Evaluation of Nano Zinc Oxide feed additive on tilapia Growth and Immunity

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Abstract: Aquaculture is the last frontier to solve the problem of the global deficiency of white protein. Some studies reported that, nano-particles have enormous potential in controlling the pathogens, improve the immune and growth functions in aquaculture. The present investigation was carried out to evaluate nano-ZnO (nZnO) in comparison to its conventional form as a fish feed additive in growth promoting and immunostimulation of Nile tilapia (Oreochromis niloticus). Zinc oxide nanoparticles were prepared using the chemical method and mixed with the fish feed. 405 Oreochromis niloticus were fed for 120 days on Zinc oxide conventional bulk scale (ZnO) and nanoscale (nZnO) supplemented feed in different concentrations (15, 30, 45 and 60 mg/kg of the feed) in addition to the control which was fed on ZnO free feed. nZnO (15mg/kg) achieved specific growth rates like the higher concentrations of bulk ZnO (60mg/kg). The 60mg/kg nZnO gave the highest rates of Specific growth rates(4 folds than control). growth hormone was higher in serum of fish fed on nZnO supplemented feed than the bulk form. Immunity was assessed through the measurement of total protein and IgM titer and IL1-beta (IL-1β) gene expression. Total protein and IgM titers revealed high values increasing with the increase of concentration of ZnO feed additive in its two forms compared to the control, but nZnO showed better results than conventional ZnO. Analysis of IL1-beta gene expression revealed that 60mg/kg conventional ZnO was the best concentration for up-regulating the IL1-beta followed by the concentration 30mg/kg in the two forms; the nano and the conventional form. The inorganic conventional ZnO up-regulated the IL-1β better than the nZnO form. Zinc was concentrated higher in muscles of fish fed on nZnO supplemented feed more than conventional ZnO fed fish but still within the permissible limits. One way ANOVA statistical analysis was used in all analyses with $p<0.05$.

Keywords: Zinc Oxide Nanoparticles, Interleukin 1-beta Expression, Growth, *Oreochromis niloticus*.

Introduction

Fish feed is essential component of the inputs in any fish farm. The feed ingredients 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.

Feed additives in nano forms have been reviewed to have different effects from enhancing growth and immunity through antioxidant effect to their use in less amount than its bulk counterparts which enhances ration criteria (Rather *et al.*, 2011; Rajendran, 2013). Nanoparticles have enormous potential in controlling the pathogens in aquaculture. Different metal and metal oxide nanoparticles were screened for their antimicrobial activities against a wide range of bacterial and fungal agents including certain freshwater cyanobacteria (Swain *et al*., 2014). Among different nanoparticles, synthesized copper oxide (CuO), zinc oxide (ZnO), silver (Ag) and silver doped titanium dioxide (Ag-TiO2) showed broad spectrum antimicrobial activity (Swain *et al*., 2014). Since CuO, ZnO and Ag nanoparticles showed higher antimicrobial activity, they may be explored for aquaculture use. Zinc oxide nano-particles as one of metal oxides are versatile because they enter in a wide variety of applications ranging from sensing, catalysis, energy storage, electronic devices and biomedical applications. Chemically, zink oxide (ZnO) and nano-zink oxide (nZnO) have the same chemical formula which suggests similar Zinc to oxygen ratio, but at the nano scale atoms are arranged with a wider energy level confinement and smaller size Zn, that could lead to more reactive atoms as the surface is increased (Zhong, 2004).
Interleukin-1 (IL-1) is apleotropic paracrine endocrine signaling molecule and is produced by a variety of cell types. Phagocytes are important sources for the synthesis and release of IL- 1 for stimulation of T cell activation showing that within fish; these molecules can be quite divergent. IL1-β is the most important one in IL-1 group. The major functions of IL1-β are activation of the proliferation of such lymphocytes as T cells and B cells, activation of cytotoxic activity in macrophage and natural killer (NK) cells, and induction of immunoglobulin (Ig)

secretion. Thus IL1-β is an important member of the immune system (Sebastián *et al*., 2012).

This research work studied the evaluation of Zinc oxide (ZnO) as a feed additive in two forms; the conventional and the nano forms in enhancing the processes of growth and immunity in tilapia fish (*Oreochromis niloticus*). Growth was evaluated through some growth parameters and immunity was assessed after *Pseudomonas fluorescence* bacterial infection (challenge test) through measurement of total protein, immunoglobulin M and gene expression of one of the most important pro-inflammatory cytokine; Interleukin-1-beta (IL1-β).

Materials and Methods

Fish: 405 mixed sex, 35-45 gm, apparently healthy tilapia (*Oreochromis niloticus*) were brought from Rahil Aquaculture Farm, Fayoum, Egypt, June 2014. Fish was weighed after arrival and treated with potassium permanganate 4 mg/L for 10 minutes before distribution in eighteen plastic tank supplied with Dechlorinated water and automatic aerator. Fish was reared for 120 days and samples were collected in November 2014.

Nanoscale Zinc Oxide (nZnO)

nZnO was prepared using chemical co-precipitation method according to Li *et al*. (2006). XRD and TEM were used for characterization of nZnO.

Experimental design

Four concentrations (15,30,45 and 60mg/kg) nZnO and conventional ZnO were added to the feed in two different treatments in triplicates in addition to the control (non treated). All groups were duplicated. Fish was fed two times daily 3% body weight for 120 days.

Estimation of Growth performance

Growth performance was calculated through two assays; a- Weight gain (**WG %**) , b- Specific Growth Rate (**SGR**)

Estimation of Growth Hormone in Blood

Growth Hormone (GH) was measured using ELISA kit Catalog No: MBS701414_48T (MyBiosource, Vancouver, British Columbia)

Determination of Zn concentrations in muscles

Zinc concentration in muscle samples was analyzed according to FAO Technical Paper No. 212 (FAO, 1983).

Evaluation of Nonspecific immunity function

A challenge test was carried out. *Pseudomonas fluorescence* bacteria was used to immunize *Oreochromis niloticus* fish in a sub lethal 0.2 ml dose (1×10^6 CFU/ml) to examine tilapia nonspecific immune response through quantization of total protein, immunoglobulin M and interleukin 1 beta gene expression using real-time PCR assay. Samples were collected 3 and 5 days after intra-peritoneal injection of the bacteria.

Total protein

Serum of the studied fish were subjected for calorimetric analysis of total proteins content using total proteins quantification kit (Spectrum, Egypt), a colorimetric method (Biuret reagent).

Serum immunoglobulin M (IgM):

IgM value was determined by IgM ELISA kit (Catalog No: MBS700823, MyBiosource, Vancouver, British Columbia).

IL1-β Gene Expression and immune response

Fish livers were isolated and flash-frozen immediately in liquid nitrogen and stored at -70^{o} C for further extraction of RNA, RNA was extracted using Gene Jet RNA Purification Kit, Two µg RNA were reverse transcribed with Revert Aid First Strand cDNA Maxime RT PreMix Kit using hexanucleotides and used as templates for Real-time PCR. Reaction was performed using Bio-Rad thermo-cycler machine; at 45 ^{o}C for 60 minutes then the reaction was stopped by rising the temperature to 70^{o}C. Primers and probes were designed using NCBI Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and GenScript (http://www.genscript.com/). Real-time PCR (Rotor-Gene Q - QIAGEN) was performed by methods described previously by Zhang *et al.* (2008) using TaqMan universal master mix II, with UNG for quantification of gene expression in both target and housekeeping genes. Serial dilution for primer, probe and cDNA samples were made. Concentrations of 50 picomol primer, 5 picomol prope, 1/100 for HK gene and 1/10 for IL1b gene gave the best results (standard concentrations).

Table (1): The sequences of IL1- β and 18s rRNA genes

Gene	Sequence	Strand*	Label**
IL1-β	TCTTCTACAAACGCGACACC	F	L1
	TCTGGAGCTGGATGTTGAAG	R	R1
	CATCAGCACAGCGCAGGACG	P	P1
18s rRNA	TTAGTTGGTGGAGCGATTTG	F	L2
	GGACATCTAAGGGCATCACA	R	R2
	TGGCGTTCAGCCACACGAGA	F	P2

*F denotes to a forward, R denotes to reverse. ** L denotes to forward primer, R denotes to reverse primer, P denotes to Probe. Probes are modified with5'Fam - 3'Tamra as (reporter- quencher).

Results

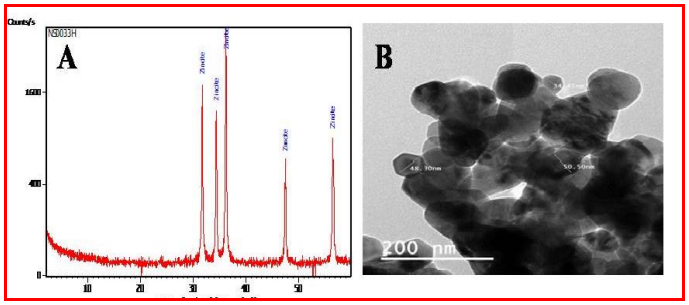

Figure (1): Characterization of ZnO nanoparticles; A:XRD and B; TEM

Characterization of Zinc Oxide Nanoparticles

The calculated crystallite size of nZnO ranged from 32-57 nm in diameter as revealed from The XRD image and 34.4-50.5 nm using TEM analyzer. Fig. 1(A&B).

Growth performance

Weight gain and specific growth rate (SGR)

The weight gain of tilapia fish increased gradually and in a positive relationship with the increase in the concentration of ZnO, with observation that nZnO supplementation enhanced the growth rates more than the conventional ZnO, which were sometimes double the weight gain. The two treatments gave fold changes of the weight gain and enhanced the growth more than the control. In this study, ZnO (15 and 30mg/kg/kg) fed fish SGRs were 0.31 and 0.37% per day compared to the control (0.27% day) with a fold differences 1.15 and 1.37, respectively. The magnitude of difference increased to 2.37 and 2.4 with the concentrations 45 and 60 mg/kg ZnO, respectively. Concerning nZnO, the magnitude of difference between the tested and control fish from concentration 15mg/kg to 45 mg/kg was nearly steady and near the value achieved in ZnO 45 and 60mg/kg (2.5, 2.2 and 2.5), respectively. nZnO gave a greater fold difference from the control equals 4 folds (Table 2). **Growth hormone in serum**

Both ZnO and nZnO supplemented feed fish groups showed a higher growth hormone level in serum than the control. Among conventional ZnO group, as the concentration of ZnO treatment increased, the growth hormone level was increasing (0.16, 0.19, 0.25 and 0.27ng/ml, respectively) with the highest values of growth hormone obtained at 60mg/kg concentrations of ZnO added to the feed. Similarly, in nZnO groups there were a positive correlation between the growth hormone level in blood and treatment concentration (mg/kg), where the values for growth hormone level in blood for nZnO different treatments were (0.2, 0.24, 0.398 and 0.402 ng/ml, respectively) Fig. 2. Overall, the value of the growth hormone was noticed to be higher in fish serum in case of feeding on the nZnO compared to bulk ZnO within the same concentration.

Table (2): Weight gain (WG) and specific growth rate (SGR) in tilapia *Oreochromis niloticus* after four months feeding on feed supplemented with ZnO bulk and nano-particles.

Treatments	Weight gain (mean ± SD)	SGR% Mean±SD
Control	5.8433 ± 2.35	0.2763 ±0.136
ZnO 15mg/kg	7.4128 ± 5.37	0.3190 ± 067
ZnO 30mg/kg	9.1711 ± 1.53	0.3705 ± 0.085
ZnO 45mg/kg	18.0133 ± 4.4	0.6474 ± 0.068
ZnO 60mg/kg	17.3089 ± 1.68	0.6547 ± 0.079
nZnO 15mg/kg	19.1767 ±0.96	0.6902 ± 0.079
nZnO 30mg/kg	19.7993 ± 1.57	0.6061 ±0.105
nZnO 45mg/kg	21.1211 ± 3.48	0.6973 ± 0.160
nZnO 60mg/kg	41.0033 ± 0.57	1.1017 ± 0.147

WG and SGR among the tested and control groups are all significantly different (P≤0.001).

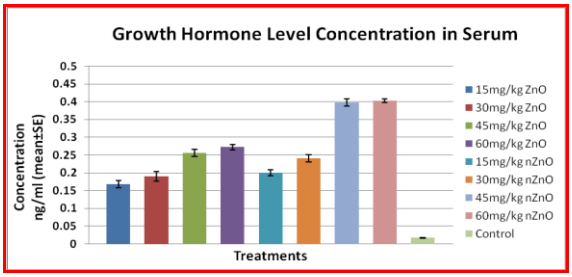

Figure (2): Growth Hormone level in blood of *O. niloticus* fed test diets for 4 months, ZnO= Zinc oxide and nZnO = Nano Zinc oxide

Table (3): Mean values of serum protein, immunoglobulin (IgM) of Nile tilapia fed diets containing ZnO and nZnO for 120 days.

Treatment	Total protein Mean (mg /dl)	Immunoglobulin M (IgM) Mean (µg/ml)
ZnO (15mg/kg)	48.76±0.032* (+0.059%)	20.47±0.003
ZnO (30mg/kg)	49.65±0.038* (+0.8%)	20.82±0.024
ZnO (45mg/kg)	51.11±0.13* (+0.11%)	21.96±0.053*
ZnO (60mg/kg)	51.85±0.034* (+0.128%)	20.99±0.172*
nZnO (15mg/kg)	55.72±0.054* (+0.21%)	22.43±0.022*
nZnO (30mg/kg)	55. 89±0.243* (+0.216%)	22.392±0.332*
nZnO (45mg/kg)	57.13±0.017* (+0.24%)	24.03±0.211*
nZnO (60mg/kg)	57.90±0.042* (+0.27%)	24.41±0.037*
Control (C)	45.95±0.163	20.03±0.023*

Data are represented as means +S.E, *significantly different from control (P<0.05). Data in parentheses are percent of change from control.

Zinc contents in muscles

In all treatments Zn concentration in muscle showed acontrol, but still within the range of permissible limit (40ppm) higher values compared to the set by the FAO (1983). Figure 3.

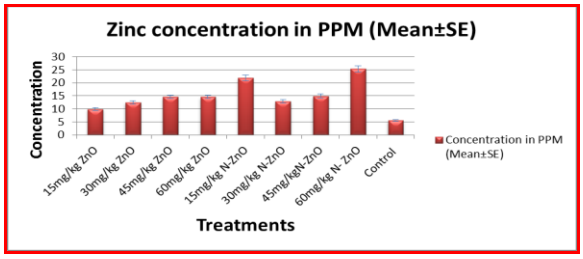

Figure (3): Zinc concentration in muscles tissue of *O. niloticus* fed for 4 months on conventional ZnO and nZnO supplemented feed in four concentrations.

Nonspecific immune responses

Serum total protein and total immunoglobulin

Serum total protein concentration of fish fed on the conventional ZnO supplemented diet was significantly lower than that of fish fed on the nZnO supplemented feed with observation that its values and Immunoglobulin M (IgM) values were in a positive direct relationship with the additive concentration (Table 3).

IL1-β gene expression

Immunization with sub lethal dose of Pseudomonas fluorescence bacteria showed increase in the IL1-β expression after three and five days of the experimental infection. Figure 4 (A-D) illustrates the levels of expression of IL1-β gene compared to 18sRNA internal control in all groups under investigation. Raw data of real time threshold cycle (Ct) showed an increase in the expression in the experimental than the control and the internal control 18sRNA.

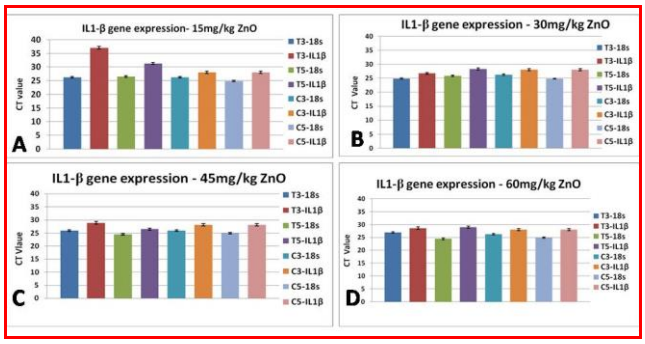

Figure (4): Expression of Interleukin 1 beta (IL1-β); experimental (T) , control (C) and house keeping genes (18sRNA), 3&5 dayes after bacterial challenge in 15, 30, 45 and 60 mg/kg (A-D) ZnO supplemented feed tilapia.

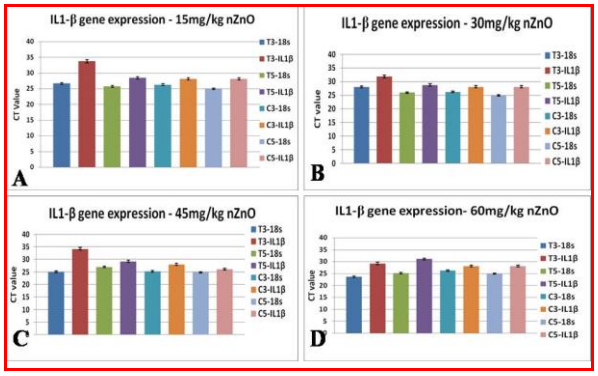

Figure (5): Expression of Interleukin 1 beta (IL1-β); experimental (T) , control (C) and house keeping genes (18sRNA), 3&5 days after bacterial challenge in 15, 30, 45 and 60 mg/kg (A-D) nZnO supplemented feed tilapia

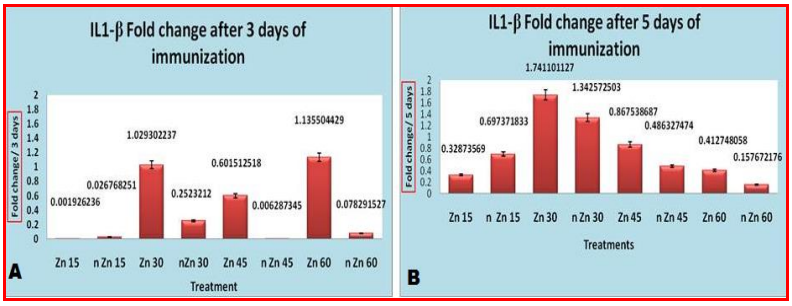

Figure (6): IL1-β gene expression fold change calculated as value of 2^-ΔΔCt in tilapia fish fed 4 months on conventional ZnO and nano-ZnO supplemented feed and challenged with bacterial infection (sampling after 3& 5 days of infection).

Discussion

The expression of the IL1-β gene was always higher than the controls under utilization of the four ZnO concentrations, but the increase in the expression differed with time (3 & 5 days) in irregular manner. The fold change in gene expression among different groups is expressed as value of 2^-ΔΔCt in tilapia fish fed 4 months on ZnO and nZnO supplemented feed and challenged with bacterial infection. Results of the fold change indicated that, the lowest concentration of nZnO (15mg/kg) in the tested diet elevated the expression of the target gene than the conventional form of ZnO after the 3 and 5 days of challenge, but was more after 5 days but still lesser than the other concentrations.(Fig. 6- A&B). It is well established that Zn is vital for the growth, immunity and development of animals in certain amount (Hao *et al*., 2013). ZnO-NP has been reported to enhance growth performance, improve feed utility and provide economic benefits in weaning piglets and poultry (Yang and Sun, 2006 and Mishra *et al*., 2014); encouraging results in average daily gain was obtained by feeding basal diets supplemented with 200, 400, 600 mg/kg nZnO or 3,000 mg/kg ZnO (Hongfu, 2008). Lina *et al*. (2009) in her study indicated that ZnO-NP has been found to improve the production performance and dressing performance of broilers on 42 days of feeding at the level of 40 mg/kg in the diet. Buentello *et al*. (2009) in his study reported that, differences exist in growth rate in response to different dietary sources of Zn and different chemical forms of Zn showed differential bioavailability in fish. In hybrid striped bass Zn proteinate was about 1.7 more efficiently utilized than ZnSO4. Faiz *et al*. (2015) reported that, Growth performances of juvenile *C. idellain* response to different dietary inorganic sources of Zn showed that the highest %WG, SGR and FCE were obtained in the group of fish fed ZnO-NPs diet (different concentrations), while other diets supplemented with zinc in sulfated at both levels and oxide at lower level showed depressed growth, what means that, nZnO promoted the growth more than other inorganic conventional forms and the growth performance of fish fed ZnO-1 diet was statistically comparable to control group of fish.

The present study indicated also that, fish fed diet supplemented with Zn in nano-form at the rate of 30 mg Zn/kg diet (Zn-NP) showed significantly ($p<0.05$) high %WG. This level lies within the range reported by many investigators for different fish species (Clearwater *et al*., 2002) but somewhat lesser than that reported for hybrid striped bass (Buentello *et al*., 2009), juvenile abalone (Tan and Mai, 2001).

90-day feeding trial of juvenile grass carp on Zinc oxide (nano and conventional forms) and Zinc Sulphate, each of which has two doses; lower and higher level (30 and 60mg/kg diet). Zinc oxide nanoparticles were found to promote growth (%WG, SGR and FCR) at its two levels (lower>higher) and enhanced RBCs count and MCHC value significantly ($p<0.05$) at its lower level more than other inorganic forms. However, Zinc Sulphate at both levels and conventional Zinc Oxide at higher level had a negative effect on both growth performance and hematological parameters (RBCs & MCHC) (Faiz *et al*., 2015).

Our study revealed that *Oreochromis niloticus* weight gain increased gradually and in a positive relationship with the increase in the concentration of ZnO in its two forms, with observation that nano-ZnO supplementation enhanced the growth rates more than conventional ZnO, which were sometimes double the weight gain. The highest WG and SGR (41.0 and 1.1% respectively) observed in the group of fish fed on 60mg/kg ZnO-NP supplemented feed compared to fish fed on the same concentration of conventional ZnO (17.3 and 0.65), the magnitude of difference in growth rates from the control increased with the increase in the bulk ZnO concentration in the feed., Concerning nZnO, the magnitude of difference between the tested and control fish from concentration 15mg/kg to 45 mg/kg was nearly steady (2.5, 2.2 and 2.5, respectively) and near the value achieved in ZnO 45 and 60mg/kg. Nano-ZnO 60mg/kg concentration gave a greater fold difference from the control equals 4 folds. Overall; it was observed that low concentrations of added nZnO (15mg/kg) to fish feed achieved specific growth rates like the higher concentrations of conventional ZnO (60 mg/kg) and the 60mg/kg nano-ZnO gave the highest rates of SGR; 4 folds than control. This results may be attributed to reduction of macromolecule to nanoscale changed their properties and increased their application (Rather *et al.,* 2011). A significantly ($p<0.05$) higher %WG and SGR were observed in fish fed on nZnO supplemented diet compared to conventional ZnO enriched diet at the same concentration which may be due to small particles size (32 to57 nm) of nZnO, the higher intestinal absorption, bioavailability and catalytic activities as reported by Alishahi *et al*. (2011). also it may be attributed to somatic growth by stimulation of DNA and RNA synthesis and growth hormone protein synthesis (Siklar *et al*., 2003).

Serum growth hormone measurment revealed that, both ZnO and nZnO supplemented feed induced a higher growth hormone level in serum than the control, with a highest and comparable values for 45 and 60mg/kg concentrations. This may be attributed to the natural function of the endocrine system in physiological processes, as when the body is saturated with a hormone induced under specific stimuli, the induction will begin to decrease because DNA will stop the transcription process with consequent translation and synthesis of certain proteins. However, the value of tilapia growth hormone was noticed to be increased more in serum in case of fish fed on the nZnO compared to conventional zinc oxide within the same concentration. These results which were in accordance with that of Hina *et al*. (2015), who found that nZnO promoted the growth performances of juvenile *C. idellain* more than other inorganic conventional forms.

Physicochemical properties of nanoparticles was found to influence the immunological effects of nanoparticles (Rather *et al*., 2011). Luo *et al*. (2015) reported that, nanoparticles can stimulate innate and adaptive immune response depending on their physicochemical properties; however, it is still unclear how nano-particles affect the immune response. Moreover, it is well documented that Zn deficiency reduces immune responses and disease resistance in human and animals (Chesters, 1997).

Zinc oxide nanoparticles are being used in the food industry as additives and during packaging due to their antimicrobial properties (Gerloff *et al*., 2009 and Jin *et al*., 2009), the sudden rise in the demand of zinc oxide nanoparticles (ZnO NP) is mostly attributed to its better antibacterial properties than the conventional ZnO (Padmavathy and Vijayaraghavan, 2016). Antimicrobial activity was demonstrated using nZnO against *L. monocytogenes*, *S. enteritidis*, and *E. coli* O157:H7 (Jin *et al.,* 2009; Costa *et al*., 2011; Incoronato *et al*., 2011 ;Singh *et al*., 2014) which was attributed to disruption of cell membrane of the bacteria according to these studies. In this study, serum total protein and IgM were affected by dietary levels of ZnO in its two forms, but it was clear that nZnO enhanced the total protein and IgM, which may be 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 as well as these proteins are highly sensitive to metal poisoning (Sakr *et al*., 2005). The parallel elevation of serum protein and immunoglobulin M with dietary ZnO indicate the important role of the protein during Zn transportation. This is in agreement with Gopal *et al*. (1997), who stated that analysis of serum globulin level of *Cyprinus carpio* fish showed a sharp increase for varying periods of treatment with nZnO.

Some studies reported that, when nano-particles enter the body, they can interact with immune cells and trigger inflammatory response, which is accompanied by the secretion of signaling molecules (cytokines, chemokines) that provide communication between immune cells and coordinate molecular events. Interlekin 1 beta (IL1-β) is a member of the interleukin 1 family of cytokines, this cytokine is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE). This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. The study of nanoparticles that induce IL-1b via inflammatory signaling pathways mechanism is an emerging theme (Reisetter *et al*., 2011) The more knowledge we have of cytokine profiles induced by nanoparticles, the better we can utilize IL1-β a biomarker to the immune response. In the present study, results of RT -PCR showed that; in the fish groups fed on conventional ZnO supplemented feed the lowest concentration of ZnO (15mg/kg) induced the highest increase in IL1-β gene expression after 3 days of challenge, which decreased after 5 days but it was still higher than the control (non treated) and the 18sRNA internal control. Concerning the fish groups fed on nZnO supplemented feed, the 45mg/kg nZnO concentration in the fish feed gave the highest increase in the expression of the target gene under study (34.24), followed by the concentration 15mg/kg supplemented feed (33.7). All concentrations of nZnO induced the IL1-β expression after 3 days, then there was obvious observation that after 5 days of immunization, the expression was decreased except in the last concentration (60mg/kg). Concerning the fold change of the gene expression, what means how many times the expression of the target gene increased compared to the internal constitutive housekeeping gene (18sRNA) Figures 13and 14 showed that, after 3 and 5 days of the bacterial immunization there was four important points of observations which could be reported here; the first was that, in the group of the nZnO the lowest concentration (15mg/kg) of adding nZnO to the feed induced IL1-β gene

fold change of expression more than the same concentration of conventional ZnO added in the group of ZnO supplementation. So the same picture of immune response represented in the IL1β gene fold change of expression appeared when ZnO and nZnO were compared at the lowest concentration of the additive; nZno was the higher inducer with this low concentration with observation that the two types of ZnO; conventional and nZnO induced the higher expression after 5 days of immunization. The second point was that, in the other three concentrations (30, 45 and 60mg/kg) the inorganic conventional ZnO was reported to induce and up-regulate the IL1-β fold change of expression more than the nZnO form within the same concentration (Fig. 13&14). The third point of observation was that, the concentration which gave good and better results of induction to the target gene was 30mg/kg in its two forms; the conventional and nano more than the other treatments, but results also indicated that conventional ZnO in this concentration (30mg/kg) was better than nano-ZnO in the process of IL1-β gene expression induction and regulation. The fourth and final point of observation was that after 5 days of experimental infection the expression of the target gene was better than after 3 days, thus the immune response represented in this gene improved after 5 days.

Collectively it could be concluded that the inorganic conventional zinc oxide induced the target gene better than the nano form in the higher concentrations and the sustainability for inducing the gene expression was for the concentration of 30 mg/kg after the 3 and 5 days, thus this concentration could be considered as the best for inducing the immune function in the form of IL1-beta. Reisetter *et al.* (2011) stated that, the study of nanoparticles that induce IL1-β via inflammasome signaling pathways mechanism is an emerging theme. In their in vitro experiment, Lucarelli *et al.* (2004) exposed human macrophages to non-toxic concentrations of different SiO2, TiO2, ZrO2, and Co nanoparticles and observed increased expression of TLR receptors and production of inflammatory cytokines, the experiment showed that different nanoparticles triggered inflammatory response in different ways. SiO2 nanoparticles induced the production of inflammatory cytokines IL-1β and TNF-α. Yang *et al*. (2012) found that, Silver nanoparticles induced inflammasome formation and triggered IL1-β release and subsequent caspase-1 activation. Inflammasome-activation-associated IL1-β production by dendritic cells (Sharp *et al*, 2009). These studies support our results concerning the effect of nZnO on IL1-β gene expression.

Concerning zinc concentration in muscles, it was determined in ptesent investigation that Zinc concentration showed higher value in all fish groups fed on ZnO supplemented feed compared to the control, but still within the range of permissible limit (40ppm) set by FAO (1983). It was noticed that as the concentration of ZnO increases the Zn concentration in muscle increased. Among all fish fed on ZnO (Bulk and Nano), in the group of fish fed on 60mg/kg nZnO supplemented feed, the value of Zn in muscles was the highest. This could be explained by the fact that nano particles possess a smaller size which enables them for higher absorption and higher bioavailability (Zaboli *et al.*, 2013). Also Feng *et al*., (2009) indicated the same suggestion that due to their small size, nano minerals are easier to be taken up by the body, these suggestion were in accordance with our results.

Conclusion

From this study it could be concluded that, supplementation of nZnO to fish feeds can possibly improve the growth rates exemplified here by the weight gain, the specific growth rates and growth hormone in blood. This could be better than the conventional zinc oxide, so it could be used in fish farms and aquaculture with its low concentrations and this could improve the economics of farming.

Concerning the immunity, results indicated that, Total protein as well as immunoglobulin M was increased in fish groups fed on ZnO supplemented feed with indication that nZnO enhanced these immune items synthesis more than the bulk form. Results indicated also that the concentration of 60mg/kg conventional ZnO was the best concentration for up-regulating the IL1beta, followed by the concentration 30mg/kg in the two forms; the nano and the conventional form. However, there was an observation that totally the inorganic conventional ZnO was reported to induce and up-regulate the IL-1β better than the nanoZnO form.This may inform that conventional form of ZnO could be better and cheaper to use in aquaculture as immuno-stimulant feed additive. The higher values of zinc in muscles in fish fed on nano zinc compared to conventional ZnO supplemented feed may be due to higher absorption and bioavailability of the small sized particles of nZnO.

Acknowledgment

This work was supported by the project no. 9040205 funded by National Research Centre, Egypt.

Declaration of interest

Conflicts of interest: none

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