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Original Article

Biochemical and physiological effect of dietary supplements of ZnO nanoparticles on common carp (*Cyprinus carpio*)

Mahdi Banaee*[1], Maryam Vaziriyan[1], Azam Derikvandy[2], Behzad Nematdoost Haghi[1], Mohammad Mohiseni[1]

[1]Department of Aquaculture, Faculty of Natural Resources and Environment, Behbahan Khatam Al-anbia University of Technology, Iran.
[2]Department of Environment, Faculty of Natural Resources and Environment, Behbahan Khatam Alanbia University of Technology, Iran.

Abstract: ZnO-NPs, like other macromolecule sources, may provide the fish with sufficient amounts of zinc and be effective in regulating the biochemical function of cells in organisms. This study aimed to assess the possibility of using nanoparticles in common carp diet by evaluating alterations in blood biochemical parameters, as a clinical marker of fish health. In this study, fish were fed diets supplemented with 0 (control), 5, 10 and 15 mg kg^{-1} ZnO-NPs for 21 days. The results showed that after 21 days admiration of ZnO-NPs, 10 and 15 mg kg^{-1} concentrations significantly increased aspartate aminotransferase (AST) activity and glucose, cholesterol, triglyceride and creatinine levels in plasma of fish (P<0.05). Also, the administration of 15 mg kg^{-1} ZnO-NPs significantly (P<0.05) increased alanine aminotransferase (ALT), Lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) activities. No significant changes were observed in plasma total protein, albumin and globulin levels (P>0.05). In conclusion, the results showed that diets containing high concentrations of ZnO-NPs supplement (10 and 15 mg) is caused severe cytotoxic effects, including changes in blood biochemical parameters. The primary toxic mechanism of ZnO-NPs was possibly increasing the cellular oxidative stress and disrupting the biochemical function of cells.

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Introduction

Zinc (Zn) is the second trace element in organisms and cannot be stored in the body (Kuma et al., 2018), therefore it needs to be provided regularly through the diet for physiological activities. The importance of zinc in fish and crustaceans health has been verified (Olmedo et al., 2013; Ma et al., 2014). Also, it is a significant component of many enzymes, the cellular antioxidant system and hormones, and an essential element for many physiological functions (Taheri et al., 2017; Domínguez et al., 2019). Zinc is involved in the natural growth, reproduction (Uriu-Adams and Keen, 2010), regulating the reverse transcription and DNA synthesis, cellular division and gene expression (Swain et al., 2016; Frassinetti et al., 2006), photochemical processes of the visual system (Swain et al., 2016), recovering injuries (Frassinetti et al., 2006), bone formation (Swain et al., 2016), increasing the immune system (Gharekhani et al., 2015; Beitsayah et al., 2019) through energy production, protein synthesis, protecting cell membranes against bacterial endotoxin and increasing the lymphocytes proliferation rate and producing antibodies (Swain et al., 2016; Velazquez-Carriles et al., 2018).

Zinc absorption is very variable in different species of fish and differs depending on the organisms' age, place of absorption in the digestive tract, and individual needs. Zinc in diet can be found as mineral salts such as zinc oxide or zinc sulfate or as organic chelators, including zinc propionate and zinc acetate. Although the bioavailability of inorganic zinc sources is more than inorganic zinc salts, the high cost of providing organic forms of zinc chelate has limited their use in the diet of the organism (Frassinetti et al., 2006). In commercial diets of farmed fish species, the amount of zinc is proportional to the fish needs; however, the bioavailability of zinc in feedstuffs is usually low (Davis and Gatlin, 1996; Bilandžić et al.,

*Correspondence author: Mahdi Banaee
E-mail: mahdibanaee@yahoo.com
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2014). Therefore, using the additional zinc in nutritional supplements (100 to 150 mg Zn per kg feed) may overcome the inhibitory effect of compounds such as calcium phosphate in fish meal and phytate or phytic acid in soybean meal, and other oilseeds and grains (Davis and Gatlin, 1996; Ma et al., 2014; Bilandžić et al., 2014).

The increased excretion of zinc in animals treated with zinc-supplemented diets has increased concerns about environmental pollutions (Musharraf and Khan, 2019). Therefore, researchers try to find a source of zinc with a higher bioavailability to reduce zinc levels in food supplements for animals. Among all possible strategies, using nanotechnology to produce nanoparticles could be a potential alternative for both organic and inorganic zinc sources. Using zinc nanoparticles compared to conventional Zn sources, as well as Zn nanoparticles are more efficient and less toxic (Taheri et al., 2017). Due to their small size, zinc oxide nanoparticles (ZnO NPs) can easily be absorbed by the digestive tract and are more effective in lower concentrations compared to the conventional ZnO (Chupani et al., 2018). In the organisms' body, mineral nanoparticles interact more effectively with organic and inorganic materials which are due to their larger surface area (Chupani et al., 2018). The effect of ZnO NPs on improving the growth, as well as enhancing the efficiency of the consumed feed and economic sources produced in farms for different species of farmed animals are reported (Hongfu, 2008; Lin et al., 2009; Mishra et al., 2014). The results of studies show that administration of low concentration of nano-zinc oxide in enhancing the growth rate of different farmed animals can have similar results as administration of high concentrations of ZnO (macromolecules). Therefore, this can be one of the benefits of ZnO NPs (Hongfu, 2008; Taheri et al., 2017). Since the physiological function of Zn is influenced by its mode of transfer and storage in the aquaculture (Bilandžić et al., 2014), using zinc supplement as nanoparticles can affect the physiological indicators and fish health.

There is little information on the adverse effects of zinc oxide nanoparticles (NPs) on farm animals such as fish (Connolly et al., 2016). In most cases, the base of toxicological studies on metal NPs is fish exposure with the soluble phase of NPs (Chupani et al., 2017, 2018; Dekani et al., 2019) and there is still a lot to learn about the toxicology and potential hazards of different doses of zinc oxide NPs in foodstuff of fish (Swain et al., 2016). Meanwhile, depending on the concentration, way, and duration of exposure, the cytotoxicity of zinc oxide NPs can lead to oxidative stress (Wang et al., 2014), lipid peroxidation, damage to cell membranes, and oxidative damage to DNA (Dekani et al., 2019). Studies conducted so far are on the oral administration of zinc oxide NPs 300 and 1000 mg Kg^{-1} feed (Connolly et al., 2016) and zinc oxide NPs 30, 50, 100 and 500 mg Kg^{-1} feed (Chupani et al., 2018; Dekani et al., 2019) in fish. Therefore, investigating the probable toxicity of oral administration of these NPs in lower doses seems necessary. Therefore, this study aimed to evaluate oral administration of zinc nanoparticles on biochemical factors of blood, as the fish health assessment index, and investigating the possibility of using zinc nanoparticles in the feedstuff of common carp, *Cyprinus carpio*.

Materials and Methods

Fish: One hundred forty four immature common carp (mean weight: 20.5±2.5 g) were obtained from a local fish farm (Ahvaz, Khuzestan Province, Iran) and randomly distributed into 12 circular tanks of 80 L capacity (12 fish per each tank) at the Department of Aquaculture (Khatam Alanbia University of Technology). The experiment was conducted following the National Ethical Framework for Animal Research in Iran (Mobasher et al., 2008). Before the experiment, fish were acclimated in aerated freshwater (24±2°C; pH, 7.4±0.2; 50% water exchange rate/day) for two weeks. The fish were subjected to artificial light (16L/8D). During the acclimatization period, fish were fed with commercial pelleted feed (Beyza Feed Mill, Shiraz, Iran) by the manufacturer's recommendations.

Diet preparation: Since oral administration of 50 and 1000 mg zinc oxide NPs to common carp and rainbow trout proved to have adverse effects (Connolly et al.,

Table 1. Zinc oxide nanoparticles physicochemical proprieties, according to the Iranian nano-materials pioneer's manufacturer.

Zinc Oxide	ZnO
Purity	+99.9 %
Average Primary Particle Size (D50)	10-30 nm
Specific surface area (SSA)	60 m^2 g^{-1}
Color	White
Bulk density	5.606 g cm^{-3}

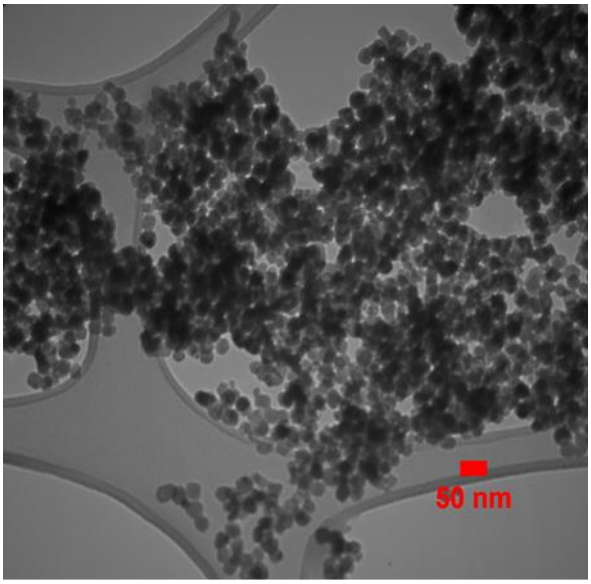

Figure 3. TEM micrographs of the Nano-ZnO powders (Adapted from Iranian Nano-materials Pioneers Company's catalog).

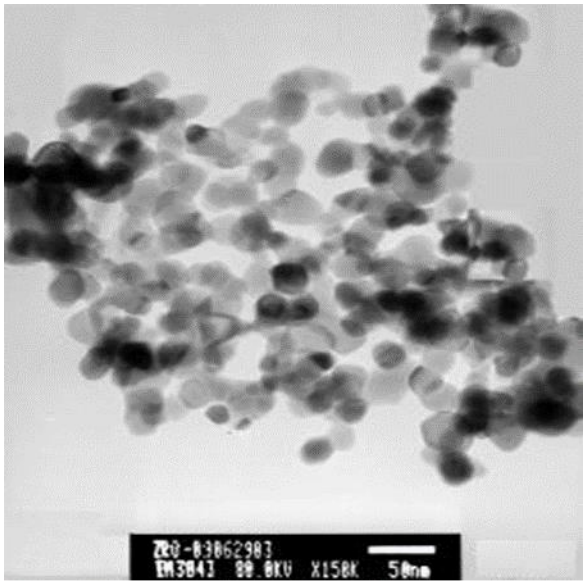

Figure 2. SEM micrographs of the Nano-ZnO powders (Adapted from Iranian Nano-materials Pioneers Company's catalog).

2016; Chupani et al., 2017), this study used 5, 10 and 15 mg Kg^{-1} zinc oxide NPs which might have lower toxicity. The formulated fish feed was enriched with nanoparticles of zinc oxide (Dekani et al., 2019).

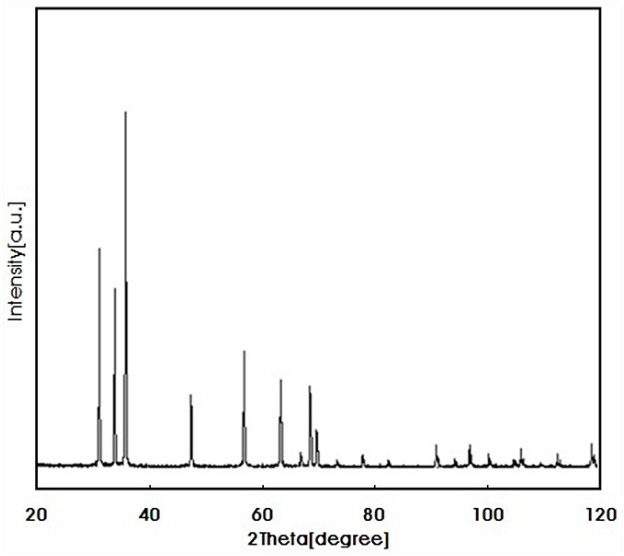

Figure 3. The X-ray powder diffraction (XRD) curves of Nano-crystalline ZnO (Adapted from Iranian Nano-materials Pioneers Company's catalog).

Nanoparticles of zinc oxide were used at 5, 10 and 15 mg per kg feed for a total of three treatments. Commercial ZnO nanoparticles, with an average primary particle size of 50 nm in the powder form, were purchased from Iranian Nano-materials Pioneers Company, Iran (Table 1). The TEM, SEM micrographs and the X-ray powder diffraction (XRD) curves of Nano-crystalline ZnO are presented in Figures 1-3.

ZnO nanoparticles were prepared using distilled water and then ultrasonicated (10 min, 35 KHz, 100/400W) using an ultrasound bath (Elma, Germany). Then, solutions were added to powdered feed to obtain nominal concentrations of 5, 10 and 15 mg ZnO NPs per kg. Each supplemented diet was mixed in a mixer for 30 minutes and then homogenized into a paste by adding fish oil (20 mL kg^{-1}) and distilled water into the food mixer. The amount of distilled water required for pelleting (20-

40% of feed weight) was then added to the mixture and further homogenized. This mixture was passed through a meat grinder, producing string shapes, which were dried in an oven at 55°C for 12 h and then broken to produce 10 mm long pellets. The pellets were packed and stored at -20°C in a freezer. The control diet was prepared by the same process, although no supplement was added.

Experimental design: During the experimental period, fish fed commercial pelleted feed enriched with 0 (control), 5, 10 and 15 mg kg^{-1} nanoparticles of zinc oxide supplement following the manufacturer's recommendations for three weeks. At the end of the experiment, 12 fish per treatment were captured using a scoop net and anesthetized with clove powder solution (1:5,000). Anesthetized fish were bled from the caudal artery/vein using 2 ml heparinized syringes. The collected blood was transferred into 2 ml micro-centrifuge tubes. The blood sample was centrifuged for 15 min at 6000 g at 4°C. Plasma samples were immediately stored at -25°C before biochemical analysis.

Sampling and analysis of blood biochemical parameters: All blood biochemical parameters were determined using a UV-visible spectrophotometer (UNICO 2100) and standard biochemical reagents (Pars Azmun Company, Tehran, Iran). Each biochemical blood parameter was measured by a particular method. Total plasma protein concentration was measured at 540 nm by the Biuret reaction. The albumin assay is based on the dye-binding properties of plasma albumin with a bromocresol green. An increase in the blue-green color was measured at 630 nm. The plasma globulin was calculated based on the ratio of albumin to total protein (Johnson et al., 1999). Plasma glucose was measured by the glucose-oxidase method at 500 nm (Sacks, 1999). Plasma cholesterol levels were measured by the CHOD-PAP enzymatic method at 510 nm, triglyceride levels by GPO-PAP enzymatic method at 546 nm (Rifai et al., 1999) and creatinine by the JAFFE method at 510 nm (Foster-Swanson et al., 1994). The activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma was determined by NADPH consumption and its conversion to NAD+ at 340 nm. Lactate dehydrogenase (LDH) in plasma was determined based on the conversion of pyruvate to lactate at 340 nm, alkaline phosphatase (ALP) based on converting nitrophenol phosphate into nitrophenol and phosphate at 405 nm, and based on optical density (OD) absorption and the formula presented in the kits' manual (Moss and Henderson, 1999).

Data analysis: The significant difference in the biochemical parameters of fish fed enrich diet with different concentrations of ZnO nanoparticles was examined using one-way ANOVA. All data were checked for normality (Kolmogorov-Smirnov test). Means were compared by Duncan's test and a $P<0.05$ was considered statistically significant. Statistical analyses were performed using SPSS (IBM, 19) software. Data are presented as mean (SD).

Results

During the experiment, mortality was not observed in the control group, and fish fed ZnO NPs supplement. On day 21 of the experiment, the results indicated that AST activity at 10 mg kg^{-1} and 15 mg kg^{-1} ZnO NPs supplement increased compared to the control ($P<0.05$). The findings demonstrated that ALT, LDH, and ALP activities statistically increased in the plasma of fish fed with 15 mg kg^{-1} ZnO NPs supplement compared to the control group ($P<0.05$). However, ALT, LDH and ALP activities on day 21 of the study did not show any significant difference in fish fed with 5 mg kg^{-1} and 10 mg kg^{-1} ZnO NPs supplement compared to the control group ($P>0.05$) (Fig. 4).

On day 21, in all the groups fed with ZnO NPs supplement showed no significant differences in plasma total protein, albumin and globulin levels compared to the control group ($P>0.05$). On day 21, a statistically significant increase in plasma glucose, cholesterol, triglyceride, and creatinine levels was seen compared to the control group ($P>0.05$) in fish fed with 10 mg kg^{-1} and 15 mg kg^{-1} ZnO NPs supplement (Table 2).

Discussions

The required zinc concentration in a feed of farmed

Table 2. Alterations in the blood biochemical parameters of common carp, *Cprinus carpio* oral exposure to ZnO Nanoparticles.

Biochemical parameters	Concentrations of ZnO Nano-particles (mg) per 1 kg feed			
	0.0	5.0	10.0	15.0
Total protein	4.6±0.5^{b}	4.2±0.5^{b}	4.4±0.4^{b}	3.8±0.4^{a}
Albumin	2.8±0.5^{a}	2.7±0.3^{a}	2.7±0.4^{a}	3.2±0.4^{b}
Globulin	1.7±0.3^{b}	1.5±0.4^{b}	1.8±0.4^{b}	0.7±0.2^{a}
Cholesterol	193.9±22.0^{a}	180.9±30.2^{a}	260.6±25.9^{b}	244.0±30.3^{b}
Triglycerides	281.6±54.8^{a}	354.9±51.7^{b}	446.9±52.0^{c}	375.6±44.1^{b}
Glucose	41.1±3.5^{a}	89.7±21.6^{b}	89.8±5.9^{b}	95.7±12.0^{b}
Creatinine	0.2±0.1^{a}	0.2±0.0^{a}	0.4±0.1^{b}	0.5±0.1^{c}

Significant differences between values when compared with control groups were characterized by alphabet symbol (P<0.05). Values represent mean ±S.D.

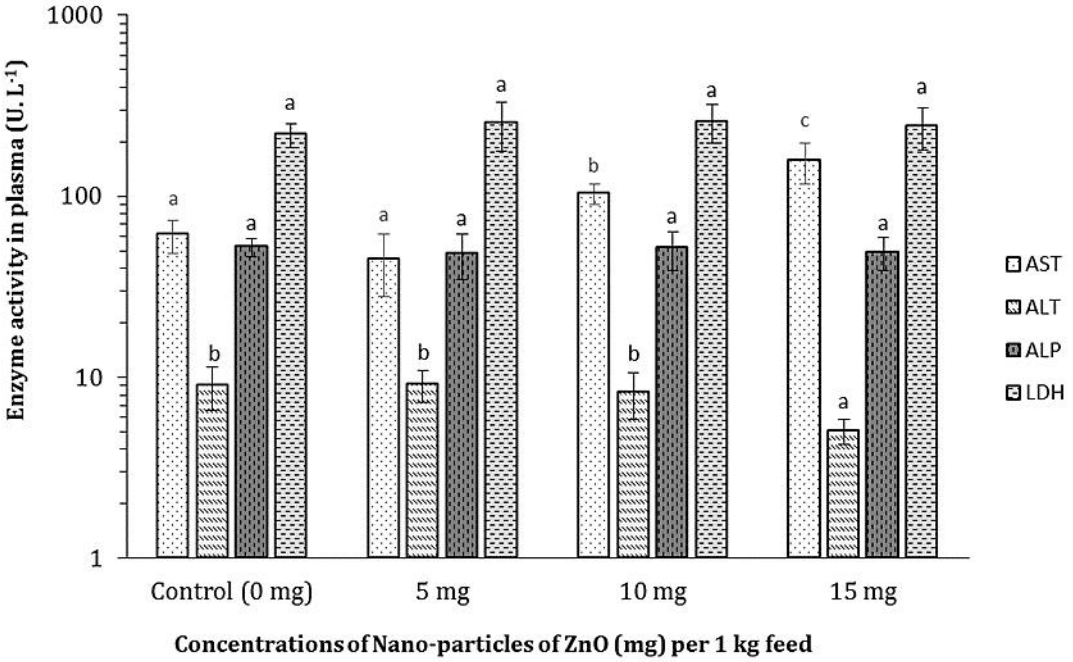

Figure 4. Changes in the enzyme activities in plasma of fish with oral exposure to ZnO Nanoparticles (Significant differences between values when compared with control groups were characterized by alphabet symbol (P<0.05). Values represent mean±S.D).

common carp is between 15-30 mg per kg feed (Davis and Gatlin, 1996; Wang and Wang, 2015). Apparently, zinc content (as mineral salts such as zinc oxide or zinc sulfate) in the formulated diet of common carp and produced in most factories of aquaculture feed in Iran is 30 mg per kg feed; however, due to the use of oilseeds in the base diet, the bioavailability of zinc may reduce for fish (Ma et al., 2014). That is why this study investigates the possibility of using ZnO NPs in foodstuff of common carp to prevent zinc deficiency in the long term. This study aimed at evaluating the blood biochemical parameters, as a general health indicator, in common carp, treated with ZnO NPs in 5, 10 and 15 mg kg^{-1} feed in a 21-day experiment.

As far as we know, there are few studies on oral administration of zinc oxide NPs in fish (Connolly et al., 2016; Chupani et al., 2018; Dekani et al., 2019). Evidence suggests that diets containing zinc oxide NPs could provide a path to transfer this compound to upper levels of the food chains in aquatic organisms such as fish. Therefore, adding zinc oxide NPs may be useful to guarantee fish needs to zinc. However, it can have several potential complications, such as being intoxicated with zinc oxide NPs. Therefore, this study aimed at investigating the effects of oral

administration of zinc oxide NPs on specific blood biochemical parameters in common carp.

In this study, oral administration of 5, 10 and 15 mg kg^{-1} ZnO NPs did not cause any mortality during the experiment which indicates that ZnO NPs (< 15 mg per kg feed) were not acutely toxic to the survival of common carps. Ma et al. (2014) and Connolly et al. (2016) also showed low toxicity of ZnO NPs to *Scophthalmus maximus* and *Oncorhynchus mykiss*.

In the present study, we analyzed changes in blood biochemical parameters of common carp. AST, ALT, LDH, and ALP are found in cells of different organs such as the heart, kidneys, liver, skeletal muscles, brain, intestine, and gills as well as erythrocytes. The release of intercellular enzymes into the blood and their increased activity in plasma are the most important clinical signs in diagnosing damage to cell membranes (Rezaei Shadegan and Banaee, 2018; Hatami et al., 2019; Banaee et al., 2019). AST activity significantly increased in plasma of fish fed diets containing 10 mg kg^{-1} and 15 mg kg^{-1} ZnO NPs, whereas its activity remained near the control level in fish fed with 5 mg kg^{-1} ZnO NPs. Thus, oral administration of more than 5 mg kg^{-1} ZnO NPs increased the plasma AST activity which may reflect damage to liver tissue. ZnO NPs may indirectly cause oxidative stress and damage hepatocytes (Dekani et al., 2019). Moreover, the results indicate the increasing probability of oxidative stress with increased concentration of ZnO NPs in the diet. Previous studies show that oral exposure to ZnO NPs caused a significant increase in AST activity in plasma (Fazilati, 2013; Chupani et al., 2018).

An increase in AST and ALT in the liver, kidney, and blood of fish treated with ZnO NPs is reported (Taheri et al., 2017; Chupani et al., 2018; Dekani et al., 2019). The activities of increased ALT, LDH, and ALP in plasma of common carp significantly increases (P<0.05) indicating that liver damage might be induced by high levels of ZnO NPs (15 mg kg^{-1}) in the diet. The increased activities of LDH and ALP in plasma of fish fed with 15 mg kg^{-1} ZnO NPs may confirm the effect of ZnO NPs supplements on the cellular metabolic functions. However, plasma ALT, LDH and ALP activities were not affected in fish fed with 5 and 10 mg kg^{-1} ZnO NPs compared to the control group. Therefore, due to antiradical and antioxidant properties of ZnO NPs, its administration at doses lower than 15 mg kg^{-1} might prevent lipid peroxidation of cell membranes and inhibit the release of the enzymes described above into the plasma (Swain et al., 2016). Our findings support the previous results of Sharma et al. (2012), Fazilati, (2013), Najafzadeh et al. (2013), and Ansari et al. (2015). These authors observed a significant increase in the activities of liver enzymes in the blood of mice after oral exposure to ZnO NPs.

Changes in biochemical parameters such as glucose, total protein, albumin, globulin, creatinine, cholesterol, and triglyceride are indices of the physiological functions in different organs, including the liver, kidneys, intestine, and gills of fish. Therefore, any alterations in these clinical indices may indicate physiological disorders (Ahmadi et al., 2014; Nematdoost Haghi and Banaee, 2017). Although more than 60% of Zn binds albumin and is transported in the blood (Suttle, 2010), the present study shows that diets containing ZnO NPs have no obvious effects on total protein, albumin and globulin levels in plasma of common carp.

The results also showed that administration of ZnO NPs supplement had no significant effect on the plasma total protein, albumin and globulin levels. Sobhanirad and Naserian (2012) found that zinc supplement administration may have no increasing or decreasing effects on protein synthesis in the liver or total protein, albumin and globulin level (Sobhanirada and Naserian, 2012).

Our results also showed that 10 and 15 mg kg^{-1} ZnO NPs in the diet caused a significant increase in plasma glucose levels in plasma of common carp. Wijesekara et al. (2009), and Chabosseau and Rutter, (2016) found that zinc plays an important in insulin biosynthesis and secretion, and is concentrated in the pancreas. Thus, adequate Zn is essential for the regulation of blood glucose (Wijesekara et al., 2009; Chabosseau and Rutter, 2016; Olechnowicz et al., 2018). However, administration of high doses of Zn in

the diet can be toxic and increase blood glucose levels (Wijesekara et al., 2009). Moreover, an increase in blood glucose of fish may reflect an increased need for energy to counteract the effects of stress caused by ZnO NPs toxicity. Hyperglycemia or elevated blood glucose levels indicate impaired glucose uptake and lipid metabolism and degradation of glycogen in liver (Banaee et al., 2019).

The results of this study showed that the administration of 10 and 15 mg kg^{-1} ZnO NPs had a significant effect on cholesterol and triglyceride levels in plasma of common carp. High doses of zinc in the foodstuff may increase cholesterol level by decreasing HDL-cholesterol and apolipoprotein (apo) A-1 (Foster et al., 2010; Olechnowicz et al., 2018). A decrease in plasma Cu due to oral consumption of zinc may increase blood cholesterol level. Cu deficiency increases the activity of HMG CoA reductase and consequently increases cholesterol concentration (Foster et al., 2010).

Zinc is effective in lipids catabolism and therefore in providing energy from stored fat (Olechnowicz et al., 2018). A significant increase in plasma triglyceride in fish fed diets containing ZnO NPs may be the result of a disturbance in lipoproteins biosynthesis, increased rate of lipids catabolism, severe liver and kidney damage, and an increased rate of cell membrane lipid peroxidation. Degradation of fats stored in tissues to provide energy to deal with ZnO NPs toxic effects may be another reason for blood triglyceride level in fish.

Any increase in plasma creatinine levels is a biomarker of kidney damage because creatinine is usually removed from the blood and then excreted from the body (Soleimany et al., 2016; Banaee et al., 2017). The administration of a high dose of ZnO NPs (15 mg kg^{-1}) for 21 days caused a significant increase in creatinine levels in plasma of fish, indicating damage in the function and structure of the kidney. Other researchers reported similar results after ZnO NPs oral exposure (Ansari et al., 2015).

Conclusion

Following oral administration of ZnO NPs for 21 days, our findings demonstrated that ZnO NPs at 5 mg per kg feed did not have any adverse effects on the clinical characteristics of fish health. However, with an increase in ZnO NPs (10 and 15 mg kg^{-1}), significant changes were observed in specific blood biochemical parameters which may be due to ZnO NPs cellular toxicity. Therefore, by administering ZnO NPs and the consequent increase in the bioavailability of Zinc, disturbances may be found in the physiological function of cells. Further research should be done on the effects of ZnO NPs in nontoxic concentrations on other physiological indices such as growth, reproduction, the immune system in fish before using ZnO NPs as a food supplement in fish foodstuff.

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