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Research Article

Effects of dietary selenium and zinc nanoparticles on growth performance, immune responses, and antioxidant enzymes activities of white leg shrimp (*Litopenaeus vannamei*)

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Abstract

In the present study selenium nanoparticles (Se-NPs) and zinc nanoparticles (Zn-NPs) included in the diets of white shrimp at 0 mg (control), 0.3 mg Se-NPs/ kg feed (T1), 0.15 mg Se-NPs+15 mg Zn-NPs/kg feed (T2) and 30 mg Zn-NPs/kg feed (T3). After 8 weeks, shrimps fed both Se-NPs and Zn-NPs showed higher (p<0.05) final body weigh, weigh gain, and SGR than shrimp fed the control diet. On the other hand, the FCR was decreased by Se-NPs and Zn-NPs when compared to the control whereas both Se-NPs+Zn-NPs revealed lower FCR than the other groups (p<0.05). Shrimp fed Se-NPs or Se-NPs +Zn-NPs revealed higher survival rate than shrimp fed the control and Zn-NPs while shrimp fed Zn-NPs showed higher survival rate than shrimp fed the control (p<0.05). The total haemocyte count, large granular cells, semi granular cells, and semi granular cells revealed higher counts in shrimps fed Se-NPs and Zn-NPs than the control (p<0.05). Shrimp fed Se-NPs or Se-NPs+ Zn-NPs revealed higher catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidas (GPX) activities than shrimp fed the control and Zn-NPs while shrimp fed Zn-NPs showed higher catalase activity than shrimp fed the control (P<0.05). The total protein, lysozyme, and phenoloxidase activities increased in shrimp fed Se-NPs or/and Zn-NPs with the highest being in shrimp fed both in shrimp fed Se-NPs and Zn-NPs followed by those fed Se-NPs then Zn-NPs when compare to the control (p<0.05). In conclusion, the co supplementation of Se-NPs and Zn-NPs showed higher performances, antioxidant status, and immune responses in white shrimp than the individual supplementation of Se-NPs or Zn-NPs.

Keywords: *Litopenaeus vannamei*, Zinc, Selenium, Growth, Immunity, Antioxidant

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Introduction

The aquaculture industry is expanding day by day to afford the human food chain with a healthy and cheap animal protein source (Moss *et al*., 2019). The shrimp and crustaceans are among the aquatic animals which can be cultured under intensive conditions (Kongnum and Hongpattarakere, 2012). The nutritional requirements can be affected by the rearing conditions, and scientific efforts are required to maintain the essential dietary nutrients for normal growth and production (Adel *et al*., 2017). The trace minerals are essential in regulating several functions in the shrimp body (Oliva-Teles, 2012).

Selenium is a vital trace element associated with regulating the growth rate, antioxidant status, and immune responses in shrimp (Chiu *et al*., 2010). Selenium is required for selenoproteins formation that is important for the production of glutathione peroxidase (Watanabe *et al*., 1997; Hoffmann and Berry, 2008). Glutathione peroxidase is antioxidative enzymes which protect the cells from the overoxidation of free radicals (Lawrence and Burk, 1976). Selenium also is required for the regulation of the thyroid glands, which secrets the growth hormone (Shenkin, 2006). Hence, the deficiency of selenium would impair the growth rate, antioxidative, and immune responses. It has been reported that the enrichment of the shrimp diet with selenium resulted in enhanced growth rate and health condition (Chiu *et al*., 2010).

Zinc is another microelement that is existed in the content of body fluids, tissues, and organs (Shenkin, 2006). Zinc is a cofactor required for the regulation of several functions in the body, including osmoregulation, metabolism of nutrients, antioxidative enzymes, and feed efficiency (Salahuddin *et al*., 2017). Additionally, zinc regulates the protein, lipid, energy, and vitamin A synthesis. Several studies displayed the potential role of zinc in the diets of shrimp when included at adequate levels (Muralisankar *et al*., 2015).

It is well documented that normal zinc and selenium levels in freshwater and seawater are insufficient to meet the requirement of growing aquatic species. Therefore, zinc and selenium are considered as essential nutrients in fish and shrimp feed (Wei *et al*., 1999; Nasr-Eldahan *et al*., 2021).

Although the requirement of mineral has been fixed for shrimp feeding; however, the enrichment of more than one element in shrimp diets is still required more efforts. The co supplementation of selenium and zinc enhanced the growth rate, antioxidative status, and immunity of several aquatic animals (Rider *et al*., 2010; Swain *et al*., 2019).

The recommended dosage of SeNP dietary inclusion ranges from 0.15 to 4 mg/kg depending on the fish and shrimp species (Dawood *et al*., 2021). Due to its high bioavailability and lower toxicity, the nano form of selenium is a novel type that receives more interest than inorganic and organic forms (Nasr-Eldahan *et al*., 2021; Sun *et al*., 2022). However, in the literature, no data were

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reported to reveal the influences of including both selenium and zinc in their nano form in diets of white shrimp.

Consumers highly demand the white shrimp (*Litopenaeus vannamei*) because of the delicious flesh properties, high nutritious value, and reasonable cost (Lee and Lee, 2018). The basal ration should be fortified with all the nutrients, including trace minerals to produce healthy shrimps. Hence the study was conducted to assess the influence of including Se-NPs or/and Zn-NPs on white shrimp by the evaluation of the growth rate, antioxidative status, and immune response.

Materials and methods

Preparations of zinc and selenium nanoparticles

In this study, selenium nanoparticles (Se-NPs) prepared by Kimiagaran Nano Mavad Company (purity of 99%) with an average size of less than 50 nanometers and also, zinc nanoparticles (Zn- NPs) purchased from *Iran's Pishgaman*-e*Nano Mavad Company* (with a purity of 98%) with an average size of 25-30 nm. The particle size of selenium and zinc was confirmed using a scanning *electron microscope* (*SEM*).

Experimental diets

Ingredients and proximal composition of the basal diet are given in Table 1. Selenium and zinc nanoparticles were dissolved in sterile phosphate-buffered *saline* (PBS) and was added to the basal diet at 0 (control), 0.3 mg Se-NPs/ kg feed (T1), 0.15 mg Se-NPs+15 mg Zn-NPs/kg feed (T2) and 30 mg Zn-NPs/kg feed (T3) (Ashouri *et al*., 2015; Taheri *et al*., 2017) and allowed to dry and stored at 4°C until use.

Table 1: Ingredients and proximal composition of the basal diet.

Ingredients	g/kg	Proximate composition	(%)
Kilka fish meal	230	Moisture	8.2
Fish oil	30	Crude protein	38.2
Squid meal	70	Crude lipid	8.1
Soybean meal	205	Ash	12
Wheat flour	300		
Liquid lecithin	20		
Mineral premix[a]	20		
Vitamin premix	5		
Anti-fungal[b]	1		
Binder[c]	20		
Anti-oxidant[d]	5		
Shrimp meal	50		
Cholesterol	2.5		

[a]Premix detailed by Kongnum and Hongpattarakere (2012) without Se and Zn.
[b]ToxiBan antifungal (Vet-A-Mix, Shenan- doah, IA).
[c]Amet binder (MehrTaban-e- Yazd, Iran).
[d]Butylatedhydroxytoluene (BHT) (Merck, Germany).
[e]Dry matter basis.

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Shrimp

A total of 300 Juvenile *L. vannamei* with an initial body weight of 5.1 ±0.2 g were purchased from hatchery center of Bandar Abbas province (Iran) and transferred to private shrimp farm in Bandar Abbas (Bandar Abbas province, Iran). Shrimp were acclimated to the experimental conditions and fed the basal diet for 2 weeks before the experiment started thrice a day with commercial feed (Beyza Feed Mill 21, Iran). Shrimp were distributed randomly into 12 aquarium tank (300 L, 3 tanks per diet, 25 shrimp per tank). During the experimental period, the water physicochemical parameter was 30.2±1.0 °C temperature, dissolved oxygen 6.3±0.1 mg L^{-1}, pH 8.1±0.2, electrical conductivity 5826.3±213.0 MM cm^{-1}, and salinity 30.0±2.1 g^{-1}. Shrimp were initially fed 3-4% of total body weight daily. The feeding frequency was three times per day at 8:00, 14:00 and 20:00 hours and lasted for eight weeks. During the period, all residual/uneaten feed, faeces were siphoned from the floor of tanks and about 50% of the water in each tank was replaced.

Growth performance

All Shrimps were deprived of food for 24 h before weighing (Gao *et al*., 2016) and sampling and growth indices were calculated according to the following formulas were measured (the shrimp in all treatments were biometried once every 2 weeks days during the 56-day experiment) at the end of feeding trial (day 56):

Weight gain = W2 (g) - W1 (g)

Specific growth rate (SGR) = 100 (ln W2 - ln W1) T^{-1}

Feed conversion ratio (FCR) = Total dry feed intake (gr) / wet weight gain (gr)

Survival rate %= (Final number of shrimp/initial number of shrimp) × 100.

Where W1 is the initial weight, W2 is the final weight and T is the number of days in the feeding period.

Sampling of hemolymph

At the end of the feeding trial (56 days), 10 shrimp from each individual tank were selected randomly. 300 µL of haemolymph was drawn from the ventral sinus of individual shrimp using 1 ml sterile syringe containing anticoagulant solution (115 mM glucose, 336 mM sodium chloride, 27 mM trisodium citrate and 9 mM EDTA, at a pH of 7) at a 1:1 (v/v) ratio (Kakoolaki *et al*., 2014). Diluted hemolymph was centrifuged at 5000×*g* for 10 min (4°C*)* and stored at -20ºC until use.

Hemolymph indices

Total haemocyte count (THC) was conducted as described by Sapcharoen and Rengpipat (Sapcharoen and

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Rengpipat, 2013). A drop of the haemolymph (20 µ) was placed on a haemocytometer and the total heamocyte counts (THC) were determined, using light microscope with ×400 magnification. THC count was expressed as log cell/ml haemolymph. Also, hyaline cell (HC) count, large granular cells (LGC) and semi- and large granular cell (SGC and LGC, respectively) count via hemolymph extension at room temperature, fixation at methanol for 10 min, staining by the method of May-Grunwald-Giemsa, and then counting with light microscope (Nikon, Japan) (Muralisankar *et al*., 2015).

Antioxidant enzymes activities

Superoxide dismutase (SOD) activity in hemolymph samples was estimated using the Nitro blue tetrazolium chloride dye reduction test (NBT) (Yasuda *et al*., 2002). Briefly, 0.1 mL aliquots of haemolymph were added to 2 mL of reaction mixture (containing 0.20 mM xanthine, 0.12 mM NBT, 0.049 IU xanthine oxidase (Sigma Company, USA) and 0.1 M phosphate buffer, pH 7.0). The mixture was incubated for 20 min at 37°C. Then, the absorbance was read at 560 nm. SOD activity was expressed as the percentage inhibition of reduction of NBT.

The catalase (CAT) activity was determined according to the method described by Góth (1991). Briefly, 0.1 mL of hemolymph was incubated for 60 s with 1 mL reaction mixture consisting of 50 mM potassium phosphate buffer (pH 7.0) and 10.6 mM H_2O_2 (Merck, Germany) freshly prepared at 37°C. The reaction was terminated by adding 0.5 mL of 32.4 mM ammonium molybdate solution (Merck, Germany). A yellow complex of ammonium molybdate and hydrogen peroxide was created. The absorbance of the complex was measured at 405 nm with a spectrophotometer and compared with a blank (distilled water). One unit of CAT activity was defined as the amount of enzyme that catalyses the decomposition of 1 µmol of H_2O_2 per minute.

Glutathione peroxidase (GPX) activity was measured according to the method described by Lawrence and Burk (Shenkin, 2006). Briefly, samples of 0.9 mL of the reaction mixture containing 50 mM potassium phosphate buffer pH 7.0, 1 mM EDTA, 1 mM sodium azide, 0.2 mM NADPH (Merck, Germany), 20 mU glutathione reductase and 1 mM GSH were incubated for 5 min at 25°C. Then, 0.1 ml of 0.25 mM H_2O_2 (0.25 mM/mL) and 50 µL hemolymph were added to the mixture, and absorbance was read at 340 nm for 60 s. Samples of distilled water were used as a blank. One unit of GPX activity was defined as µmol NADPH consumed per min per mg serum protein.

Lysozyme activity

Lysozyme activity was measured as described by Sotelo-Mundo *et al*. (2003), with some modifications. Briefly, 100 µl of diluted haemolymph was added to 1 mL of a suspension of Micrococcus lysodeikticus (0.2 mg/ mL) in a 0.05 M sodium phosphate buffer (pH 6.2). Absorbance was measured at

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530 nm after 240 s and 540 s by spectrophotometer (Biophotometer Eppendorf).

Phenoloxidase activity

The phenoloxidase activities was measured using a spectrophotometer by recording the formation of dopachrome from L-dihydroxyphenylalanine (L-DOPA) at the final reading of the medium at 490 nm (Safari *et al*., 2014).

Total protein

Total protein were estimated on shrimp hemolymph by using commercial kits (Pars Azmoon Company, Tehran, Iran) (biuret method) and a biochemical auto analyzer (Prestige 24i, Japan) (Adel *et al*., 2015).

Statistical analysis

All the tests were performed in triplicate. The data were subjected to statistical analysis using the SPSS software version no. 20 (SPSS Inc., Chicago, IL, USA). The statistical analysis was done by using one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. *P*-value of <0.05 was considered significant.

Results

Growth performance

White shrimp fed both Se-NPs and Zn-NPs showed higher final body weigh than shrimp fed the control diet (p<0.05). The weigh gain and SGR were increased in shrimp fed Se-NPs or/and Zn-NPs when compared to the control whereas shrimp fed both Se-NPs and Zn-NPs revealed higher weight gain and SGR than the other groups (p<0.05). On the other hand, the FCR was decreased by Se-NPs or/and Zn-NPs when compared to the control whereas both Se-NPs and Zn-NPs revealed lower FCR than the other groups (p<0.05). Shrimp fed Se-NPs or both Se-NPs and Zn-NPs revealed higher survival rate than shrimp fed the control and Zn-NPs while shrimp fed Zn-NPs showed higher survival rate than shrimp fed the control (p<0.05) (Table 2).

Table 2: Growth performance of shrimp fed with different levels of Zn and Se nanoparticles.

Parameters	T0	T1	T2	T3
Initial weight (g)	5.24 ±0.62	5.21 ±0.42	5.23 ±0.50	5.24 ±0.46
Final weight (g)	9.90 ±0.31^{b}	10.82 ±0.6^{ab}	10.57 ±0.42^{ab}	11.79 ±0.5^{a}
Weight gain (%)	88.9 ±3.3^{c}	107.6 ±4.1^{b}	102.0±6.8^{b}	125.1 ±5.1^{a}
FCR	1.47 ±0.12^{a}	1.30 ±0.08^{b}	1.32 ±0.16^{b}	1.09 ±0.08^{c}
SGR (%)	1.14 ±0.07^{c}	1.32 ±0.13^{b}	1.30 ±0.09^{b}	1.45 ±0.12^{a}
SR (%)	90.8 ±1.6^{c}	97.0 ±1.8^{a}	92.1 ±2.2^{b}	98.6 ±1.8^{a}

*Values are expressed as means±SD (n=20). Data in the same row assigned with the different superscripts are significantly different (p<0.05). FCR, feed efficiency ratio, SGR, specific growth rate, SR, survival. T0: Control, T1: Se-NPs, T2: Zn-NPs and T3: Se-NPs+Zn NPs.

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Hemolymph indices

The total haemocyte count (THC) and large granular cells (LGC) revealed higher counts in shrimps fed Se-NPs or/and Zn-NPs than the control. The THC and LGC had the highest values in shrimp fed both Se-NPs and Zn-NPs followed by those fed Se-NPs then those fed Zn-NPs (p<0.05). Semi granular cells (SGC) displayed higher values in shrimp fed Se-NPs or both Se-NPs and Zn-NPs than the other groups (p<0.05). Hyaline count (HC) was increased in shrimp fed both Se-NPs and Zn-NPs followed by those fed Se-NPs when compared to the other groups (Table 3) (p<0.05).

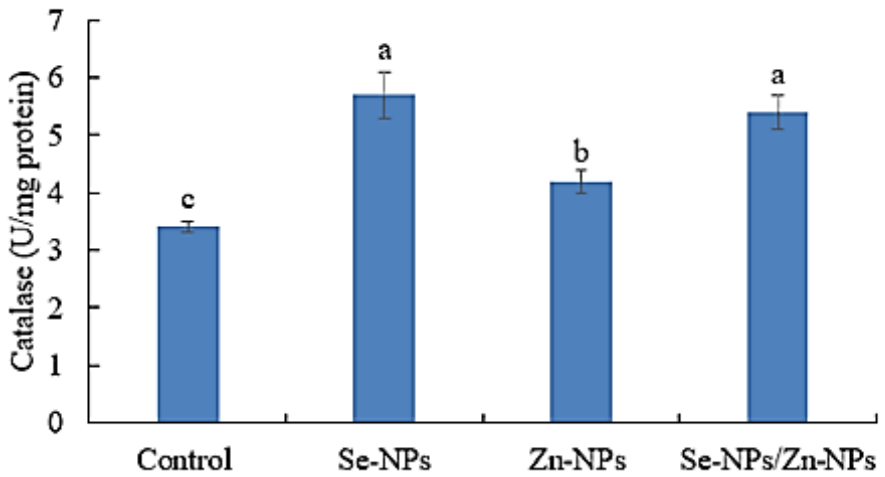
Table 3: Efects of dietary Zn and Se nanoparticles on hemolymph indices of juvenile shrimp after 56 days of supplementation.

Parameter	T0	T1	T2	T3
Total haemocyte count (THC) ($\times 10^6$ cells mL^{-1})	11.2 ±0.1^{d}	12.8 ±0.2^{b}	11.8 ±0.2^{c}	13.7 ±0.1^{a}
Large granular cells (LGC) (%)	10.7 ±0.1^{d}	15.6±0.1^{b}	13.6 ±0.2^{c}	16.5 ±0.2^{a}
Semi granular cells (SGC) (%)	29.2 ±2.2^{b}	35.1 ±2.2^{a}	30.2 ±2.4^{b}	34.1 ±2.1^{a}
Hyaline count (HC) (%)	70.1 ±0.7^{c}	73.9 ±0.5^{b}	71.1 ±0.8^{c}	77.8 ±0.7^{a}

*Values are expressed as means±SD (n=8). Data in the same row assigned with the different superscripts are significantly different (p<0.05).
T0: Control, T1: Se-NPs, T2: Zn-NPs and T3: Se-NPs+Zn NPs.

Antioxidative status

The antioxidative status is expressed by catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidas (GPX) activities (Fig. 1). Shrimp fed Se-NPs and Se-NPs+Zn-NPs revealed higher CAT, SOD, and GPX than shrimp fed the control and Zn-NPs while shrimp fed Zn-NPs showed higher catalase, superoxide dismutase and glutathione peroxidase activities than shrimp fed the control (p<0.05).



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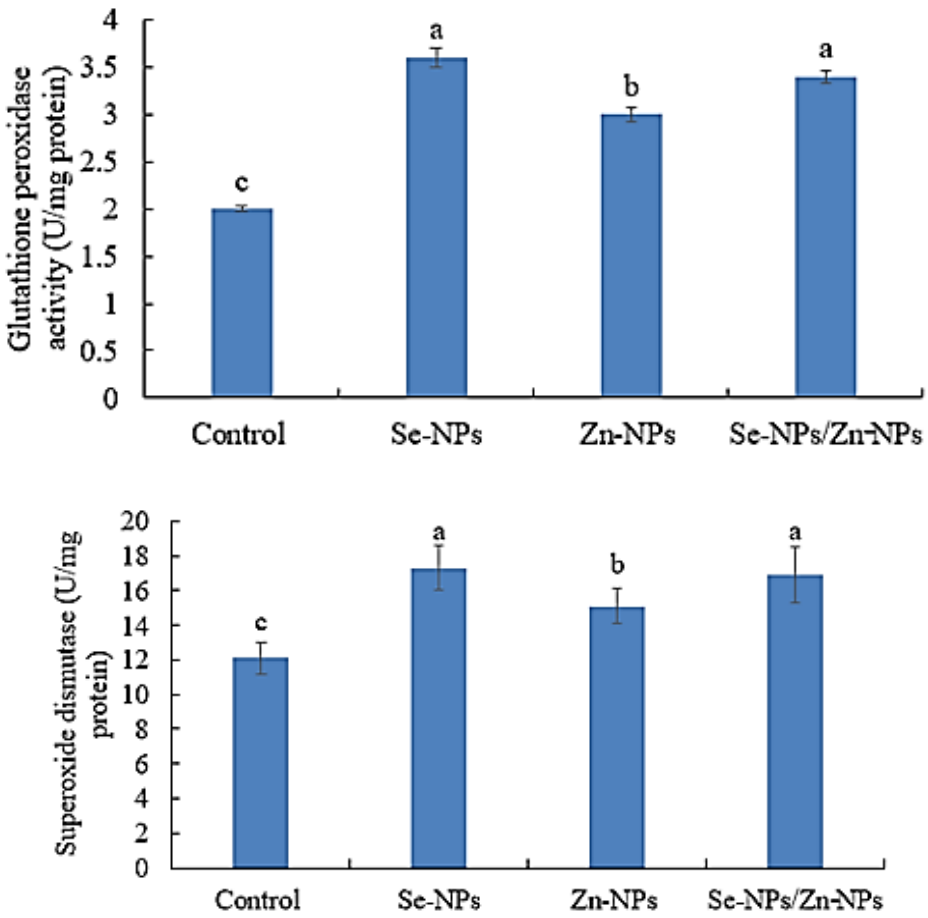

Figure 1: Antioxidant enzymes activities in shrimp fed different levels of Zn and Se nanoparticles during 8 weeks. Values are expressed as means±SD (*n*=9). Values in each row with different superscripts shows significant difference ($p<0.05$).

Immune response

The total protein, lysozyme, and phenoloxidase activities increased in experimental shrimp fed with Se-NPs, Zn-NPs and Se-NPs + Zn-NPs when compare to the control ($p<0.05$), while these activities showed lower in experimental shrimp fed Zn-NPs in compare with shrimp fed with Se-NPs and or Se-NPs+Zn-NPs. The total protein, lysozyme, and phenoloxidase activities increased in shrimp fed Se-NPs or/and Zn-NPs with the highest being in shrimp fed both in shrimp fed Se-NPs and Zn-NPs followed by those fed Se-NPs then Zn-NPs when compare to the control ($P<0.05$) (Fig. 2).

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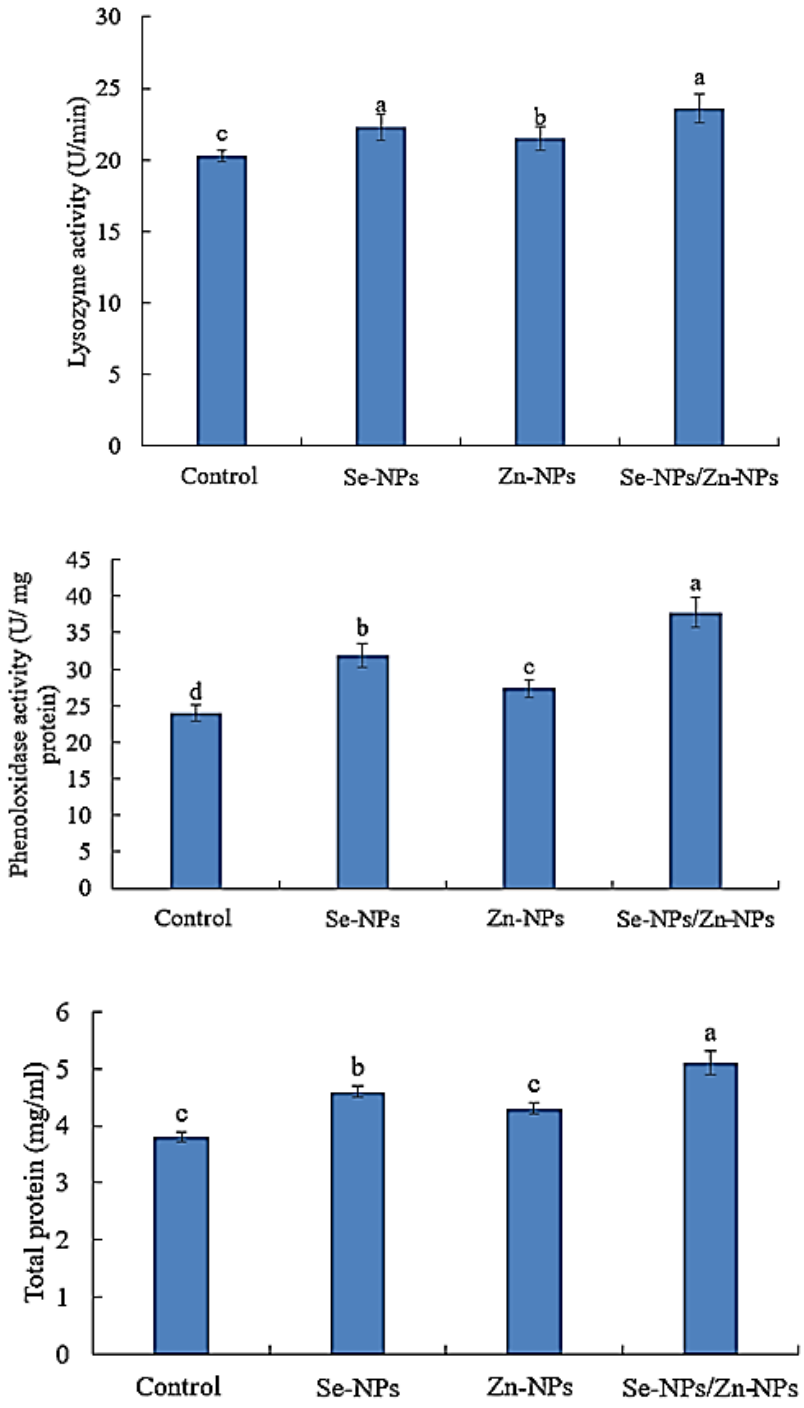

Figure 2: Effects of dietary Zn and Se nanoparticles on immunity parameters of juvenile shrimp after 56 days of supplementation. Values are expressed as means±SD (n=8). Data in the same row assigned with the different superscripts are significantly different (p<0.05).

Discussion

Selenium and zinc are trace minerals associated with several physiological and biological functions through improving the osmoregulation, fluid balance, and forming hormones and enzymes (Muralisankar *et al*., 2015; Dawood *et al*., 2019). The nanoform

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also presented a practical strategy to include trace minerals in aquafeed (Jampilek *et al*., 2019; Dawood *et al*., 2019). Nano minerals has a big surface which increases the efficacy of the element even when supplied at low levels. In this sense, nano minerals decrease the cost of mineral supplementation and the expected toxicological impacts (Shaw and Handy, 2011). The co enrichment of trace minerals (Se-NPs and Zn-NPs) is usually applied in fish feeding (Swain *et al*., 2019). The supplementation of shrimp diets with Se-NPs or Zn-NPs resulted in improving the growth rate, immune, and antioxidative status (Muralisankar *et al*., 2015; Satgurunathan *et al*., 2017). However, including both Se-NPs and Zn-NPs in the shrimp diets is not well documented.

The results displayed an increased growth rate, weight gain, and SGR in white shrimp fed Se-NPs + Zn-NPs. In line with the present study, including zinc in the diets of grass shrimp, *Penaeus monodon* (Shiau and Jiang, 2006) and freshwater prawn, *Macrobrachium rosenbergii* (Muralisankar *et al*., 2015) resulted in improved growth rate and feed efficiency. The inclusion of selenium also increased the growth rate and feed utilization in fish (Dawood *et al*., 2020); however to the authors knowledge no studies revealed the influence of selenium on white shrimp. The results revealed that both Se-NPs and Zn-NPs are efficient in improving the growth and FCR of whit shrimp. The reduced FCR in shrimps fed Se-NPs and Zn-NPs attributed to the enhanced feed efficiency. Muralisankar, Saravana Bhavan, Radhakrishnan, Seenivasan, Srinivasan and Santhanam (Muralisankar *et al*., 2015) reported that zinc (10–60 mg kg^{-1} of feed) increased the feed efficiency in shrimps by enhancing the activities of digestive enzymes. The results also indicate higher survival rates in shrimps fed Se-NPs and Zn-NPs than the control group. The enhanced survival rate is reflecting the healthy status of white shrimps and the non-stressful conditions during the trial.

The main purpose of including selenium and zinc in the diets of aquatic animals is to maintain the antioxidative status (Muralisankar *et al*., 2015; Neamat-Allah *et al*., 2019). The stressful conditions impair the cell function by deteriorating the redox of reactive oxygen species (ROS) which increase the oxidative stress and harm the cell structure and damage DNA (Al-Deriny *et al*., 2020; Harsij *et al*., 2020). Several enzymes are synthesized to breakdown the high concentration of ROS and keep the antioxidative balance (Lee *et al*., 2004; Dawood *et al*., 2020). These enzymes include CAT, SOD, and GPX, which displayed enhanced values in white shrimp, fed with Se-NPs +Zn-NPs. Similar results were obtained by zinc (Muralisankar *et al*., 2015) and selenium (Chiu *et al*., 2010) in *M. rosenbergii*. The enhanced antioxidative status is probably attributed to the role of selenium in formin the selenoproteins which required for the release of

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glutathione peroxidase (Hoffmann and Berry, 2008).

The membranes of immune cells are subjected to oxidation by ROS due to its high content of lipid (Yu, 1994). Hence, the crosstalk between the oxidative stress and the immune system is of importance in aquatic animals. The application of Se-NPs and Zn-NPs is expected to enhance the antioxidative response and prevent cellular immunity (Shaw and Handy, 2011). The positive effect of Zn-NPs on the antioxidant system could be attributed to its role as a cofactor for cytoplasm and extra-cellular SOD and the ability of Zn to maintain the activities of radical scavenging enzymes (Kumar *et al*., 2016). In parallel with the enhanced antioxidative status, the present study displayed improved immune response in white shrimp fed both Se-NPs and Zn-NPs. The analyzed immune-related variables in this study are the total protein, total hematocyte count, lysozyme, and phenoloxidase activities. In the same line, *M. rosenbergii* fed selenium revealed improved total haemocyte count (THC) and phenoloxidase activity (Chiu *et al*., 2010) whereas *P. monodon* fed zinc showed increased THC (Shiau and Jiang, 2006). Although no disease challenge was performed in the present study, however, the activated phenoloxidase and lysozyme activities suggest the protection role of Se-NPs and Zn-NPs in whit shrimp against infection. The THC and its derivitives (LGC, SGC, and HC) is the fluid storm in crustaceans with a phagocytic activity that is vital during the outbreak (Van de Braak *et al*., 2002). The high THC along with enhanced lysozyme and phenoloxidase activities in this study reflecting enhanced immune response in white shrimp fed Se-NPs and Zn-NPs. Additionally, the high total protein in white shrimp indicates the good metabolism of the diet and improved feed efficiency (Dawood, 2016). Besides, increased total protein is attributed to its high content of related immunological molecules (Schliep and Felgenhauer, 1978).

In conclusion, the present study displayed the potential role of Se-NPs and Zn-NPs in white shrimp aquaculture by improving growth performance and enhancing antioxidant and immune parameters. However, the optimal level of Se nanoparticles in the diet of shrimp needs further investigation. further studies are required to reveal the mechanistic role of the individual and combined supplementation of Se-NPs and Zn-NPs in enhancing the antioxidative and immune response. Also, the disease challenge is recommended for future studies with a particular focus on the prevalent diseases in the field for eco-friendly and sustainable shrimp aquaculture.

In summary, the inclusion of Se-NPs and Zn-NPs resulted in improved growth rate, antioxidative, and immune responses. The co supplementation of Se-NPs and Zn-NPs showed higher performances than the individual supplementation of Se-NPs or Zn-NPs.

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