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Potential effects of zinc oxide nanoparticles on growth, hematology, and serum and skin mucus biochemical parameters of common carp (*Cyprinus carpio*) juveniles

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Abstract

This study investigated the effects of zinc oxide nanoparticles (ZnO-NPs) on the growth, blood parameters, skin mucus, and serum biochemistry of juvenile common carp (*Cyprinus carpio*). A total of 300 fish with an average weight of 12.00 ± 0.33 g were randomly distributed in 12 fiberglass tanks with a capacity of 300 L and fed with diets supplemented with ZnO-NPs at concentrations of 0 (control), 10.00, 50.00, and 100 mg kg^{-1} for 60 days. The results indicated that 50.00 mg kg^{-1} ZnO-NPs supplementation significantly improved fish weight, length, specific growth rate, and feed conversion ratio. There was no significant difference in survival rates between the treatments and control group; however, the highest survival rate was observed in the treatment with 50.00 mg kg^{-1} ZnO-NPs. This treatment also led to the highest red blood cell counts, hemoglobin levels, mean corpuscular hemoglobin concentration, and lymphocyte percentages. In contrast, the highest neutrophil counts were observed at 100 mg kg^{-1} ZnO-NPs. The highest serum biochemical parameters, including total immunoglobulin, lysozyme, and Zn levels were observed in the treatment with 50.00 mg kg^{-1} ZnO-NPs. While, in the skin mucus, total immunoglobulin, lysozyme, and alkaline phosphatase significantly increased at groups fed with 10.00 mg kg^{-1} ZnO-NPs. Overall, the findings suggest that dietary inclusion of 50.00 mg kg^{-1} ZnO-NPs can effectively promote growth and enhance immune responses of *C. carpio* juveniles, indicating their potential application in aquaculture practices. However, caution is advised at higher concentrations due to the potential adverse effects on fish health.

Introduction

Fish diets frequently include vitamin and mineral supplements to mitigate deficiencies, as fish cannot synthesize minerals and must obtain them from their diet.[1] Zinc (Zn) is one of the most important trace elements for fish, and it is an essential mineral for over 300 enzymes and 2,000 transcription factors, as well as protein and carbohydrate metabolism. Zinc ions play a crucial role in the regulation of cellular signal reception, cell membrane receptors, transporters, membrane channels, protein kinases, and phosphatases, and binding of transcription factors to DNA.[2] However, its bioavailability can be affected by compounds, such as tricalcium phosphate found in fish meal and phytic acid present in soybeans and grains, which may result in reduced growth rates, increased mortality, and various health issues in freshwater fish.[3] Although increased dietary Zn intake can help address deficiency-related problems, it may also result in greater Zn excretion and environmental accumulation, potentially contributing to the pollution.[4] Researchers are thus investigating methods to improve Zn bioavailability while reducing the quantity of Zn needed in dietary supplements.[5]

Nowadays, nanoparticles (NPs) have attracted considerable interest in aquaculture for enhancing growth performance, disease resistance, and water quality management.[6] The Zinc oxide NPs (ZnO-NPs) are known for their unique properties and important roles in biological processes.[7] The small size and large surface area of ZnO-NPs increase their reactivity and availability in the body, which may improve their effectiveness in therapeutic approaches.[8] They have shown anti-oxidant,

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anti-microbial, and anti-inflammatory effects, which can be advantageous in nutritional and immunity contexts. Research has shown that ZnO-NPs can neutralize free radicals and diminish inflammation, being essential to numerous health concerns.[9]

According to the Food and Agriculture Organization report,[10] approximately 4.20 million tons of common carp (*Cyprinus carpio*) are produced annually worldwide, accounting for about 8.60% of global freshwater aquaculture production. Due to the intense monoculture systems and artificial diets, these fish are more exposed to numerous toxins, making them a crucial model for toxicity studies.[11] One study examined the acute toxicity of ZnO-NPs on common carp, revealing that high concentrations (*e.g.*, 3,500 mg L^{-1}) can be lethal, with significant histopathological changes observed in gill and liver tissues after exposure.[12] Although ZnO-NPs offer potential benefits, further research is necessary to understand their undesirable effects and hazards. Therefore, this study aimed to investigate the effects of various concentrations of ZnO-NPs and determine the optimal dosage for the diet of juvenile *C. carpio*, with a focus on growth performance and immune response.

Materials and Methods

Animal maintenance and treatment. Juvenile carps (n = 300), apparently healthy, with an average weight of 12.00 ± 0.33 g, were procured from the Sanger Dam Centre (Rasht, Iran). The fish were weighed after arrival and treated with potassium permanganate (4.00 mg L^{-1} for 10 min; Merck, Darmstadt, Germany).[13] Before treatment, the fish were acclimated for 2 weeks under controlled photo-thermal conditions (12 hr light/12 hr dark, 20.00 ± 2.00 ˚C, pH: 7.50, and dissolved oxygen: 7.00 ± 0.40 mg L^{-1}) and fed with a commercial carp pelleted feed (Etehad Guilan, Rasht, Iran) including 30.00% protein, 4.00% fat, 50.00% carbs, 10.00 - 11.00% moisture, and a particle size of 2.20 mm. The ZnO-NPs (Pilato, Valencia, Spain) with a purity of 99.98% and a particle size of 20.00 - 25.00 nm were acquired in dry powder (Fig. 1). To generate a suspension with a concentration of 1,000 mg L^{-1}, 1.00 g of nanoparticle was dispersed in 10.00 m L^{-1} of double-distilled water using an ultrasonic bath (IS-2; Intersonic, Istanbul, Türkiye) for 10 min. This suspension was maintained in a dark bottle at room temperature in the laboratory until usage.[14] The concentrations of 10.00, 50.00, and 100 mg kg^{-1} ZnO-NPs were added to the feed in three different treatments, each conducted in triplicate, alongside a control group (non-treated).[15] All the treatments were maintained separately in a 300 L fiberglass tank (n = 25) equipped with semi-static water flow conditions, with 80.00% of the water in each experimental tank changed with fresh water daily. Feeding was undertaken at a rate of 3.00% of body weight three times a day for 60 days.

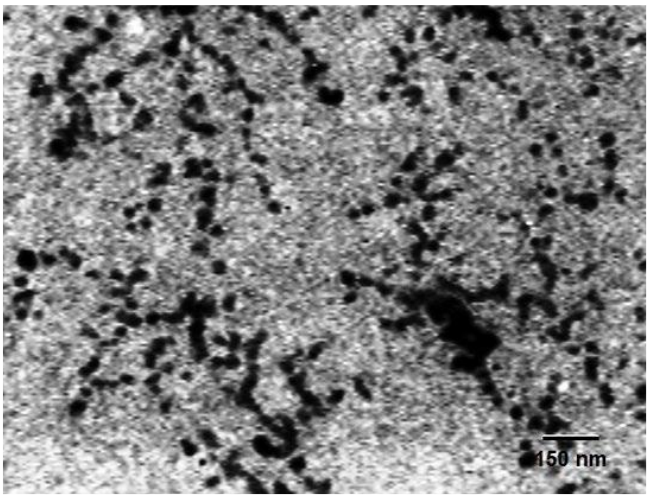

Fig. 1. Transmission electron microscopy of zinc oxide nanoparticles from stock suspension.

Fish growth performance. To evaluate the fish growth performance and feeding efficiency, individual weight and length measurements were meticulously recorded from each experimental tank at the end of the study after 150 mg L^{-1} clove powder (*Eugenia caryophyllata*; Merck) induced anaesthesia. Before sampling, feeding was suspended for 24 hr. The data were used to calculate growth parameters as follows:[16,17]

$$\textit{Body weight gain} = \textit{Final weight (g)} - \textit{Initial weight (g)}$$

$$\textit{Specific growth rate (SGR; \% per day)} = 100 \times [(\textit{Ln (Final weight)} - \textit{Ln (Initial weight)}] / \textit{days}$$

$$\textit{Condition factor (CF)} = W / L^3$$

$$\textit{Feed conversion ratio (FCR)} = \textit{Feed consumed} / \textit{Body weight gain}$$

$$\textit{Hepatosomatic index} = \textit{Liver weight} / \textit{Final weight}$$

$$\textit{Viscerosomatic index} = \textit{Visceral weight} / \textit{Final weight}$$

where, *Ln* is the natural logarithm, *W* is weight and *L* is total length (cm) and *day* is the culture periods of fish.

Mucus collection. Skin mucus was collected following the method described by Subramanian *et al*.[18] Briefly, nine fish were randomly caught from each treatment and placed in zipper storage bags, containing 10.00 mL of 50.00 mM sodium chloride. The bags were gently shaken for 2 - 3 min, and after collecting the mucus, the fish were returned to the recovery tanks. The collected mucus was centrifuged at 1,500 *g* for 10 min at 4.00 ˚C to remove debris, and the supernatant was carefully transferred to the sterile microtubes. The samples were then immediately stored at – 80.00 ˚C until biochemical analyses.

Blood sampling. For blood sampling, fish were wiped and cleaned to avoid mucus mixing into the blood. Then, 15 fish were randomly caught from each treatment, and blood was collected from the caudal vein using a plastic syringe. For the hematological assays using heparinized syringes, blood was collected into the Eppendorf tubes being pre-coated with heparin (Chemidarou, Tehran, Iran)

to prevent blood coagulation and facilitate hematological analysis. For serum separation, the blood was collected with 3.00 mL non-heparinized plastic test tubes. After blood was coagulated, tubes were centrifuged at 3,500 rpm for 15 min (Sigma, Deisenhofen, Germany), and the serum was stored below – 20.00 ˚C.[19]

Assessment of hematological parameters. The levels of red blood cells (RBCs) and white blood cells (WBCs) were determined by hemocytometers according to the Benjamine method.[20] Hemoglobin (Hb) content was determined by UV-Vis spectrophotometer (7200; Jenway, Staffordshire, UK) using Drabkin's reagent according to the van Kampen and Zijlstra.[21] The hematocrit (Hct; %) was determined by filling Hct capillary tubes, being centrifuged (8,400 *g*; 10 min) using a micro-Hct centrifuge. The Hct values were recorded using centrifuge combo reader. The leukocytic count was determined through an indirect method using blood smear stained with May-Grunwald-Giemsa, according to the method described by Lucky[22] for differential leukocytic count, and absolute values for each type of cell were calculated according to Schalm.[23] The mean corpuscular volume, mean corpuscular Hb (MCH), and MCH concentration (MCHC) were determined using the following formulas:

$$\text{Mean corpuscular volume} = (\text{Hct}/\text{RBCs}) \times 10$$

$$\text{MCH} = \text{Hb} \times 10/\text{RBCs}$$

$$\text{MCHC} = (\text{Hb}/\text{Hct}) \times 100$$

Assessment of immunological indices. The total protein concentration (g dL^{-1}) in serum and mucus samples was determined using 20.00 µL of each sample with Pars Azmoon (Tehran, Iran) diagnostic kits based on the Biuret method.[24] Lysozyme activity (U mL^{-1} *per* min) was measured by a turbidimetric spectrophotometric assay (Libra S12; Biochrom, Cambridge, UK) using *Micrococcus lysodeikticus* as a substrate. The lyophilized *M. lysodeikticus* cells were re-suspended in 0.04 M potassium phosphate buffer, and the absorbance of the bacterial suspension was adjusted to 0.60 - 0.70 at 450 nm *vs.* buffer blank. In each reaction, 250 µL of the mucus and 25.00 µL of the serum supernatant were mixed with 175 µL of *M. lysodeikticus* suspension (plus buffer to reach final volume), and the decrease in absorbance at 450 nm was followed over 10 min. One unit of lysozyme activity was defined as the amount of enzyme causing a reduction of 0.001 absorbance units *per* min at 25.00 ˚C.[25] The total immunoglobulin (IgM; mg mL^{-1}) concentration was measured using the method described by Siwicki.[25] In this procedure, 100 µL of the mucus and serum samples were mixed with 100 µL of 12.00% polyethylene glycol (Sigma-Aldrich, St. Louis, USA) and incubated for 2 hr to precipitate the immunoglobulin molecules. Subsequently, the mixture was centrifuged at 5,000 rpm for 5 min at 4.00 ˚C. The assessment of liver enzymes was conducted using the quantitative detection kit from Pars Azmoon Co. (Tehran, Iran) through a photometric method, utilizing a sample volume of 100 µL.[26] The alkaline phosphatase (ALP; U L^{-1}) enzyme measurement followed Kinetic photometric method optimized with the International German Society of Clinical Chemistry (DGKC) method. For aspartate aminotransferase (AST; U L^{-1}) enzyme, the measurements were carried out based on the method recommended by the International Federation of Clinical Chemistry and Laboratory Medicine.

Statistical analysis. The data were analyzed using SPSS Software (version 23.0; IBM Corp., Armonk, USA). The normality of data distribution was verified using the Kolmogorov-Smirnov test. Differences among treatments were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference *post hoc* test to compare means. Statistical significance was accepted at $p < 0.05$. All data are expressed as mean ± standard deviation. The optimal dietary inclusion level of ZnO-NPs was determined using a quadratic polynomial regression model.

Results

Table 1 illustrates that dietary supplementation with ZnO-NPs significantly influenced the growth performance of *C. carpio* juveniles ($p < 0.05$). At the treatment of 50.00 mg kg^{-1} of ZnO-NPs exhibited the highest final length, FW, weight gain, SGR, optimal FCR, and condition factor ($p < 0.05$). The viscerosomatic index differed significantly among treatments, being the highest at 100 mg kg^{-1} and the lowest at treatment of 10.00 mg kg^{-1} ($p < 0.05$), whereas hepatosomatic index and survival rate showed no significant differences ($p > 0.05$). Moreover, quadratic regression analyses estimated optimal dietary ZnO-NPs levels for maximizing SGR and FCR at 53.15 mg kg^{-1} and 52.25 mg kg^{-1}, respectively (Fig. 2). The ANOVA results of quadratic polynomial regression are provided in Table 2.

According to the Table 3, dietary ZnO-NPs significantly influenced hematological parameters of *C. carpio* juveniles ($p < 0.05$). The highest WBC count was observed in the control group, while fish fed 10.00, 50.00 and 100 mg kg^{-1} ZnO-NPs exhibited significantly lower values ($p < 0.05$). In contrast, RBC counts did not differ significantly among treatments ($p > 0.05$). The Hb concentration was significantly higher in fish fed 50.00 mg kg^{-1} ZnO-NPs compared to the control ($p < 0.05$), whereas Hct, mean corpuscular volume, and MCH remained statistically unchanged ($p > 0.05$). The MCHC increased significantly in the ZnO-NPs-supplemented groups, with the highest value at 50.00 mg kg^{-1} ($p < 0.05$). Regarding leukocyte differentials, lymphocyte levels peaked at 50.00 mg kg^{-1}, while monocytes were the lowest in this group and the highest in fish fed 100 mg kg^{-1} ZnO-NPs ($p < 0.05$). Neutrophil and eosinophil counts showed no significant differences among treatments ($p > 0.05$).

Table 1. Growth performance of juvenile *Cyprinus carpio* fed diets supplemented with different levels of zinc oxide nanoparticles (ZnO-NPs).

Parameters	Control	ZnO-NPs concentration (mg kg^{-1})		
		10.00	50.00	100
Final length (cm)	10.76 ± 0.06^{b}	11.45 ± 0.23^{ab}	11.74 ± 0.15^{a}	11.32 ± 0.45^{ab}
Final growth (g)	23.80 ± 0.44^{b}	27.90 ± 2.04^{ab}	30.14 ± 1.07^{a}	27.11 ± 2.82^{ab}
Weight gain (g)	11.47 ± 0.44^{b}	15.57 ± 2.19^{ab}	17.81 ± 0.98^{a}	14.78 ± 2.36^{ab}
FCR	3.81 ± 0.34^{a}	2.87 ± 0.40^{ab}	2.55 ± 0.28^{b}	3.17 ± 0.68^{ab}
SGR (% *per* day)	1.09 ± 0.03^{b}	1.35 ± 0.12^{ab}	1.48 ± 0.06^{a}	1.30 ± 0.17^{ab}
CF (% *per* day)	2.21 ± 0.29^{b}	2.43 ± 0.65^{ab}	2.56 ± 0.57^{a}	2.39 ± 0.84^{ab}
VSI (%)	6.02 ± 0.43^{ab}	4.35 ± 0.35^{b}	6.48 ± 0.57^{ab}	7.67 ± 1.71^{a}
HSI (%)	1.66 ± 0.20^{a}	1.39 ± 0.26^{a}	2.05 ± 0.65^{a}	2.21 ± 0.25^{a}
SR (%)	88.00 ± 12.00^{a}	93.33 ± 4.61^{a}	94.66 ± 4.61^{a}	94.66 ± 2.30^{a}

FCR: Feed conversion ratio; SGR: Specific growth rate; CF: Condition factor; VSI: Viscerosomatic index; HSI: Hepatosomatic index; SR: Survival rate.

^{ab} Different letters in each row indicate a significant difference ($p < 0.05$).

Table 2. Quadratic polynomial regression to optimize dietary zinc oxide nanoparticles concentration in *Cyprinus carpio*.

Parameters	Sources	Sum of squares	df	Mean square	F	Significance
SGR	Regression	0.235	2	0.118	9.919	0.005
	Residual	0.107	9	0.012		
FCR	Regression	2.583	2	1.291	7.000	0.015
	Residual	1.660	9	0.184		

FCR: Feed conversion ratio; SGR: Specific growth rate, df: Degree of freedom.

Table 3. Hematological parameters of juvenile *Cyprinus carpio* fed diets containing different levels of zinc oxide nanoparticles (ZnO-NPs).

Parameters	Control	ZnO-NPs concentration (mg kg^{-1})		
		10.00	50.00	100
WBCs (×10^{3} cell mm^{-3})	4,400.00 ± 100.00^{a}	3,416.00 ± 246.00^{b}	3,316.00 ± 160.00^{b}	3,700.00 ± 50.00^{b}
RBCs (cell mm^{-3})	1,480.00 ± 18.02^{a}	1,483.00 ± 10.40^{a}	1,563.00 ± 70.23^{a}	1,495.00 ± 37.84^{a}
Hemoglobin (g dL^{-1})	5.82 ± 0.07^{b}	5.88 ± 0.04^{ab}	6.23 ± 0.28^{a}	5.91 ± 0.10^{ab}
Hematocrit (%)	46.60 ± 0.57^{a}	46.30 ± 0.57^{a}	48.30 ± 2.08^{a}	46.30 ± 0.57^{a}
MCV (fL)	315.00 ± 2.64^{a}	312.00 ± 1.52^{a}	309.00 ± 2.51^{a}	309.00 ± 3.78^{a}
MCH (pg)	39.30 ± 0.55^{a}	39.60 ± 0.15^{a}	39.80 ± 0.05^{a}	39.50 ± 0.28^{a}
MCHC (g dL^{-1})	12.47 ± 0.07^{b}	12.69 ± 0.07^{ab}	12.90 ± 0.13^{a}	12.76 ± 0.07^{ab}
Neutrophil (%)	15.00 ± 2.00a^{a}	13.33 ± 1.52^{a}	12.66 ± 1.15^{a}	15.66 ± 0.57^{a}
Lymphocyte (%)	79.66 ± 2.51^{ab}	81.00 ± 3.00^{ab}	84.00 ± 1.00^{a}	77.66 ± 2.08^{b}
Monocyte (%)	5.33 ± 0.57^{ab}	5.33 ± 1.52^{ab}	3.33 ± 0.57^{b}	6.00 ± 1.00^{a}
Eosinophil (%)	0.00 ± 0.00^{a}	0.33 ± 0.01^{a}	0.00 ± 0.00^{a}	0.66 ± 0.05^{a}

RBCs: Red blood cells; WBCs: White blood cells; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration.

^{ab} Different letters in each row indicate a significant difference ($p < 0.05$).

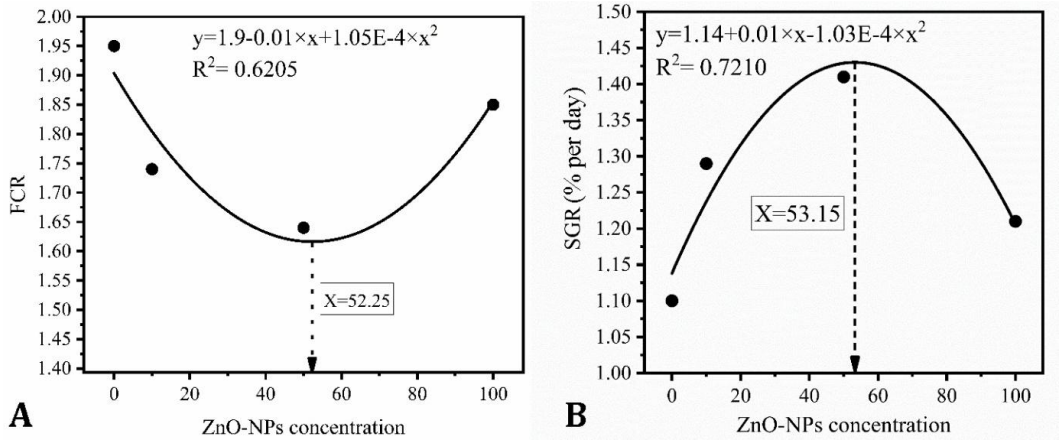

Fig. 2. Quadratic polynomial regression to optimize dietary zinc oxide nanoparticles (ZnO-NPs) concentration in *Cyprinus carpio* on **A)** FCR: Feed conversion ratio, and **B)** SGR: Specific growth rate.

Table 4 demonstrates that dietary ZnO-NPs supplementation significantly affected the serum biochemical and immunological parameters of *C. carpio* juveniles ($p < 0.05$). Fish fed 50.00 mg kg^{-1} ZnO-NPs showed the highest total protein, IgM, lysozyme activity, alternative complement hemolytic 50.00% activity, and serum Zn concentration ($p < 0.05$). The AST activity was significantly elevated only in fish fed 10.00 mg kg^{-1} ZnO-NPs ($p < 0.05$), whereas ALP activity decreased markedly in all ZnO-NPs-supplemented groups compared to the control ($p < 0.05$).

As shown in Table 5, dietary ZnO-NPs supplementation significantly affected the mucus biochemical and immunological parameters of *C. carpio* juveniles ($p < 0.05$). Fish fed 10.00 mg kg^{-1} ZnO-NPs exhibited the highest total protein, IgM, lysozyme activity, and ALP levels in skin mucus, being significantly higher than those of the control and other treatments ($p < 0.05$).

Discussion

Our results indicated that a concentration of 50.00 mg kg^{-1} ZnO-NPs significantly improved *C. carpio* juveniles' weight, SGR, and FCR, suggesting a positive impact on fish growth and health. The survival rates reaching approximately 95.00% in the 50.00 mg kg^{-1} concentration indicate that these concentrations positively affect fish viability, corresponding with findings from other studies where lower concentrations of ZnO-NPs were associated with improved survival rates due to the enhanced physiological conditions.[27]

The observed decrease in WBCs in *C. carpio* received diets supplemented with ZnO-NPs suggests a potential immune-modulatory or mild suppressive effect of these NPs on hematopoietic activity.[28-30] Previous studies reported that exposure to ZnO-NPs can reduce leukocyte numbers due to the oxidative stress, accumulation of NPs in hematopoietic organs, such as the liver and spleen, and altered cytokine regulation. Tuayen *et al.*[28] showed that different dosages of ZnO-NPs in carps feed caused significant decreases in WBC counts, indicating immune system modulation without severe stress or toxicity. Rasheed *et al.*[29] confirmed dose-dependent hematological changes, including lowered WBCs in common carp exposed to ZnO-NPs. In contrast, the RBCs results indicated the favorable biological potential of ZnO-NPs, aligning with previous studies that have demonstrated their ability to exert significant physiological effects on RBCs, which may be either beneficial or detrimental depending on their size and concentration.[31] However, Hb was the highest in the treatment containing 50.00 mg kg^{-1} ZnO-NPs, suggesting an adaptive response to maintain oxygen transport.[32] The increase in Hb levels up to 50.00 mg kg^{-1} of the Zn-NPs diet may be attributed to the potent anti-oxidant properties of NPs, which help maintain cell stability and integrity within organisms, thus guarding against hemolysis. However, NPs can become toxic at higher levels, leading to adverse effects.[33] Kumar *et al.*[34] showed that Hb levels increased significantly in response to 2.00 - 6.00 mg kg^{-1} of ZnO-NPs in the *Pangasianodon hypophthalmus* diet, suggesting an adaptive mechanism to enhance oxygen transport under stressful conditions, even when RBC counts were compromised.

Previous studies have demonstrated that Zn deficiency can impair immune function, leading to decreased production of complement proteins and reduced immune

Table 4. Serum biochemical and immunological parameters of juvenile *Cyprinus carpio* fed diets supplemented with different levels of zinc oxide nanoparticles (ZnO-NPs).

Parameters	Control	ZnO-NPs concentration (mg kg^{-1})		
		10.00	50.00	100
Total protein (mg mL^{-1})	2.57 ± 0.03^{b}	2.82 ± 0.01^{b}	3.14 ± 0.13^{a}	2.81 ± 0.17^{b}
Immunoglobulin M (mg mL^{-1})	15.09 ± 0.20^{c}	16.52 ± 0.04^{b}	17.64 ± 0.24^{a}	15.20 ± 0.23^{c}
Lysozyme (U mL min^{-1})	35.80 ± 0.79^{c}	39.03 ± 0.72^{b}	41.33 ± 0.86^{a}	37.80 ± 0.78^{bc}
ACH_{50} (%)	123.00 ± 1.00^{b}	128.33 ± 1.52^{a}	130.66 ± 2.08^{a}	129.00 ± 1.00^{a}
Aspartate aminotransferase (U L^{-1})	132.66 ± 1.52^{b}	152.00 ± 5.00^{a}	137.00 ± 5.56^{b}	128.66 ± 4.93^{b}
Alkaline phosphatase (U L^{-1})	102.03 ± 7.56^{a}	84.23 ± 2.30^{b}	87.10 ± 1.67^{b}	93.93 ± 1.98^{ab}
Zinc (µg dL^{-1})	172.00 ± 3.00^{b}	175.00± 2.00^{b}	182.33 ± 2.51^{a}	170.00 ± 2.00^{b}

ACH_{50}: Alternative complement hemolytic 50.00% activity.
^{abc} Different letters in each row indicate a significant difference ($p < 0.05$).

Table 5. Mucus biochemical and immunological parameters of juvenile *Cyprinus carpio* exposed to different dietary levels of zinc oxide nanoparticles (ZnO-NPs).

Parameters	Control	ZnO-NPs concentration (mg kg^{-1})		
		10.00	50.00	100
Total protein (mg mL^{-1})	50.46 ± 0.66^{b}	59.06 ± 1.50^{a}	52.37 ± 0.01^{b}	50.90 ± 0.36^{b}
Immunoglobulin M (mg mL^{-1})	0.34 ± 0.00^{b}	0.46 ± 0.06^{a}	0.35 ± 0.01^{b}	0.34 ± 0.001^{b}
Lysozyme (U mL^{-1} *per* min)	54.66 ± 2.55^{b}	68.13 ± 2.01^{a}	58.66 ± 2.27^{b}	55.50 ± 0.75^{b}
Alkaline phosphatase (U L^{-1})	32.13 ± 1.28^{d}	80.76 ± 3.02^{a}	47.43 ± 2.05^{b}	40.10 ± 1.30^{c}

$^{a-d}$ Different letters in each row indicate a significant difference ($p < 0.05$).

responsiveness. Conversely, adequate Zn concentration is associated with enhanced immune function and increased infection resistance. The findings from this study align with these observations, suggesting that ZnO-NPs may provide a bioavailable source of zinc, supporting the activation of the complement system.[35]

Compared to control group, the total protein concentration in mucus and blood serum was higher in all treatments exposed to ZnO-NPs. The highest total protein concentration was observed in the mucus tissue of fish treated with 10.00 mg kg^{-1} of ZnO-NPs, while the blood serum exhibited 50.00 mg kg^{-1} of ZnO-NPs. This concentration may enhance protein synthesis or reduce protein degradation in low ZnO-NP concentrations compared to the higher concentrations.[36] It was indicated that the immunity parameters of *Clarias batrachus* were positively influenced by the supplementation of ZnO-NPs.[37] According to the Hasaballah *et al.*,[38] ZnO-NPs result in enhanced cellular and humoral immunological parameters in tilapia (*Oreochromis mossambicus*) by increasing resistance against the pathogens (*Aeromonas hydrophila* and *Vibrio parahaemolyticus*).

Mucosal lysozyme activity and serum IgM levels were notably elevated in treatments containing 10.00 and 50.00 mg kg^{-1} ZnO-NPs, respectively. Low levels of ZnO-NPs may promote antibody production and the ability to combat microbial infections, thereby strengthening the fish defence mechanisms.[39] Our finding aligns with previous studies that demonstrated enhanced immune responses in fish exposed to appropriate concentrations of NPs.[31,34,39] Sherif *et al.*,[39] have demonstrated that including 30.00 and 60.00 µg *per* g ZnO-NPs in the diet of *Oreochromis niloticus* significantly enhances the immune response and increases resistance to *A. hydrophila* infection.

The alternative complement hemolytic 50.00% activity level significantly increased in all ZnO-NPs-treated groups compared to the control, suggesting that ZnO-NPs may stimulate the complement system. Zinc, as an essential element in numerous biochemical pathways, supports immune function by activating complement-related enzymes and proteins.[35,40] Moreover, NPs, such as ZnO-NPs, can trigger complement activation through mechanisms affected by their physicochemical properties, including particle size and surface charge, thereby enhancing opsonization and immune responses.[41] In contrast, additional Zn does not lead to further increases in complement activity.[42] Kim *et al.*,[42] have demonstrated that optimal Zn concentration is necessary for effective immune responses, and exceeding these levels may not provide additional benefits. This phenomenon could be attributed to several factors, including (1) the complement system may reach a saturation point where adding Zn does not lead to further increases in activity. Once optimal Zn concentration is achieved, additional supplementation yields no additional benefits, (2) each organism has physiological limits regarding how much nutrient it can utilize effectively. Excessive concentrations may not be necessary for optimal immune function and could lead to diminishing returns, and (3) individual variability in response to ZnO-NPs among subjects may also contribute to the lack of significant differences between treatments. Furthermore, genetic predisposition, health condition, and environmental factors can affect the response of organisms to NPs exposure.[43,44] Also, Xu *et al.*,[35] reported that ZnO-NPs could prime protective immune responses in *Galleria mellonella*, indicating their potential role in enhancing host immunity by activating immune pathways.

Both AST and ALP are essential enzymes in clinical diagnostics. The AST is primarily associated with liver function, as it is released into the bloodstream when hepatocytes are damaged. Its elevated levels can reflect damage to multiple organ systems, but it is primarily used as a marker for liver health.[45] However, ALP is critical in various physiological processes, including digestion and bone mineralization. Elevated ALP levels can indicate issues related to the liver, bile ducts, or bone disorders.[34] The increased blood serum AST activity at treatments containing ZnO-NPs, especially at low concentrations, suggests potential liver stress or damage due to the ZnO-NPs exposure. Studies have demonstrated that exposure to ZnO-NPs leads to significant elevations in liver enzymes, such as AST, indicating liver damage.[46]

Furthermore, the hepatotoxic effects of ZnO-NPs have been attributed to their ability to generate reactive oxygen species, leading to cellular damage and inflammation within the liver. This oxidative stress is a critical factor in the pathogenesis of liver injury related to the NPs exposure, reinforcing that elevated AST levels can be a biomarker for liver stress or damage.[47] Studies indicated that after five days of treatment with ZnO-NPs at a concentration of 10.00 mg kg^{-1}, there was a significant increase in AST levels in the livers of male rats, suggesting hepatic dysfunction and morphological changes in the liver tissue. Additionally, exposure to ZnO-NPs led to oxidative stress in the liver tissue and increased levels of AST in female rats, further supporting the potential for liver damage.[48] Another study highlighted that sub-chronic exposure to ZnO-NPs caused biochemical alterations, including elevated serum AST levels in Sprague-Dawley male rats, and noted that higher concentrations of NPs were linked to the metabolic changes and potential liver impairment.[49]

Serum ALP levels decreased in all ZnO-NPs-treated groups compared to the control, showing an upward trend at higher concentrations that may reflect metabolic alterations or liver dysfunction. In contrast, mucus ALP levels significantly increased in all treatments but declined at higher ZnO-NP doses, indicating enhanced metabolic activity at moderate levels and possible toxicity at excessive concentrations.[50] Similarly, Al-Ragi *et al.*,[50]

reported that ZnO-NPs (100 - 200 mg kg^{-1}) exposure in male albino mice induced hepatic dysfunction and histological damage, including hepatocyte necrosis and membrane destruction, confirming the potential hepatotoxicity of ZnO-NPs accumulation in biological systems.

The more elevated accumulation of Zn in the serum of fish subjected to 50.00 mg kg^{-1} of ZnO-NPs suggests that these fish effectively absorb and utilize Zn from the ZnO-NPs. The Zn is crucial for numerous biological functions, including enzyme activity and immune function. Therefore, dietary supplementation with 50.00 mg kg^{-1} ZnO-NPs can enhance Zn bioavailability, potentially improving health outcomes.[8,37]

Feeding juvenile *C. carpio* with 50.00 mg kg^{-1} ZnO-NPs led to significant increases in FW, length, SGR, FCR, and survival rate (about 95.00%). This group exhibited the lowest WBC count and monocyte percentage, yet the highest RBCs, Hb, MCHC, and lymphocyte levels. Serum analysis showed the highest total protein, IgM, lysozyme, and Zn in fish fed 50.00 mg kg^{-1} ZnO-NPs, while mucus samples from the 10.00 mg kg^{-1} group had the greatest total protein, IgM, lysozyme, and ALP. These results suggest that dietary ZnO-NPs (10.00 - 50.00 mg kg^{-1} can significantly improve growth and immune parameters in juvenile *C. carpio*, though caution is advised at higher concentrations due to the potential health risks.

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Conflict of interest

The authors declare no conflict of interest.

References

1. Amara S, Ben-Slama I, Mrad I, et al. Acute exposure to zinc oxide nanoparticles does not affect the cognitive capacity and neurotransmitters levels in adult rats. Nanotoxicology 2014; 8(Suppl 1): 208-215.
2. Ahmadifard N, Ghaderpour S, Agh N, et al. Long-term incorporation of Selenium and Zinc in microalgae *Isochrysis galbana* and *Nannochloropsis oculata* and its effects on rotifer. Aquac Res 2022; 53(8): 3188-3201
3. Wang J, Wang WX. Optimal dietary requirements of zinc in marine medaka *Oryzias melastigma*: importance of daily net flux. Aquaculture 2015; 448: 54-62.
4. Feng M, Wang ZS, Zhou AG, et al. The effects of different sizes of nanometer zinc oxide on the proliferation and cell integrity of mice duodenum-epithelial cells in primary culture. Pak J Nutr 2009; 8(8): 1164-1166.
5. Nematzadeh K, Ahmadifard N, Samadi N, et al. The effects of zinc-enriched *Saccharomyces cerevisiae* on the growth and mineral composition of marine rotifer, *Brachionus plicatilis*. Int J Aquat Biol 2018; 6(2): 88-94.
6. Khosravi-Katuli K, Prato E, Lofrano G, et al. Effects of nanoparticles in species of aquaculture interest. Environ Sci Pollut Res Int 2017; 24(21): 17326-17346.
7. Hanifi E, Ahmadifard N, Atashbar B, et al. Effects of zinc oxide nanoparticles on photosynthetic pigments, zinc accumulation, and activity of antioxidant enzymes of *Dunalilla salina* [Persian]. Aquat Anim Nutr 2023; 8(4): 31-42.
8. Motelica L, Vasile BS, Ficai A, et al. Antibacterial activity of zinc oxide nanoparticles loaded with essential oils. Pharmaceutics 2023; 15: 2470. doi: 10.3390/ pharmaceutics15102470.
9. Zhou XQ, Hayat Z, Zhang DD, et al. Zinc oxide nanoparticles: synthesis, characterization, modification, and applications in food and agriculture. Processes 2023; 11: 1193. doi: 10.3390/ pr11041193.
10. Sadeghi S, Mousavi-Sabet H, Hedayati A, et al. Copper-oxide nanoparticles effects on goldfish (*Carassius auratus*): lethal toxicity, haematological, and biochemical effects. Vet Res Commun 2024; 48(3): 1611-1620.
11. FAO. The State of World Fisheries and Aquaculture 2022. Towards Blue Transformation. Rome, 2022.
12. Kakakhel MA, Wu F, Sajjad W, et al. Long-term exposure to high-concentration silver nanoparticles induced toxicity, fatality, bioaccumulation, and histological alteration in fish (*Cyprinus carpio*). Environ Sci Eur 2021; 33: 14. doi: 10.1186/s12302-021-00453-7.
13. Hashim AG, Al Fatlawy YFK. Study the acute toxicity of zinc oxide nanoparticles on *Cyprinus carpio's* tissues. JWSM 2023; 16(2): 26-39.
14. Tawfik M, Moustafa M, Abumourad IMK, et al. Evaluation of nano zinc oxide feed additive on tilapia growth and immunity. In Proceedings: 15th International Conference on Environmental Science and Technology. Rhodes, Greece 2017; 1-9.
15. Deepa S, Murugananthkumar R, Raj Gupta Y, et al. Effects of zinc oxide nanoparticles and zinc sulfate on the testis of common carp, *Cyprinus carpio*. Nanotoxicology 2019; 13(2): 240-257.
16. Sharyfpoor A, Soltani M, Abdullahi H, et al. Anaesthetic effect of clove (*Eugenia caryophyllata*) essential oil under different pH and temperature conditions in common carp (*Cyprinus carpio*) [Persian]. Iran Fish Sci J 2003; 11(4): 59-74.
17. Shepherd CJ, Bromage NR. Intensive fish farming. 1st ed. New Jersey, USA: Wiley-Blackwell 1992; 325-345.
18. Subramanian S, MacKinnon SL, Ross NW. A comparative study on innate immune parameters in

the epidermal mucus of various fish species. Comp Biochem Physiol B Biochem Mol Biol 2007; 148(3): 256-263.
19. Blaxhall PC, Daisley KW. Routine haematological methods for use with fish blood. J Fish Biol 1973; 5(6): 771-781.
20. Benjamin, MM. Outline of veterinary clinical pathology. 1st ed. New Delhi, India: Kalyani 1985; 330-350
21. van Kampen EJ, Zijlstra WG. Standardization of hemoglobinometry. II. The hemiglobincyanide method. Clin Chim Acta 1961; 6: 538-544.
22. Lucky Z. Methods for the diagnosis of fish diseases. New Delhi, India: Amerind Pub 1977; 50-100
23. Schalm O. Hematology of Birds. In: Jain NC (Ed). Schalm's Veterinary Hematology. 4th ed. Philadelphia, USA: Lea & Febiger 1986; 257.
24. Gornall AG, Bardawill CJ, David MM. Determination of serum proteins by means of the Biuret reaction. J Biol Chem 1949; 177(2): 751-766.
25. Siwicki AK, Anderson DP, Rumsey GL. Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis. Vet Immunol Immunopathol 1994; 41(1-2): 125-139.
26. Shahsavani D, Mohri M, Gholipour Kanani H. Determination of normal values of some blood serum enzymes in *Acipenser stellatus* Pallas. Fish Physiol Biochem 2010; 36(1): 39-43.
27. Lin Y, Wang J, Dai H, et al. Salinity moderated the toxicity of zinc oxide nanoparticles (ZnO NPs) towards the early development of *Takifugu obscurus*. Int J Environ Res Public Health 2023; 20(4): 3209. doi: 10.3390/ijerph20043209.
28. Tuayen MJ, AL-Humairi KO, Fadhel AA. Impact of iron oxide and zinc oxide nanoparticles supplementation on some biochemical and hematological parameters in *Cyprinus Carpio* L. EJAS 2025; 17(1): 514-521.
29. Rasheed A, Iqbal KJ, Safdar A, et al. Toxicological effects of zinc oxide nanoparticles on hemato-biochemical profile of common carp (*Cyprinus carpio*). J King Saud Univ Sci 2023; 35(7): 102835. doi: 10.1016/j.jksus.2023.102835.
30. Khan GB, Akhtar N, Khan MF, et al. Toxicological impact of zinc nano particles on tilapia fish (*Oreochromis mossambicus*). Saudi J Biol Sci 2022; 29(2): 1221-1226.
31. Chahardeh Baladehi E, Hedayati SAA, Kolangi Miandar H, et al The hematological improvement of rainbow trout (*Oncorhynchus mykiss*) during dietary supplementation with vitamin C after exposure to zinc nanoparticles. Iran J Fish Sci 2017; 16(1): 162-169.
32. Rahman HS, Othman HH, Abdullah R, et al. Beneficial and toxicological aspects of zinc oxide nanoparticles in animals. Vet Med Sci 2022; 8(4): 1769-1779.
33. Khan KU, Zuberi A, Nazir S, et al. Effects of dietary selenium nanoparticles on physiological and biochemical aspects of juvenile *Tor putitora*. Turk J Zool 2016; 40(5): 704-712.
34. Kumar N, Singh DK, Chandan NK, et al. Nano-zinc enhances gene regulation of non-specific immunity and antioxidative status to mitigate multiple stresses in fish. Sci Rep 2023; 13: 5015. doi: 10.1038/s41598-023-32296-y.
35. Xu MN, Li L, Pan W, et al. Zinc oxide nanoparticles prime a protective immune response in *Galleria mellonella* to defend against Candida albicans. Front Microbiol 2021; 12: 766138. doi: 10.3389/fmicb.2021.766138.
36. Beghin M, Lambert J, Sturve J, et al. Immuno-modulatory effects of single and combined exposure to ZnO and TiO_2 nanoparticles on rainbow trout challenged with *Aeromonas salmonicida achromogenes*. Aquat Toxicol 2024; 272: 106981. doi: 10.1016/j.aquatox.2024.106981.
37. Jewel AS, Haque A, Akter N, et al. Effects of dietary supplementation of Zn-nanoparticles on the growth performance and nutritional quality of Asian catfish, *Clarias batrachus*. Front Sustain Food Syst 2024; 8: 1410557. doi: 10.3389/fsufs.2024.1410557.
38. Hasaballah AI, El-Naggar HA, Abd-El Rahman IE, et al. Surf redfish-based ZnO-NPs and their biological activity with reference to their non-target toxicity. Mar Drugs 2023; 21(8): 437. doi: 10.3390/md21080437.
39. Sherif AH, Abdelsalam M, Ali NG, et al. Zinc oxide nanoparticles boost the immune responses in *Oreochromis niloticus* and improve disease resistance to *Aeromonas hydrophila* infection. Biol Trace Elem Res 2023; 201(2): 927-936.
40. La-Beck NM, Islam MR, Markiewski MM. Nanoparticle-induced complement activation: implications for cancer nanomedicine. Front Immunol 2021; 11: 603039. doi: 10.3389/fimmu.2020.603039.
41. Ray P, Haideri N, Haque I, et al. The impact of nanoparticles on the immune system: a gray zone of nanomedicine. J Immunological Sci 2021; 5(1): 19-33
42. Kim CS, Nguyen HD, Ignacio RM, et al. Immunotoxicity of zinc oxide nanoparticles with different size and electrostatic charge. Int J Nanomedicine 2014; 9(Suppl 2): 195-205.
43. Mohammadi S, Ahmadifard N, Atashbar B, et al. Long-term effect of zinc oxide nanoparticles on population growth, reproductive characteristics and zinc accumulation of marine rotifer, *Brachionus plicatilis*. Int J Aquat Biol 2021; 9(5): 333-343.
44. Sharma P, Jang NY, Lee JW, et al. Application of ZnO-based nanocomposites for vaccines and cancer immunotherapy. Pharmaceutics 2019; 11: 493. doi: 10.3390/pharmaceutics11100493.
45. McGill MR. The past and present of serum aminotransferases and the future of liver injury biomarkers. EXCLI J 2016; 15: 817-828.
46. Pei X, Jiang H, Xu G, et al. Lethality of zinc oxide

nanoparticles surpasses conventional zinc oxide via oxidative stress, mitochondrial damage and calcium overload: a comparative hepatotoxicity study. Int J Mol Sci 2022; 23(12): 6724. doi: 10.3390/ijms23126724.
47. Aboulhoda BE, Abdeltawab DA, Rashed LA, et al. Hepatotoxic effect of oral zinc oxide nanoparticles and the ameliorating role of selenium in rats: a histological, immunohistochemical and molecular study. Tissue Cell 2020; 67: 101441. doi: 10.1016/j.tice.2020.101441.
48. Hosseini SM, Amani R, Moshrefi AH, et al. Chronic zinc oxide nanoparticles exposure produces hepatic and pancreatic impairment in female rats. Iran J Toxicol 2020; 14(3): 145-153.
49. Ramadan AG, Yassein AA, Eissa EA, et al. Biochemical and histopathological alterations induced by subchronic exposure to zinc oxide nanoparticle in male rats and assessment of its genotoxicity. J Umm Al-Qura Univ Appl Sci 2022; 8: 41-49.
50. Al-Ragi MJ, Karieb SS, Fathallah N, et al. Effect of zinc oxide nanoparticles on liver functions in albino mice. Cureus 2024; 16(2): e54822. doi: 10.7759/cureus.54822.