The hematological improvement of rainbow trout (*Oncorhynchus mykiss*) during dietary supplementation with vitamin C after exposure to zinc nano-particles

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Abstract

The aim of this study was to examine the adverse effects of zinc nanoparticles on hematological indices of trout and investigate the improvement of these indices after vitamin C treatments. This study assesses the protective role of vitamin C in fish exposed to ZnO NPs. Two concentrations of ZnO-NPs (40 and 80 mg L^{-1}) and two doses of vitamin C (400 and 800 mg per kg of feed) were used to treat 162 specimens of *Oncorhynchus mykiss*. No mortality was observed during the test. After 5 and 10 days of exposure, hematological data were analyzed according to routine clinical methods. Statistical analysis showed significant changes in WBCs and RBC on day 10 (p<0.05). Values for HT and MCH were significantly higher in treatment 2 (normal diet+40 mg L^{-1} ZnO-NPs) and 9 (800 mg/kg Vit + 80 mg L^{-1} ZnO-NPs), and lower in treatment 3 (normal diet+80 mg L^{-1} ZnO-NPs) in comparison with the control group (normal diet+0 mg L^{-1} ZnO-NPs) (P<0.05). No significant differences of MCV and MCHC were observed (p>0.05), while significant increase in neutrophils and monocytes, and decrease in lymphocyte cells were recorded (p<0.05). ZnO-NPs stimulated the immune system of *O. mykiss*, but this effect did not have any lethality on this species at 40 and 80 mg L^{-1}. Vitamin C in different concentrations could help to prevent rainbow trout from the toxic effects of this nano metal.

Keywords: Fish, Hematology, Nano particle, Toxicity, Vitamin C.

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Introduction

Zinc, an essential element, is one of the most common heavy metal pollutants which are wildly used in industrial products. In addition, today with increasing nanotechnology, Zinc oxide Nanoparticles (ZnO-NPs) are one of the most widely used nano materials due to their unique advantages in industrial and medicinal products. The potential effects of ZnO-NPs on aquatic ecosystems have attracted special attentions (Collins *et al*., 2012). The adverse effects of zinc to fish such as changes in ventilatory and heart physiology, depressive effect on tissue respiration leading to death by hypoxia and adverse effects on hemathological indices were cited by many researchers (Kori-Siakpere and Ubogu, 2008). Ascorbic acid (AA) known as vitamin C; is an essential micronutrient for many fish species. Vitamin C cannot synthesize D-glucose due to the lack of L-gulonolactone oxidase that is responsible for the synthesis of vitamin C de novo. The important role of vitamin C in iron metabolism and hematology and immune response of fish is well known (Ibrahem *et al*., 2010). *Oncorhynchus mykiss* (Rainbow trout) belongs to the family Salmonidae, native to cold-water tributaries of the Pacific Ocean in Asia and North America. Although, many researchers studied the effects of metal nanoparticles on blood parameters of *O. mykiss* (Boyle *et al.*, 2013; Khabbazi *et al*., 2015) data on hematological indices of vitamin C supplementation after exposure to metal nanoparticles in limited for this species. Therefore, the aim of this study is to examine the adverse effects of zinc nanoparticles on hematological indices of *O. mykiss* and investigate the improvement of these indices after vitamin C treatments.

Materials and methods

A total of 162 specimens of *O. mykiss* were obtained (mean weight 100±3.5) from aquaculture farms and transferred to the laboratory in Gorgan University of Agricultural and Natural Resources. Fish were accumulated in 70 L plastic aseptic tank for 2 weeks then were stocked in tanks which were filled with de-chlorinated water and aerating system. Water temperature was fixed at 15±2°C and the photoperiod was adjusted based on 11 hours daylight. pH was measured with a portable device (Model TS) as 7.2±0.4 and dissolved oxygen measured with a digital measuring oxygen device (DOE Model -5510), as 6.74±0.2 and hardness was 185±16 mg/L. Two concentration of ZnO-NPs (40 and 80 mg L^{-1}) and two doses of vitamin C (400 and 800 mg per kg of feed) were used in this study. Zn Nano colloid was obtained from Nonaka Company, Iran (Antimicrobial Product 2 brand, mean particle size of 20-30 nm, Fig. 1). In addition, vitamin C powder was purchased from ACROS ORGANICS company (L (+)-Ascorbic acid, 99%, MW=176.13 $C_6H_6O_6$).

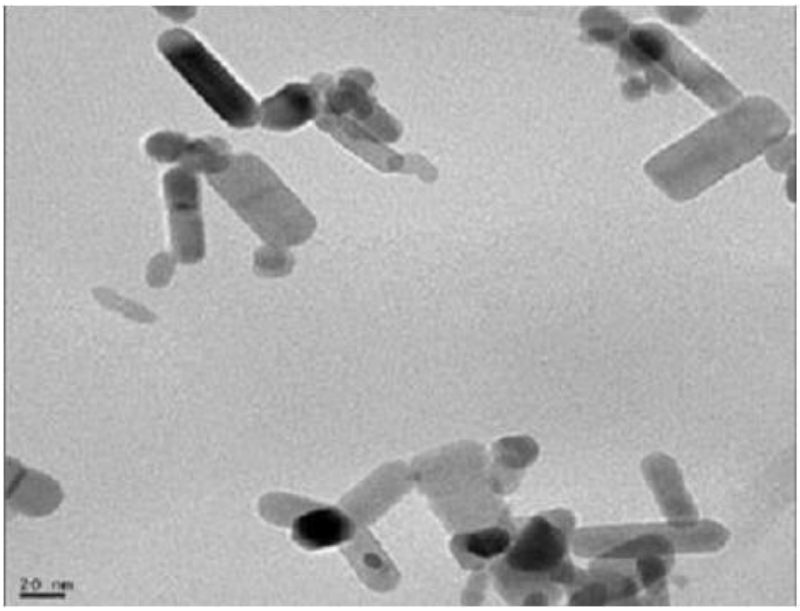

Figure 1: Particle size of Zn nanoparticles used in this study

Canola oil was used to add vitamin C powder to the diet (In order to combine vitamin C with diet, vitamin C was mixed in 10 cc of pure water and then to prevent separation of vitamin C, it has added with some canola oil and water solution, and then water were sprayed on diet). Oil coating method was employed to mix vitamin C with the diet (Adel and Khara, 2016).

At the end of the acclimation period, fish were treated in 9 experimental groups (6 fish in each treatment; replicates with 2 individuals). Treatments were chosen according to the previous references as treatment 1: normal diet+ no ZnO-NPs, treatment 2: normal diet+ 40 mg L^{-1} ZnO-NPs, treatment 3: normal diet+ 80 mg L^{-1} ZnO-NPs, treatment 4: 400 mg vitamin C diet+ no ZnO-NPs, treatment 5: 400 mg vitamin C diet+ 40 mg L^{-1} ZnO-NPs, treatment 6: 400 mg vitamin C diet+ 80 mg L^{-1} ZnO-NPs, treatment 7: 800 mg vitamin C diet+ no ZnO-NPs, treatment 8: 800 mg vitamin C diet+ 40 mg L^{-1} ZnO-NPs, and treatment 9: 800 mg vitamin C diet+ 80 mg L^{-1} ZnO-NPs. Normal diets with no ZnO-NPs were appointed as control treatment.

Fish were fed 2% of body weight per day. Faces were siphoned from aquaria after feeding and concentrations of toxicants were renewed after that. Blood samples were taken after 5 and 10 days of exposure from fishes. Hematological parameters were estimated according to routine clinical methods (Hedayati and Niazi, 2015; Alizadeh *et al.*, 2011). Mean cell hemoglobin (MCH), mean corpuscular volume (MCV), and mean cell hemoglobin concentration (MCHC) were calculated based on Decie and Lewis (Landis and Yu, 2004). To analyze hemoglobin percentage, Cyan-

met hemoglobin method in spectrophotometer was used and Naeubaur's double hemocytometer was used to study erythrocytes (Mukherjee, 1988). One-way analyses of variance (ANOVA) were used to analyze hematological parameters. Differences between means were determined using Tukey's multiple range tests at 5% probability level.

Results

No mortality was observed during the test. Fish were analyzed 5 and 10 days after exposure and hematological data were pooled. Analysis of hematological indices after 5 days showed several significant changes in blood parameters in treatments. The results showed a significant increase in in WBC (White Blood Cells) in treatment 9 in comparison with that in the control group. Analysis of RBC (Red Blood Cells) showed significant decrease in treatments 2, 3, 4, 5, 6, and 8. There were significant decreases in HB in treatments 3, 4, 5, 6, and 8. Results indicated that Ht (haematocrit) in treatment 2 was significantly different as compared to all treatments. Changes in MCV (Mean Corpuscular Volume) were only significant in treatment 3. MCH (Mean Corpuscular Hemoglobin) showed several significant changes in different treatments while there was no significant difference in MCHC (Mean Corpuscular Hemoglobin Concentration) between treatments. Moreover, results showed significant increase in types of WBCs after 5 days exposure to ZnO-NPs (Table 1).

10 days results of exposure to ZnO-NPs for hematological data are represented in Table 2. Statistical analysis revealed that significant changes in WBC occurred in treatments 2, 3, 4, 6, and 7. However, a significant increase in RBC occurred in treatment 2; while treatment 3, 6, 8 and 9 showed significant decrease. Values for Ht and MCH were significantly higher in treatments 2 and 9, and lower in treatment 3 in comparison with that in the control group. No significant differences were observed with control group in MCV and MCHC. Finally, analysis of leucocyte cells showed significant increase in neutrophils and monocytes, and decrease in lymphocyte cells after 10 days experiment.

Table 3 shows analysis of differences between blood parameters on days 5 and 10. Values for WBC and RBC count were significantly different between 5 and 10 days analyses in treatments 8 and 4, respectively. However, MCV, MCH and MCHC did not show significant differences.

Table 1: Hematological changes in *Oncorhynchus mykiss* exposed to Zn nanoparticles after 5 days. Different uppercase letters show significant difference between treatments in rows (p<0.05).

Hematological indices	Treatments (mean±SE)								
	1	2	3	4	5	6	7	8	9
WBC	10400± 173.20^{ab}	11183± 158.98^{ab}	9950± 592.31^{b}	10783 ±72.64^{ab}	11033± 202^{ab}	11466± 33^{ca}	10216± 7212^{ab}	11033± 176^{ab}	12500± 305^{c}
RBC	5.63± 0.03^{a}	4.3± 0.2^{b}	4.6± 15^{bc}	4.16± 0.08^{b}	4.5± 0.2^{b}	4.3± 0.1^{b}	5.1± 0.21^{cad}	4.9± 0.05^{abd}	5.4± 0.15^{da}
Hb	15.43± 0.31^{ae}	11.53± 0.66^{bf}	8.6± 0.38^{c}	12.16± 0.64^{dbef}	14.33± 0.38^{e}	10.63± 0.14^{fc}	13.03± 0.57^{gf}	10.73± 0.17^{bcd}	17.2± .7^{a}
Ht	45.66± 1.3^{ag}	36± 1.5^{ba}	26.33± 2.3^{cb}	37± 1.5^{bce}	33± 1.5^{cbe}	32.66± 2.3^{cbf}	39.66± 4^{aefd}	33.66± 0.6^{ac}	49.33± 4.2^{dg}
MCV	81.12± 2.8^{ab}	84.27± 6.5^{a}	57.71± 7.1^{b}	89.01± 5.3^{a}	73.94± 6.5^{a}	75.22± 2.6^{a}	78.23± 11^{a}	68.69± 0.7^{a}	91.93± 10^{a}
MCH	27.39± 0.7^{acd}	27.06± 2.6^{acde}	18.77± 0.5^{be}	29.16± 0.9^{acd}	31.91± 0.7^{c}	24.59± 0.7^{de}	25.38± 0.08^{de}	21.91± 0.5^{e}	31.86± 0.3^{ac}
MCHC	33.87± 1.6^{a}	32.01± 1.5^{a}	33.4± 3.7^{a}	33.11± 2.9^{a}	43.72± 5.48^{a}	32.81± 1.85^{a}	33.83± 4.7^{a}	31.92± 1.11^{a}	35.57± 4.1^{a}
neutrophils	13± 0.5^{a}	3.6± 0.3^{a}	8.3± 1.2^{a}	6.6± 1.3^{a}	19.6± 6.8^{a}	38.6± 23^{a}	14.3± 0.8^{a}	6.6± 1.6^{a}	14± 2.8^{a}
lymphocyte	81.3± .3^{abcf}	87± 0.5^{b}	84± 0.5^{ab}	86±1^{ab}	68.6± 1.2^{e}	76.6± 1.3^{c}	80.6± 0.6^{adc}	86± 2.08^{ab}	76.3± 1.4^{fc}
eosinophil	0±0^{a}	2.6± 0.3^{b}	1.3± 0.3^{ab}	0.3± 0.3^{a}	1.3± 0.3^{ab}	1.3± 0.3^{ab}	0.3± 0.3^{a}	1±0^{a}	1± 0.5^{a}
monocyte	5.6± 0.6^{a}	6.6± 0.6^{ac}	6.3± 0.3^{ac}	7±0^{ac}	5.6± 0.3^{a}	6.6± 0.6^{ac}	4.6± 0.6^{a}	6.3± 0.6^{ac}	8.6± 0.8^{bc}

Table 2: Hematological changes in *Oncorhynchus mykiss* exposed to Zn nanoparticles during 10 days. Different uppercase letters show significant difference between treatments in rows (p<0.05).

Hematological indices	Treatments (mean±SE)								
	1	2	3	4	5	6	7	8	9
WBC	13683± 174^{a}	11666± 218^{b}	12350± 160^{be}	9366± 176^{c}	14166± 33^{ad}	14966± 284^{d}	12733± 33^{e}	13366± 272^{ae}	13833± 145^{a}
RBC	5.26± 0.06^{ac}	6.233± 0.26^{b}	4.4± 0.25^{c}	5.73± 0.12^{ab}	5.63± 0.18^{ab}	4.76± 0.08^{c}	5.1± 0.23^{ac}	4.5± 0.15^{ac}	4.8±0.1^{c}
Hb	13.4± 0.15^{a}	16.6± 0.24^{b}	6.8± 0.11^{c}	15.06± 0.4^{de}	14.7± .02de	13.3± 0.2^{a}	13.8± 0.3^{ad}	15.16± 0.2^{e}	16.8± 0.08^{b}
Ht	41.3± 1.2^{abc}	49.3± 2.7^{b}	31±5^{c}	43.66± 1.3^{ab}	43.6± 1.4^{ab}	40±1.1^{abc}	39.3± 1.2^{abc}	43±0.5^{abc}	48±3.4^{ab}
MCV	78.5± 1.8^{ab}	79.07± 1.8^{ab}	71.6± 14^{b}	76± 0.8^{ab}	77.8± 4.7^{ab}	84.06± 3.9^{ab}	77.23± 1.3^{ab}	95.7± 3.1^{ab}	101.2± 5.3^{a}
MCH	25.45± 0.2^{a}	26.86± 1.4^{a}	15.5± 0.6^{b}	26.2± 0.1^{a}	26.2± 1.1^{a}	28±0.1^{a}	27.1± 0.8^{a}	33.7± 0.7^{c}	35.1± 0.8^{c}
MCHC	32.4± 0.6^{a}	34±2.3^{a}	23±3.4^{b}	34.5± 0.3^{a}	33.8± 0.6^{a}	33.5± 1.4^{a}	35.1± 0.5^{a}	35.2± 0.4^{a}	34.9± 2.6^{a}
neutrophils	9±0.5^{a}	18.3±1.2^{b}	15.6± 0.3^{bc}	6±1.5^{a}	7.6± 0.8^{a}	13.6± 0.8^{c}	6.6±0.6^{a}	7.6± 0.8^{a}	6.3± 0.8^{a}
lymphocyte	85.6± 1.4^{ade}	72.3± 1.2^{bcd}	70.3± 3.1^{c}	91.6± 1.8^{ae}	88± 1.7^{ae}	80.6± 0.3^{db}	87.3± 2.3^{ade}	91.3± 0.8^{e}	93± 1.1^{ae}
eosinophil	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
monocytes	5.3±1.3^{ab}	9.3±2.3^{ab}	11± 4.1^{b}	2.3± 0.6^{ab}	4±1^{ab}	5.6±06^{ab}	6±1.7^{ab}	2.3±1.3^{ab}	0.6±0.3^{a}

Table 3: Difference between hematological indices after 5 and 10 days analysis. (*) shows significant level at 0.05 %.

Hematological indices	*p* value (0.05 %)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
WBC	0.0*	0.1	0.01*	0.0*	0.0*	0.0*	0.0*	0.0*	0.01*
RBC	0.08	0.04*	0.5	0.0*	0.01*	0.09	0.9	0.07	0.03*
Hb	0.04*	0.02*	0.01*	0.1	0.3	0.0*	0.2	0.0*	0.4
Ht	0.07	0.1	0.3	0.3	0.0*	0.4	0.9	0.0*	0.9
MCV	0.9	0.9	0.7	0.9	0.9	0.9	0.9	0.8	0.9
MCH	0.9	0.9	0.7	0.9	0.8	0.9	0.9	0.8	0.9
MCHC	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	1
neutrophils	0.08	0.0*	0.0*	0.7	0.04*	0.3	0.0*	0.6	0.06
lymphocyte	0.04*	0.0*	0.01*	0.05*	0.0*	0.04*	0.05*	0.07	0.0*
eosinophil	N/A	0.01*	0.01*	0.3	0.01*	0.01*	0.3	N/A	0.1
monocytes	0.8	0.3	0.3	0.02*	0.1	0.3	0.5*	0.05*	0.0*

Discussion

Hematological data showed that ZnO-NPs had a certain influence on some blood indices in this study. Decreases in HB concentration or RBC count may be indicators of anemia (Li *et al*., 2011).

Changes of differential leukocyte counts are recognized as sensitive indices of xenobiotics or dysfunction in hematological tissues or certain infectious diseases (Khabbazi *et al*., 2015).

Lymphocyte percentage was lower than normal lymphopenia, and it can be a suitable marker of immune system deficiency and xenobiotic substance treatments that can also decrease the body's supply of lymphocytes (Banaee *et al*., 2008). In addition, increase in eosinophil and monocyte and depletion in lymphocytes were also observed as a result of changes in WBC in leucocytes cells (Table 1). Therefore, according to the results, combination of 800 mg Vitamin C diet+ 80 mg L^{-1} ZnO-NPs caused a kind of infection in rainbow trout after 5 days exposure. Banaee *et al*. (2008) stated that most infections cause neutrophilia. The degree of elevation often indicates the severity of the infection. Tissue damage from other causes raises the neutrophile for similar reasons. Poisonings, and severe disease, like kidney failure all cause neutrophilia (Abarghuei *et al.,* 2016).

The decrease in RBC count and HT levels observed in this study may be indicators of acute anemia. In fact, analysis after 5 days exposure showed that ZnO-NPs treatments (2 and 3) had more effect on erythrocytes than leucocytes. Reduction in RBC count and HT levels in toxicant-treated fish may be due to erythropoiesis disorder and the formation of RBC (Shaluei *et al*., 2013). In addition, following erythropoiesis alterations, MCV, MCH and MCHC levels might change because they are calculated based on HT, HB and RBC values. Some authors cited that the decrease in RBC count and HT levels could be related to the stress after short exposure to nanometals (Shaluei *et al*., 2013; Khabbazi *et al*., 2015).

The potential hematological improvement role of Vitamin C, treatments 2 and 3 compared with that in 5, 8, 6, and 9 was analyzed. The results indicated significant improvements in blood indices (Tables 2 and 3). Improvements observed in treatment 6 in contrast to 3 for WBC, 9 in contrast to 2 and 3 for RBC, 5 in contrast to 2 for HB, HT and lymphocyte. Overall, treatment 9 (800 mg Vitamin C diet) showed great amelioration of hematological indices. This shows that Vitamin C successfully irritated the immune response system of rainbow trout and decreased the adverse effects of ZnO-NPs on hematological indices after 5 days exposure.

Analysis after 10 days exposure showed that 400 mg Vitamin C was not sufficient for immune response to ZnO-NPs toxicity (treatments 5 and 6). According to Table 2, most improvements of hematological indices belonged to 800 mg diet Vitamin C (treatments 8 and 9). In this step, low amount of Vitamin C might be useful to protect rainbow trout from toxic materials. After long exposure (10 days) destruction process of ZnO-NPs would be greater due to bioaccumulation and higher amount of vitamin C is essential to improve hematological indices which are affected by ZnO-NPs.

In this study, blood parameters were significantly associated with high vitamin content. Elevation of vitamin C leads to hematocrit and hemoglobin increase, which corresponds with results obtained by others (Fracalossi *et al*., 2001). Vitamin C is expressed as the vitamin that has a positive role in improving the effectiveness in stressful conditions on the fish. This vitamin can increase antibody production and prevent the negative impacts of nanoparticles and the protective role of vitamin C can prevent the conversion of unsaturated fatty acids to cholesterol esters in stress conditions (Gabaudan and Verlhac, 2001). In our study, hemoglobin and RBCs increased in vitamin treatments, similar to the results of (Ibrahem *et al*., 2010).

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