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Original Article

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The toxicity of zinc oxide nanoparticles on blood factors of Huso huso and Cyprinus carpio

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Abstract

In this research, acute toxicity (LC) of zinc oxide nanoparticle on Beluga (Huso huso) and common carp (Cyprinus carpio) was studied. The experiment was conducted using the Organization for Economic Co-operation and Development (No. 202) method. For both species, 96hLC10 was determined to be more than 200 mg/l. To assess the effects of zinc oxide nanoparticles on blood factors, leukocyte index count (Lymphocyte, Monocytes, Neutrophils, Eosinophil, and Basophils) was performed, and the results were analyzed using IBM SPSS software version. According to the results and based on the ANOVA test, there is no significant difference among mean values of leukocyte index responses ($p>0.05$), and one should note that the amount of lymphocyte had decreased while that of neutrophils and monocytes had increased. Furthermore, with regard to concentration levels, this decrease or increase has not followed a systematic trend. In Beluga based on ANOVA test, there is a significant difference between mean values of lymphocytes and neutrophils ($p<0.05$). Again, compared to control, lymphocyte count increased while that of neutrophils and eosinophil decreased, and this too, did not follow a systematic trend. Finally, concentration to 200 mg/l ZnO did not cause mortality in Huso huso and Cyprinus carpio. Eventually, decline of body resistance against nano zinc oxide was observed in these species through blood cells study.

Keywords: Nano zinc oxide, Huso huso, Cyprinus carpio, Blood factors, Acute toxicity

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INTRODUCTION

Advances in science and technology has enabled mankind to gain increasing ability to take advantage of biological or non-biological resources in nature, which as a side effect, has led to ecological imbalance and environmental pollution (Moazam Payan, 1991). Human efforts for pollutants removal is hampered by the pace of population growth and ever increasing need of crucial resources (Askash, 1993). Sturgeon is the most valuable and expensive fish species in Iran. Its caviar has the highest quality among other countries, and exporting this product is one of the significant sources of the country's national income. In recent years, environmental pollution has led to the reduction of population of this species. But for now, in addition to the attitude and policy, what can be effective in preventing population reduction of this kind of fish are artificial propagation and breeding, and reduced environmental pollution when using water for artificial propagation and releasing fish in rivers. Human beings produce wide variety of pollutants, and a large portion of these materials may enter directly or indirectly into aquatic ecosystems. Some pollutants like most organic material decompose during biological process. However, other materials such as pesticides and heavy metals are resistant to decomposition and remain persistent in the environment for a long time (Esmaili Sari, 1990).

Nano particles have shown a growing trend of use in the industry during recent years (Chen et al. 2008). Nano zinc oxide is an industrially important nanoparticle that has found extensive industrial uses such as development of semi-conductors, sensors, and emitting devices. Despite these advantages, however, un-controlled use and distribution of this substance in the environment may result in serious side effects.

Pollution can also affect physiology, growth, and reproduction of fish, and even lead to fish death. Wei et al. reported zebra fish embryo death, and delay in hatching time because of exposure to zinc nanoparticle (Wei et al. 2009). Some other effects are reported including titanium dioxide nano particles' effect on rainbow trout and zebra fishes (Fent, 2010); sorption of cadmium and lead natural sediment particles on Cyprinus carpio (Zhang, 2007); titanium dioxide nano particles' effect on rainbow trout fish (Federici, 2007), and acute toxicity (LC50) of copper nano particles on zebra fish and its embryos (Hydutsky, 2007).

Research shows that silver nano particle with brand name LS-2000 has an acute toxicity effect on Barbus grypus (Alishahi, 2009). In addition, Soltani (2009) studied the toxicity of silver nanoparticles suspensions on rainbow trout fish. The 96-hour LC50 of silver nanoparticles was calculated on four fish species including Cyprinus carpio, Barbus barbulus, Rainbow Cichlid, and Poecilia reticulate (Alishahi, 2012). Toxicity of silver and gold nanoparticles in embryos of zebra fish has also been studied (Bar-Ilan, 2009). It should be noted that nano particles had destructive effects on plankton, algae, and bacteria.

Cyprinus carpio is the most common fresh water fish in aquaculture. Huso huso is the biggest and most expensive sturgeon fish in fresh water. Huso huso is endangered, Cyprinus carpio is vulnerable to extinction, and both of them are included in the red list of the International Union for Conservation of Nature (IUCN). Despite all the efforts made by relevant agencies like annual breeding, artificial reproduction, and release of fingerling fishes such as Acipenser nudiventris, Acipenser persicus, and bony fishes like Rutilus rutilus caspicus, Rutilus frisii kutum, and Cyprinus carpio in the Caspian Sea, the amount of fishing and fish stocks have declined drastically. One of the main causes of the extinction is environmental pollution due to urban sewage and industrial and agricultural waste.

This is the first study in Iran to examine the toxicity of nano zinc oxide on two fish species with breeding values (Huso huso as the main and most valuable fish species having the best ability for culture and Cyprinus carpio as the oldest species in aquaculture). Considering the growing widespread use of this substance in industrial effluents followed up by their entry into the water, we aimed to determine lethal concentration and maximum allowable concentration of the

substance in two fish species in order to set a limit for its entry to the environment so that it exerts minimal damage to living organisms. The aim of this study was to determine the potential toxicity of ZnO nanoparticles on blood factors of sturgeon and common carp.

MATERIAL AND METHOD

The research was conducted from early May to late August 2012 in the Shahid Beheshti Hatchery (Guilan Province — South Caspian Sea). Zinc nano oxide (20 nm, 99% purity) was purchased from Iranian Pioneer of Nano Materials Company.

Preparation and Adaptation of Fish

To perform the test, fingerling fish weighing 2.0±0.5 g were transported to the test site from Shahid Beheshti Hatchery. Before starting the test, Cyprinus carpio fish were acclimatized to experimental condition for duration of eight days. Huso huso was bred in the Shahid Beheshti Hatchery; therefore, it did not need adaptation. The fish were fed on a daily basis, but feeding was stopped during the trial period. Temperature, water hardness, pH, and dissolved oxygen were measured on a daily basis. For fish exposure, 12 plastic tanks containing 20 liters of water were used. In order to keep fishes close to the natural conditions, low hardness water from Sefidroud River channel was used.

Preparing Test Concentrations

A standard stock solution (7.5 g/l nano zinc oxide for high concentration and 2 g/l nano zinc oxide for low concentration) was prepared. Initial experiments were carried out with five treatments (0.1, 1, 10, 50, and 100 mg/l) and two replicates in each treatment compared to control in order to determine the range of experiment.

Exposure of Fish to Nanoparticle

The experiment was conducted using the OECD[1] (No. 202) method. Therefore, ten fish were transferred to each tank. Density of fish in each tank was at least 1 g/l (TRC; 1984). During the experiment, operational conditions including temperature, dissolved oxygen, water hardness, and pH were measured. Aeration was carried out using a central aeration system, and dissolved oxygen was maintained above 9 mg/l. At the end of each day, the number of fish death was recorded, and dead fish were accurately examined by their appearance signs. Symptoms caused by these compounds were also examined.

Exposure of Cyprinus carpio to Zinc Oxide Nano Particle

The experiment was carried out with the same concentrations on common carp. Due to no observed deaths during the experiment period, the fish were fed after 96 hours; the water tank was renewed and fish were exposed to those concentrations of nano zinc oxide for the next four days to study long-term effects of exposure to nano zinc oxide. After the initial testing, because the concentration did not resulted in fish death, the concentration was increased to 50, 100, 150 and 200 mg/l in subsequent experiments. At this stage, like the previous four days, no death was observed; and at the end of the experimental period, no dead fish were observed. In order to discover the effects of nano zinc oxide, feeding was done and tanks were refilled the next day, and again the fish were exposed to nano zinc oxide.

Exposure of Huso huso to Zinc Oxide Nano Particle

The range-finding experiment was carried out with five treatments and two replicates per treatment concentrations of 0.1, 1, 10, 50, and 100 mg/l, which were then compared with the control fish. The experiment was conducted within four days and during the experiment, temperature, dissolved oxygen, pH, and water hardness were measured. Aeration was carried out using a central aeration system, and dissolved oxygen was maintained above nine mg/l. In order to obtain higher confidence in results, the experiment was repeated three times. After this initial testing because the

1. Organization for Economic Co-operation and Development Guideline on testing of chemicals

concentration did not result in fish death, the concentration was increased to 50, 100, 150, and 200 mg/l in the next experiments. Due to no death during the experiment period, fish were fed after 96 hours. The water tank was renewed and fish were exposed to those concentrations of nano zinc oxide to study long-term effects of exposure to nano zinc oxide. All chemical and physical factors in Table 1 are referenced from the Shahid Beheshti Hatchery.

Table 1: physical and chemical factors of water tanks

Factor	Value
Temperature (C°)	2.5± 23
(mg L-1 CaCO3) Total hardness	0.1±204
(mg L-1 CaCO3) Total alkalinity	10.0±202.0
pH	0.2±8.1
(mg L-1) DO	0.2 ± 6.4
(mg L-1) Nitrite	0.0002±0.003
(mg L-1) Nitrate	0.3±3.6
(mg L-1) Phosphate	0.4±8.5

Blood Smear and Count of (Cyprinus carpio) and (Huso huso)

At the end of the experiments, blood was taken from each fish species. By cutting off caudal fins from three replicate fishes, a small piece of blood was placed on clean and sterile counting slide. Afterward, blood expansion was provided by means of a second slide and with quick movement and angle of 45 degrees. Immediately, by using methanol, blood expansions were fixed and stained with clinical dye. In order to stain, blood slides were placed in glass bottles containing 10% Giemsa solution for duration of 30 minutes. Thereafter, the slides were washed and placed in room temperature in order to dry. For reading and counting Leukocytes (Lymphocytes, Neutrophils, Eosinophils, Monocytes, and Basophils) timer device and light microscope (OLYMPUS U-ACAD – Japan) were used, and results were analyzed using SPSS Software version.

RESULT

Effects of Nano Zinc Oxide on Cyprinus carpio

No significant difference in mortality rate between test and control fish was observed for concentrations 0.1, 1, 10, 50, 100, 150, and 200 mg/l ($p>0.05$).

Symptoms resulting from exposure of Cyprinus carpio to Nano Zinc Oxide

Fish exposed to nano zinc oxide initially had no abnormal symptoms. However, after eight days, whitening skin and congestion in the head area were observed. Then, lateral deviation in the backbone was observed in one fish, while exophthalmos was observed in other fishes. In addition, some dead fish had blurred abdomen.

Effects of Nano Zinc Oxide on Huso huso

No significant difference in mortality rate of test and control sturgeons was observed at concentrations of 0.1, 1, 10, 50, 100, 150, and 200 mg/L ($p>0.05$). The long-term effects were studied, and the results are as follows.

Symptoms resulting from Huso huso embryos exposure to Nano Zinc Oxide

In fish with 48 hours age, quick turning around, pale skin, dizziness, and imbalance on the water surface are symptoms observed on fishes exposed to nano zinc oxide after 96 hours. On day eight, the mortality of this fish population had started. In high concentrations, mucus skin in some fish was observed, while others had congested gills, and thus, their body turned pink in color, yet some other fish showed bent spines.

Statistical analysis of white blood cell count in Common carp (Cyprinus carpio)

Statistical analysis of lymphocyte number in Cyprinus carpio

According to Table 2, and ANOVA test, the significance level is 0.149. Although there was no significant difference ($p>0.05$) among the number of lymphocytes in different concentrations of nano zinc oxide in common carp (Cyprinus carpio), comparison with control fish showed that the number of lymphocytes had decreased at different concentrations; however, this decrease had not followed a systematic trend (Figure 1).

Table 2: Results of Cyprinus carpio white blood cell count according to ANOVA test

ANOVA lymphocyte					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	270.607	4	67.652	1.887	.149
Within Groups	788.800	22	35.855		
Total	1059.407	26			
Monocyte					
Between Groups	7.413	4	1.853	1.147	.361
Within Groups	35.550	22	1.616		
Total	42.963	26			
Neutrophile					
Between Groups	53.283	4	13.321	1.015	.421
Within Groups	288.717	22	13.123		
Total	342.000	26			
Basophile					
Between Groups	121.185	4	30.296	2.212	.101
Within Groups	301.333	22	13.697		
Total	422.519	26			

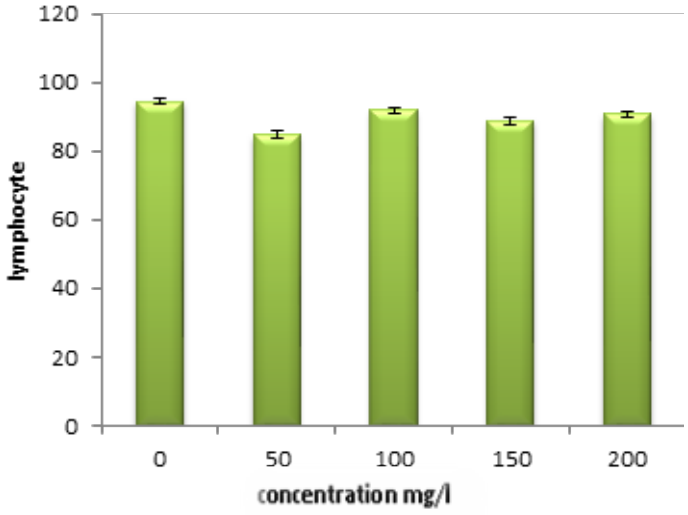

Figure 1: Number of Cyprinus carpio lymphocytes in different concentrations of nano zinc oxide

Statistical analysis of monocytes number in Common carp (Cyprinus carpio)

According to the Table 2 and ANOVA test, significant level is 0.361. Although there was no significant difference ($p>0.05$) among the number of monocytes in different concentrations of nano zinc oxide in common carp (Cyprinus carpio), comparison with control fish indicated that the number of monocytes increased at different concentrations; however, this increase did not follow a systematic trend (Figure 2).

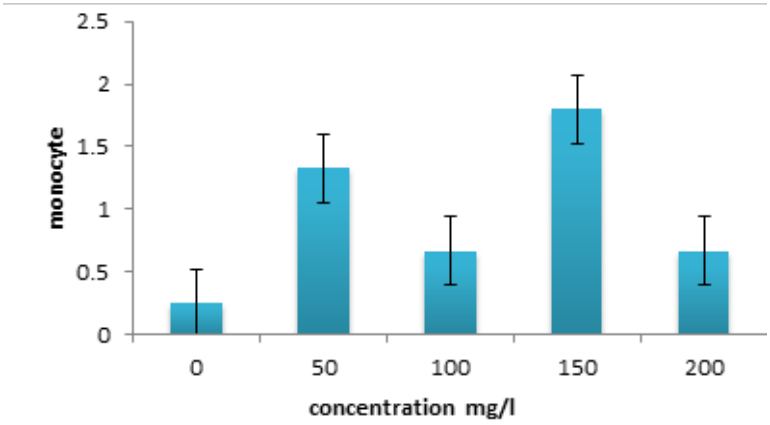

Figure 2: Numbers of Cyprinus carpio monocytes in different concentration of nano zinc oxide

Statistical analysis of neutrophils number in Cyprinus carpio

According to Table 2 and ANOVA test, the significance level is 0.421. Although, there was no significant difference ($p>0.05$) among the number of neutrophils in different concentrations of nano zinc oxide in common carp (Cyprinus carpio), comparison with control fish showed that the number of neutrophils increased at different concentrations; however, this increase did not follow a systematic trend (Figure 3).

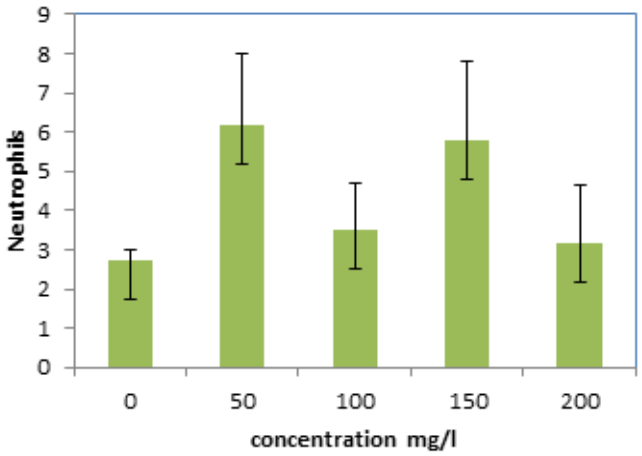

Figure 3: Numbers of Cyprinus carpio neutrophils in different concentrations of nano zinc oxide

Statistical analysis of number of basophile in (Cyprinus carpio)

According to Table 2 and ANOVA test, the significance level is 0.101. Although there was no significant difference ($p>0.05$) among the number of basophiles in different concentrations of nano zinc oxide in common carp (Cyprinus carpio), comparison with control fish showed no significant difference ($p>0.05$).

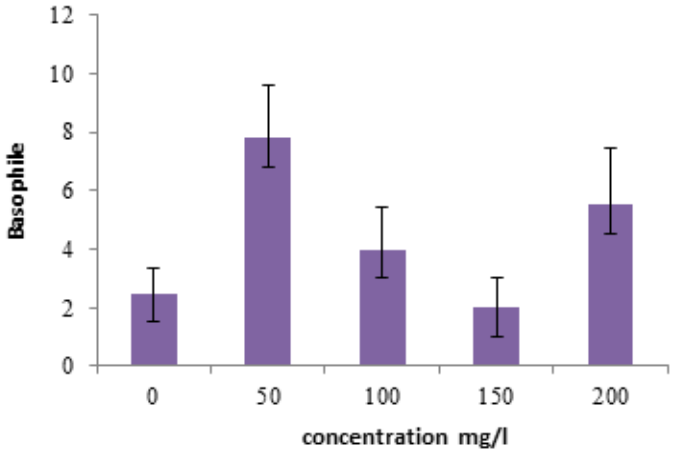

Figure 4: Number of Cyprinus carpio basophiles in different concentrations of nano zinc oxide

A Statistical analysis of white blood cells count in Huso huso

Statistical analysis of lymphocyte in Huso huso

According to Table 3 and ANOVA test, the significance level is 0.001. There was significant difference ($p<0.05$) among the number of lymphocytes in different concentrations of nano zinc oxide in Huso huso, and comparison with control fish indicated that the number of lymphocytes increased at different concentrations; however, this increase did not follow a systematic trend (Figure 5).

Table 3: Results of white blood cell count according to ANOVA test in Huso huso

ANOVA lymphocyte					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6979.125	4	1744.781	7.425	.001
Within Groups	4464.833	19	234.991		
Total	11443.958	23			
Monocyte					
Between Groups	6.800	4	1.700	.973	.445
Within Groups	33.200	19	1.747		
Total	40.000	23			
Neutrophile					
Between Groups	5121.700	4	1280.425	4.226	.013
Within Groups	5756.300	19	302.963		
Total	10878.000	23			
Eosinophils					
Between Groups	106.958	4	26.740	2.248	.102
Within Groups	226.000	19	11.895		
Total	332.958	23			

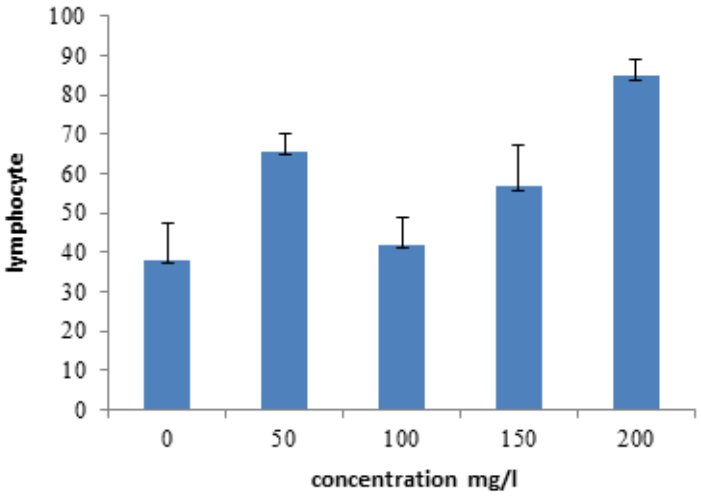

Figure 5: Number of Huso huso lymphocytes in different concentrations of nano zinc oxide

Statistical analysis of number of monocytes in Huso huso

According to Table 3 and ANOVA test, the significance level is 0.445. Therefore, there was no significant difference ($p>0.05$) among the number of monocytes in different concentrations of nano zinc oxide in beluga (Huso huso) and in comparison with the control fish (Figure 6).

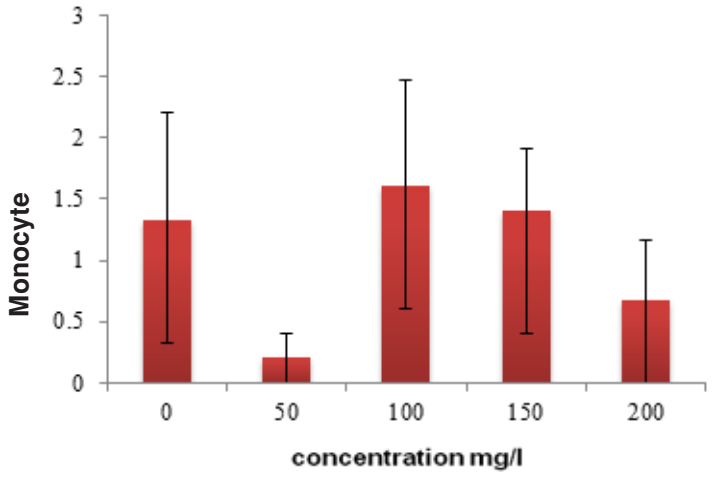

Figure 6: Number of Huso huso monocytes in different concentrations of nano zinc oxide

Statistical analysis of number of neutrophils in Huso huso

According to Table 3 and ANOVA test, the significance level is 0.013. There was a significant difference ($p<0.05$) among the number of neutrophils in different concentrations of nano zinc oxide in beluga (Huso huso). Comparison with control fish indicated that the number of neutrophils decreased at different concentrations; however, this decrease did not follow a systematic trend (Figure 7).

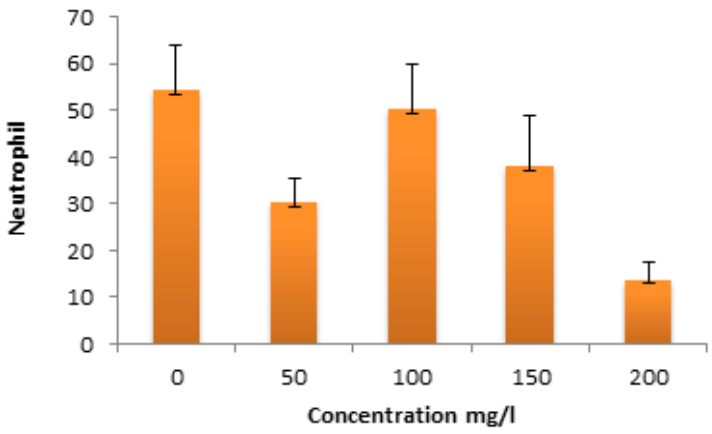

Figure 7: Number of Huso huso neutrophils in different concentrations of nano zinc oxide

Statistical analysis of number of eosinophils Huso huso

According to the table 3 and ANOVA test, the significant level is 0.102. Therefore, there was no significant difference ($p>0.05$) among the number of eosinophils in different concentrations of nano zinc oxide in Huso huso, and comparison with the control fish indicated that the number of eosinophils decreased at different concentrations; however, the trend was decreasing with increasing concentration (Figure 8).

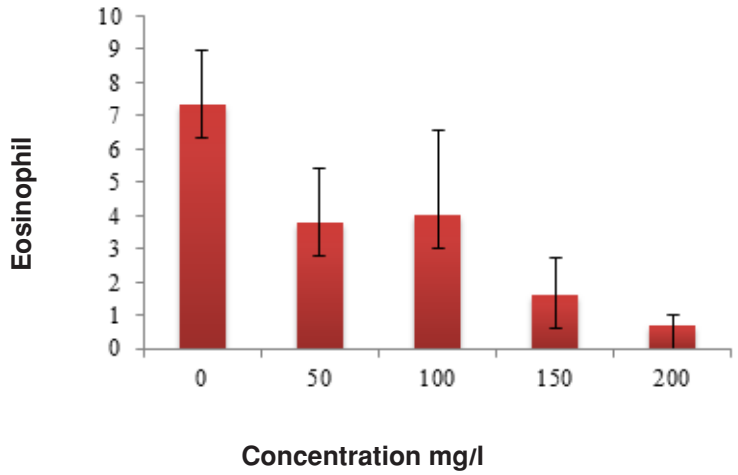

Figure 8: Number of Huso huso eosinophils in different concentrations of nano zinc oxide

Discussion and conclusion

According to the test results, concentrations of 0.1, 1, 10, 50, 100, 150, and 200 mg/l nano zinc oxide in beluga (Huso huso) and Common carp (Cyprinus carpio) are not fatal. Therefore, LC10 is greater than 200 mg/l. Increasing concentrations to more than 200 mg/l caused sedimentation of nano zinc oxide in the tanks, and since no suitable solvent was available for increasing solubility of this substance, performing experiments with concentrations above 200 mg/l were prohibited. Therefore, test fish were found resistant against nano zinc oxide, and used concentrations did not result in lethal effects. This non-effectiveness is probably due to different assimilation of these particles in different fish species. This result has been obtained since none of these species is filter feeders, and assimilation is not done through the skin. From accumulation of mucus on the skin, unusual swimming of fish and change in color, it can be concluded that nano zinc oxide is very effective on the fish nervous system. Nano zinc oxide can disturb the circulatory activity, and this can lead to color change in fish. With regard to morphological changes in common carp, it seems that the degree of resistance against nano zinc oxide is higher than that of beluga.

In addition, Wei et al. (2009) reported that exposure to nano zinc oxide in concentrations 50 and 100 mg/l on zebra fish embryo cause mortality of them. However, concentration of 1-25 mg/l can cause hatching delay in the embryo. This causes decrease in body length, and tail abnormalities after being exposed to nano particle for 96 hours.

White blood cell count showed that in Huso huso, the number of lymphocytes increased and the number of neutrophils and eosinophils decreased. Decrease in number of the latter cells indicates that nano zinc oxide has a negative effect on the immune system and resulted in reduced phagocytosis potential in the Huso huso. Decrease of lymphocytes in Cyprinus carpio is an indication of stress increase. On the other hand, increase

in monocytes shows phagocytosis increase. Furthermore, neutrophil, which is an indicator of disease and infection, increased. Finally, one can conclude that nano zinc oxide concentrations of 0.1, 1, 10, 50, 100, 150, and 200 mg/l did not cause mortality in Huso huso and Cyprinus carpio; however, decline of resistance against nano zinc oxide was observed in these species. With regard to daily industrial development, and thus, more utilization of nano zinc oxide in industrial processes, one can expect probable increase of this compound in wastewaters and waterways. Thus, appropriate decision making in this regard is necessary

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