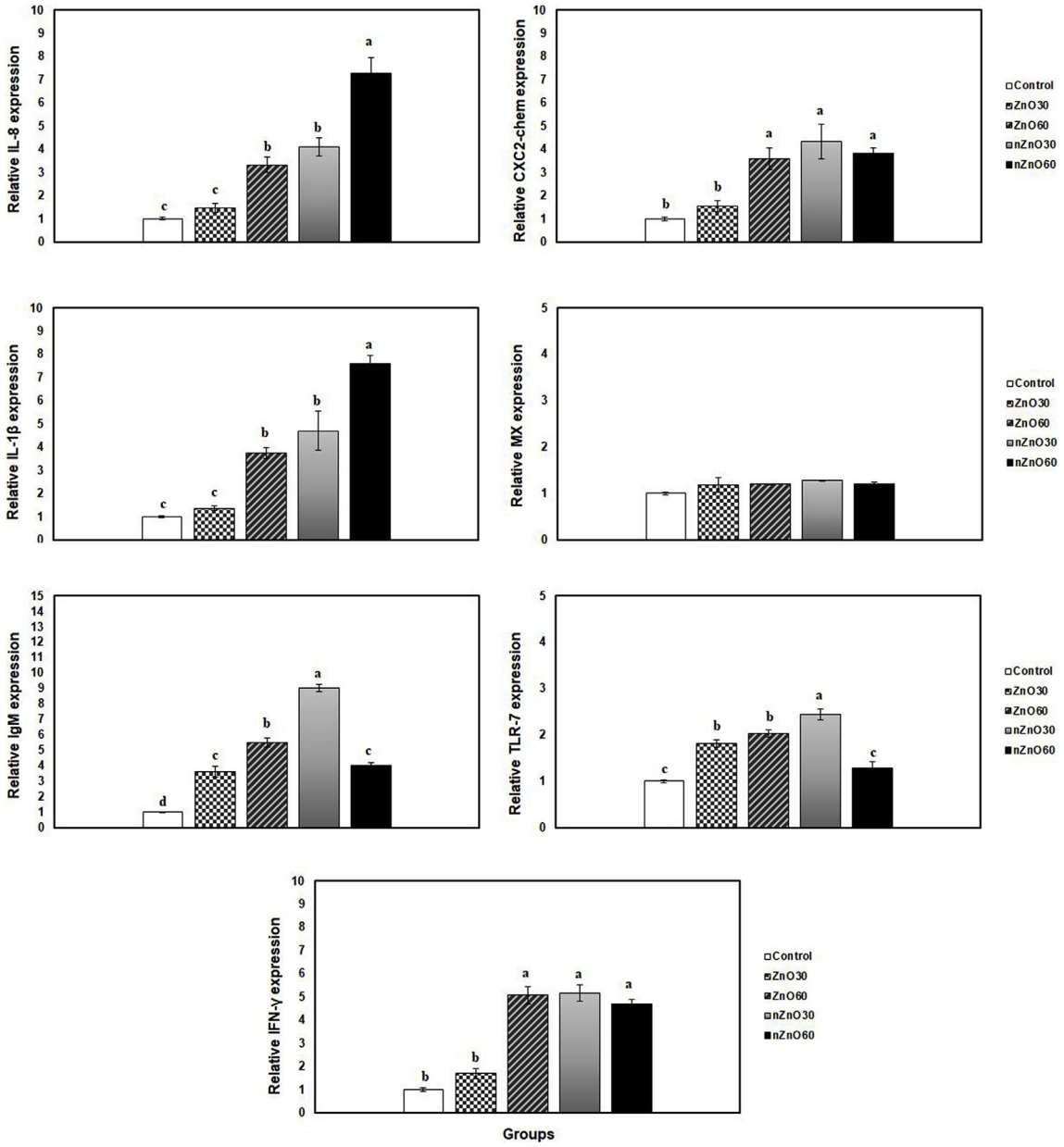

Fig. 2. Effect of dietary ZnO and nZnO supplementation for 60 days on the relative expression of interleukin1 beta (*IL-1β*), interleukin 8 (*IL-8*), immunoglobulin M heavy chain (*IgM* heavy chain), CXC-chemokine, interferon (*IFN-γ*) and myxovirus resistance (*Mx*), and Toll-like receptors (*TLR-7*) in spleen of *O. niloticus*. The expression abundance of genes mRNA was normalized against the internal control gene β-actin using quantitative real-time PCR technique. Values are mean ± SE. Bars with different letters are significantly different (at $P < 0.05$).

of ZnO_{60}-administered group showed small, rounded dark red ulcer on skin (Fig. 3C). While those of $nZnO_{30}$- supplemented group appeared apparently healthy except of mild fin rot (Fig. 3D). The highest cumulative mortality rate among *O. niloticus* challenged fish was observed in both control and $nZnO_{60}$-supplemented group, while decreased in other Zn-supplemented groups. The relative survival percentage (RSP) of *O. niloticus* in Zn-supplemented groups were high after bacterial challenge particularly in $nZnO_{30}$- and ZnO_{60} supplemented groups. On

Table 4
Effect of dietary ZnO and nZnO supplementation for 60 days on TAC, antioxidant enzymes (SOD and CAT) activity and lipid peroxide (MDA) level in *O. niloticus*

Parameters	Zn, g/kg of diet					*p*-value
	Control	ZnO_{30}	ZnO_{60}	$nZnO_{30}$	$nZnO_{60}$	
TAC ((nmol of trolox equivalent/l)	0.171^{b} ± 0.01	0.179^{b} ± 0.010	0.192^{b} ± 0.003	0.265^{a} ± 0.017	0.186^{b} ± 0.014	0.002
SOD activity (U/l)	0.056^{d} ± 0.003	0.102^{c} ± 0.003	0.198^{b} ± 0.006	0.278^{a} ± 0.01	0.120^{c} ± 0.011	< 0.001
CAT activity(U/l)	16.52^{c} ± 0.294	33.31^{b} ± 0.915	35.38^{b} ± 1.79	40.21^{a} ± 1.35	19.79^{c} ± 1.20	< 0.001
MDA (nmol/ml)	193.73^{b} ± 1.90	108.52^{cd} ± 1.63	115.66^{c} ± 1.98	104.32^{d} ± 1.70	214.20^{a} ± 1.98	< 0.001

Means bearing different superscripts (a,b,c and d) within the same row are significantly different ($P < 0.05$).

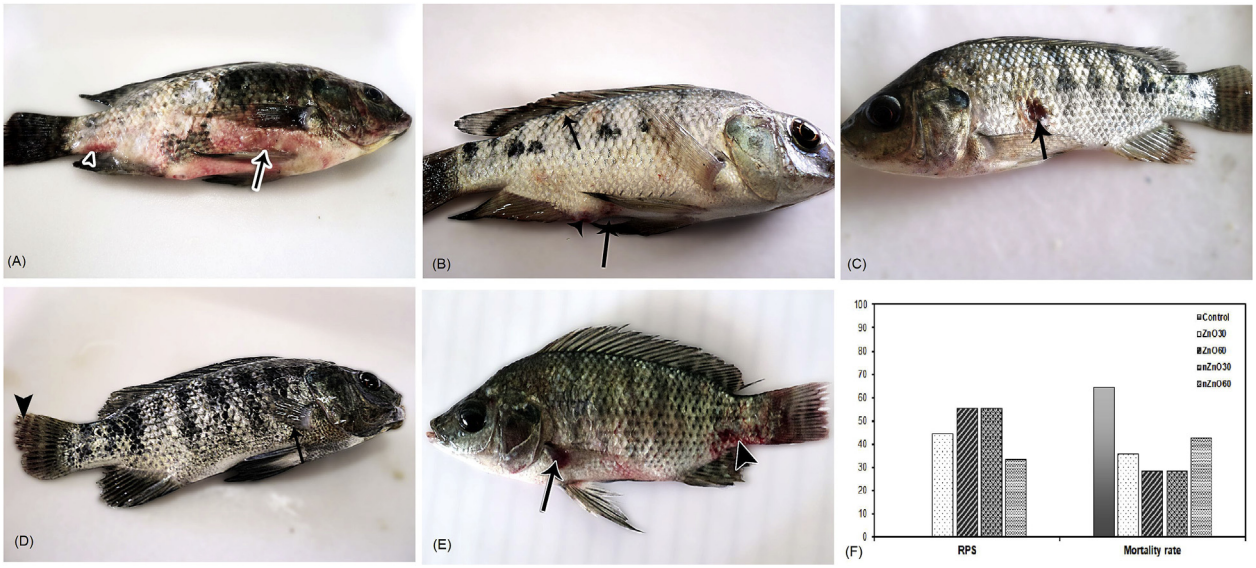

Fig. 3. Fish of different experimental groups challenged with *Aeromonas sobria* showing typical signs of infection. Signs included, A. Ascites and loss of scales with severe erythema at breast region (arrow) and caudal peduncle (arrowhead) (Control group). B. Hemorrhages on skin (arrow) with severe hyperemia at both anal opening (arrowhead) and the site of injection (arrow) (ZnO_{30}- supplemented group). C. Small, rounded dark red ulcer on skin (arrow) (ZnO_{60}-administered group). D. Mild fin rot at pectoral fin (arrow) and caudal fin (head arrow) ($nZnO_{30}$- supplemented group). E. Severe hemorrhage at the base of pectoral fin (arrow) and caudal peduncle region (arrow) ($nZnO_{60}$-supplemented group. F. Mortality rate and RPS after fish were challenged with *Aeromonas sobria*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the other hand, the lowest RSP was recorded in $nZnO_{60}$-supplemented group (Fig. 3F).

4. Discussion

Several cellular and humoral components are participated in the innate immune response system. At the cellular level, leukocytes including monocytes, macrophages, neutrophils, and T and B lymphocytes are the major cell types. Besides, lysozyme, complement, hemolysin, transferring and C-reactive protein as humoral elements [48]. Some researcher established that Zn is vital for the growth, immunity and development of animals in certain amount [49]. ZnO nanoparticle has been found to modulate growth performance, enhance feed utility and give economic benefits in piglets and poultry [21,23]. Faiz et al. [26] demonstrated that nZnO improving the growth rate and immune status in grass carp (*Ctenopharyngodon idella*). Also, Muralisankar et al. [27] stated a significant elevation in protein content, enhance antioxidant enzymes activity, and improved weight in freshwater prawn (*Macrobrachium rosenbergii*) upon feeding with diet supplemented with nZnO.

Our results revealed that *O. niloticus* immune status was improved and elevated survival rate against *Aeromonas sobria* upon supplementation of ZnO particularly in the conventional form particularly at level of 60 mg/kg diet. Similarly, low nano-ZnO supplementation enhanced the immune status in the level of 30 mg/kg diet but not in level of 60 mg/kg diet that exhibit the lowest immune responses and antioxidant status, besides mediating oxidative and inflammatory injuries.

It was observed that low concentrations of nZnO (30 mg/kg) to fish diet improved the survivability and immune status of fish more than the higher concentrations of conventional ZnO (60 mg/kg). This results may be attributed to reduction of macromolecule to nanoscale changed their properties and increased their application [13] whereas the small particles size of nZnO mediates the higher intestinal absorption, bioavailability and catalytic activities [50]. Luo et al. [51] reported that, nanoparticles could stimulate innate and adaptive immune response depending on their physicochemical properties. Moreover, it is well documented that Zn deficiency reduces immune responses and disease resistance in human and animals [52].

Serum total protein and IgM were improved by dietary supplementation of ZnO in its two forms, but it was clear that nZnO enhanced the total protein and IgM, resulting in improvement of the specific humoral adaptive immunity. It is possible that the observed increase in protein content was due to the increased protein synthesis in the liver; an important function of serum proteins is the maintenance of osmotic balance between blood and tissue spaces. The parallel elevation of serum protein and IgM with dietary ZnO indicate the important role of the protein during Zn transportation [53]. Similarly, Tawfik et al. [3] reported an increment of total protein and IgM by increasing the level of ZnO besides the nZnO supplementation showed better results than conventional ZnO.

A significant modulation of the liver function particularly serum AST concentrations was recorded as a result of the supplementation of both levels of conventional Zn and at level of 30 mg/kg diet of nano Zn particles, suggesting hepatoprotective potency of ZnO in fish liver cells. This may be attributed to cytokine production, which provides protection to the liver in various liver-injury models. In a previous study, ZnO has been shown to have a hepatoprotective activity, which possibly protects the liver of from chemical-induced injury [54]. Ahmadi et al. [55] and Liu et al. [56] found that the supplementation of diet with nZnO had no significantly affects the ALT and AST activates. On the other hand, the increased liver enzymes level in nZn_{60} group, was in agreement with Chupani et el [57]. who reported that exposure to Zn nano particles at levels of 50 and 500 mg/kg had effected liver and kidney function.

The serum bactericidal activity of fish is mediated by molecules capable of prohibiting bacterial growth [48]. Our findings stated that Zn supplementation augmented the non-specific innate immunological responses (bactericidal, lysozyme activities and CRP) in *O. niloticus* in either conventional (ZnO_{60}) or nano ($nZnO_{30}$), which strengthened the role of Zn in stimulating the non-specific immune response that attributable to the presence of immunostimulants. The efficient immune response elicited by low concentration of nano form is attributed to the small size of nanoparticles that permit the passage of them across the tissues and cell membrane more than the conventional form [58].

By ZnO supplementation, serum MDA level was decreased while the TAC and antioxidant enzyme level was improved in Zn-administered

groups, which indicated that the addition of conventional Zn and/or nano Zn enhanced the antioxidant activity of tilapia fish. These changes were significantly recorded with the dietary nano ZnO at a low dose level 30 mg/kg diet. Zinc has an important role in scavenging of free radicals and ROS and considered as an essential element in the formation and antioxidant function of Cu–Zn SOD [59]. In the same line, Fathi et al. [60] and Ibrahim et al. [61], revealed that serum concentrations of MDA in broiler chickens was significantly decreased by nano-ZnO supplementation. The obtained data are in harmony with those reported by Ahmadi et al. [55] who resulted that nZn supplementation decreased MDA level. Also, Liu et al. [56] stated that supplemental zinc decreased MDA level in the liver.

Generally, the group that was supplemented with high level of nZnO (60 mg/kg diet) not showed an enhancement in most examined immunological indices. It may be linked to bioaccumulation of Zn nanoparticles in aquatic organisms as a result of increasing the level of nano zinc form supplementation [58], leading to an adverse effect on liver function, antioxidant status and induce the oxidative stress marker. This in accordance with the higher toxic effect of nano Zn particles over the bulk Zn and ionic Zn stated in fish cell lines [62] and zebrafish embryo [63] and larva [64].

Such deleterious effects of ZnO nanoparticles were attributed to the high production of reactive oxygen species (ROS) that exhausted the intracellular antioxidant elements, mediating an initiation of a cascade of chain reactions leading to oxidative injury. The oxidative damage was indicated by overproduction of lipid peroxide (MDA) as an end-product of lipid peroxidation and decreased TAC, as well as reduction of scavenging activities of SOD and CAT [65] as recorded in our findings. The generated ROS is further react directly with the lipid bilayer of the cell membrane increasing membrane fluidity and permeability, which leads to leakage of liver enzymes into the blood circulation, and increasing their serum levels. Moreover, Ma et al. [66] have reported that the increase in cell membrane fluidity and permeability that induced by high level of ZnO nanoparticles encouraging more accumulation and disguise of ZnO nanoparticle into the cell potentiating its toxic effect. Therefore, we supposed that the supplementation with nZnO at the level of 60 mg/kg diet may mediated the incidence of oxidative damage.

Some studies detailed that, when nano-particles enter the body, they can interact with immune cells and trigger inflammatory response, which is accompanied by the discharge of signaling molecules (cytokines and chemokines) that give communication between immune cells and facilitate molecular events. Interleukin 1-beta (IL1-β) is a member of the interleukin 1 group of cytokines, it is a significant mediator of the inflammatory response, and is involved in an assortment of cellular activities, including cell proliferation, differentiation, and apoptosis.

TNF-alpha, is one of the pro-inflammatory cytokine related with early immune expressed genes and has a key role in managing inflammation [67,68]. TLRs being pattern recognition molecules are a powerful first line of defense. IgM is the principle immunoglobulin in teleost that can evoke an effective specific humoral responses against different antigens [69].

In the present study, ZnO supplementation was reported to up-regulate the IL-1β and IL-8 expression profile significantly in ZnO_{60} and $nZnO_{30}$-supplemented group, Tawfik et al. [3] reported an up-regulation the IL1-β gene expression in *O. niloticus* following nZnO and Zno as a feed additive. Abo-Al-Ela et al. [43] found that 17 alpha-methyltestosterone administration significantly increased the expression of *IFNγ, Mx, TLR-7, IL-1β, IL-8* and *IgM* -encoding genes in Nile tilapia fry. Also, Yilmaz [70] increased levels of immune expression related genes in the liver of fish fed with 5 g/kg caffeic acid, indicating a modulation of immune response. Hence, it could be concluded that the conventional ZnO at high concentration of 60 mg/kg diet and nano form in the low concentration could be considered as the best for inducing the immune function, but results also indicated that nZnO in high concentration (60 mg/kg) was markedly up-regulated indicating a high inflammatory response.

Several studies revealed a positive correlation between the antioxidant enzyme activity and innate immune response in both finfish and shellfish supplemented by various feed additives [71,72]. As reported in the present study, upon inclusion of low level of nZnO particles in the diet, fish could create antioxidant defense, as well as generate more innate components as a consequence. Subsequently, the better anti-oxidative status following supplementation with ZnO recorded in the present investigation may demonstrate higher capacity of disease prevention in fish and vice versa with the high concentration of nZnO particles.

5. Conclusion

From this study, it could be concluded that, supplementation of nZnO to *O. niloticus* fish feeds in low concentration (30 mg/kg diet) or conventional ZnO in the concentration of 60 mg/kg can possibly improve the immune and antioxidant status of fish. On the other hand, the Zn nanoform in level of 60 mg/kg diet disrupts immune responses and antioxidant status, besides mediating oxidative and inflammatory injuries. Therefore, it is better to use Zn nano-form in fish farms and aquaculture at low concentrations and this could improve the economics of farming and enhance fish production.

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