Impact of silver nanoparticles on hepatic enzymes and thyroid hormones in striped catfish, *Pangasianodon hypophthalmus* (Pisces: Pangasiidae)

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ABSTRACT

Nanotechnology is the exploitation of physical, chemical, and biological characteristics of the particles with less than 100 nanometers in size. The most of the produced nanoparticles (56%) are composed of silver. The high consumption of these materials in industry and household products has led to their frequent release in aquatic ecosystems. The median lethal concentration (LC_{50}) and the impact of silver nanoparticles on liver enzymes (ALP, LDH, AST, ALT) and thyroid hormones (T4 and T3) in *Pangasius hypophthalmus* were investigated in the present study in three steps: At first, OECD (The Organization for Economic Cooperation and Development) protocols were used to determine the fatal levels of the silver nanoparticles (Ag NPs) in striped catfish. Second, semi-lethal concentration was found as 37.32 µg L^{-1} via regression test. In the last step, 168 fish received 0, 3.37, 7.46, 18.66 µg l^{-1} Ag NPs with three replicate. Six fish were randomly selected after 14 days from each replicate. Whole fish body extraction was used to measure the liver enzymes and thyroid hormones. The results suggested that due to the lower LC_{50} of Ag NPs in striped catfish, this species is more susceptible compared to various other fish species. Exposure to the silver nanoparticles with different concentrations significantly increased the levels of liver enzymes (ALP, LDH, AST, ALT) and also significantly decreased the T3, but no effect on T4.

Keywords: Silver nanoparticles, *Pangasianodon hypophthalmus*, LC_{50}, Liver enzymes, Thyroid hormones.

INTRODUCTION

Nanotechnology is a cut-edging technology that relates to the particle size ranging from virtually one to 100 nm in at least one dimension (Roco *et al*. 2000). The small size and surface/volume ratio in the nanomaterials lead to their higher reactive attributes compared to bulk or ionic counterparts. It is estimated that nearly 60,000 tons of nanoparticles with 1628 nano-based productions are produced annually in 30 countries (Khan *et al*. 2015) The global production of nanomaterials induces ecotoxicological risks in sediments and water bodies and it is one of the key issues for ecotoxicologists. Toxicological effects of the nanomaterials have been reported on the aquatic organisms such as fish, mollusks, and others. Even though, the impacts of nanomaterial toxicity on the aquatic environments and organisms are not well known (Johari *et al*. 2013; Johari *et al*. 2016; Clark *et al*. 2018).

Ag NPs are one of the most frequently used nanomaterials that have remarkably different physicochemical and biological characteristics compared to their ionic and bulk forms. Silver has been widely used for many targets such as monetary currency, jewelry, utensils, photography, dental alloy, explosives, etc. (Chen & Schluesener 2008). Moreover, it has been using as a bactericide and therefore it is also used for drinking and swimming pool water refinement (Tugulea *et al*. 2014). Toxicological aspects of Ag NPs are briefly mentioned (Park 2014). It is

reported that silver is one of the most harmful metals to aquatic organisms. However, considerable industrial losses occur rarely, its discharge in the aquatic environments is in conjunction with side effects on the aquatic's organisms. Acute toxic effects of nano-silver have been reported on fishes such as *Cyprinus carpio, Carassius carassius* and *Perca fluviatilis* (Bilberg *et al*. 2011; Hedayati *et al.* 2012). Most of the studies are in vitro and short-term on the toxicological and environmental effects of Ag NPs in aquatic animals (Farkas *et al*. 2011). A few investigations on nano-silver particles revealed its deleterious effects on liver enzymes. It is also reported that nano silver particles can cause tissue alterations (Wu & Zhou 2013), chromosome breakage, DNA breakage and oxidation, mutation and inflammatory responses (Chae *et al*. 2009; Choi 2009; Reijnders 2009; Singh *et al*. 2009). Moreover, some studies demonstrated that silver nanoparticles-induced oxidative stress is related to the activity of enzymes such as superoxide dismutase, catalase, glutathione peroxidase and lactate dehydrogenase (Kolayli & Keha 1999; Arora *et al*. 2008; Chen & Schluesener 2008).

It is well known that some blood parameters provide reliable indicators and information on fish health (Aldrin *et al*. 1982; Serpunin & Likhatchyova 1998). Therefore, some hematological parameters can be used as a biomarker for the assessing the effects of nanomaterials such as nano-silver particles. In the present study, Ag NP effects on *Pangasianodon hypophthalmus* (Sauvage 1878) were assessed by evaluating the alterations in thyroid hormones (T3, T4), and liver enzymes change assay: ALP, LDH, AST, ALT.

MATERIAL AND METHODS
Experimental animals
In this study 350 juvenile striped catfish (with 2.15 ± 0.11 g weight, 3.35 ± 0.17 cm length) were placed and kept in 25-L tanks with aeration at Qum Reproduction and Culture Center of Ornamental Fishes, Qum, Iran. The fish adaptation period was two weeks and feeding were stopped 24 h before the experiment. Water quality parameters including dissolved oxygen, temperature, and pH were measured daily (Table 1).

Table 1. Means of the water quality parameters during the experiment.

Photoperiod	pH	Temperature	Dissolved oxygen (ppm)
12 L:12 D	8.05 ± 0.11	30.21 ± 0.25	6.94 ± 0.77

Silver nanoparticle
Silver nano-powder (spherical 20 nm-particles (Fig. 1, 2), coated with 0.2 wt % polyvinylpyrrolidone surfactant (PVP, Iranian Nanomaterial Co., Mashhad, Iran) for low oxygen content and easy dispersing. Physicochemical properties of nano-silver included: True density: 10.5 g cm^{-1}, purity: 99.5%, APS: 20 nm, SSA: $\approx$ 18-22 m^2 g^{-1}, color: black and morphology: spherical.
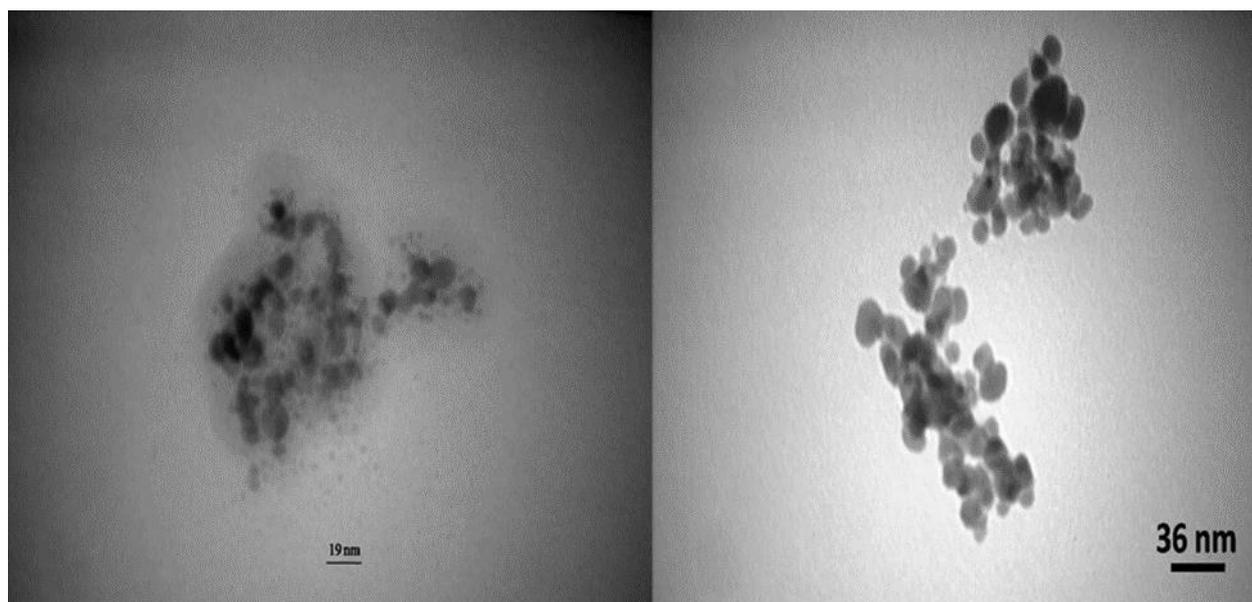
Fig.1. Representative TEM image of stock nano-silver suspension.
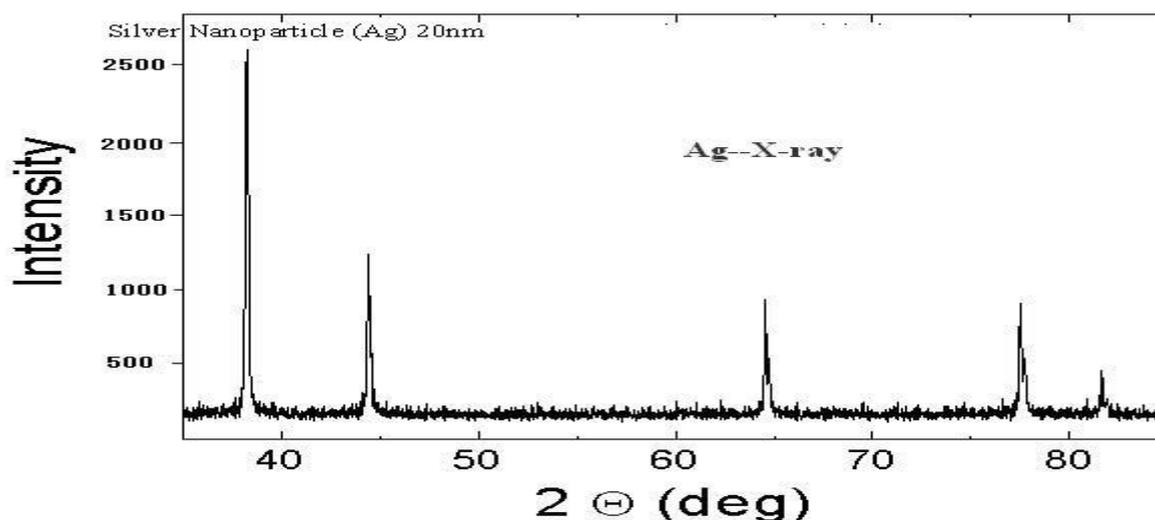
Fig. 2. Nano-silver powder X-ray diffraction pattern with the indexed diffraction lines of silver.

Experiment 1: Determination of the toxicity and fatal levels

Since the fatal levels of Ag NPs were unknown, OECD (The Organization for Economic Co-operation and Development) protocols were used to determine the fatal levels. After an adaptation period, Ag NPs with increasing concentrations of 0, 12.5, 25, 50 and 100 μg L^{-1} were added to the tanks each containing five fish. Behavioral changes and mortality rates were observed at 24, 48, 72 and 96 h after the addition of nanoparticles. The fatal range of Ag NPs was determined based on the first concentration causing the highest loss (100%), i.e. 25-50 μg L^{-1} in this study.

Experiment 2: Determination of the median lethal concentration (LC$_{50}$)

After determining the fatal range for Ag NPs, in the second step, the increasing concentrations (21, 25, 30, 36, 43 and 52 μg L^{-1}) of Ag NPs were added to aquarium fish (14 in each tank). Behavioral changes and mortality rates were observed at 24, 48, 72 and 96 h after exposure. During this period, physicochemical factors of the water were also measured and recorded. The mortality rates at each concentration were determined and the data were analyzed using the Probit regression model. 96-h LC$_{50}$ of Ag NPs was determined at 37.32 μg L^{-1}.

Experiment 3: Fish exposure to LC$_{50}$ of Ag NPs

Based on the LC$_{50}$ obtained in 96 h, 4 treatments were selected with 3 replications and 168 fish were placed in 12 reservoirs. Then, fish were exposed to the previously mentioned concentrations in 8 L- tanks for 14 days. There was not any water change and feeding during the exposure.

Treatment A (T$_{50\%}$): Addition of 50% LC$_{50}$ of Ag NPs
Treatment B (T$_{20\%}$): Addition of 1/5 LC$_{50}$ of silver nanoparticles
Treatment C (T$_{10\%}$): Addition of 1/10 LC$_{50}$ of silver nanoparticles
Control group (D): No Ag NPs added
Six fish were randomly selected, weighed and then prepared for biopsy from each replicate.

Biochemical measurements

Samples were homogenized into phosphate buffer fluid (samples were first weighed and placed in the buffer at the ratio of 1-part homogenized sample to 10 parts buffer). Then the samples were stirred with an 800 rpm electric stirrer for 1 min and centrifuged for 15 min at 4° C. The resultant extract was placed at -70°C.

Thyroid hormones

From each replicate, six fish were randomly selected, homogenized and placed into the buffer (the fish were first weighed and placed in the buffer with the ratio of 1-part sample to 10 parts buffer). The samples were crushed by sonic oscillation for 5 min and vortexed for 10 min. The samples were then centrifuged at 5000 rpm for 10 min at 4 °C. The supernatant was removed and kept at -70°C (Yu *et al.* 2010; Zhao *et al.* 2013). The T3 and T4 concentrations were quantified using ELIZA assay. The ELIZA wells were coated by the T3 or T4 antibodies

(capture antibody). The fish sample solutions (T3 and T4 antigen source) were then added to the well for incubation. A second antibody (known as detection antibody) was added and the antigen was sandwiched between two antibodies (sandwich ELIZA). Then an enzyme-linked secondary antibody was added, binding to the detecting antibody. At the next stage, substrate was added, converting by enzyme to detectable form for signal detection using ELIZA Reader.

Hepatic enzymes evaluation

Liver enzymes (AST, ALT, ALP, LDH) in 12 samples from 4 treatments (48 samples in total) were evaluated. Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assayed by the method of Reitman & Frankel (1957). Alkaline phosphatase (ALP) activity was measured according to Principato *et al.* (1985). Lactate dehydrogenase (LDH) was evaluated by the method of Cabaud & Wroblewski (1958).

Statistical analysis

Statistical analysis in this study is divided into two parts. At first, Probit regression model was used to estimate the Ag NPs LC_{50} and second, the Kolmogorov–Smirnov test was used to assess the normality of distributions. Parameters were compared using one-way analysis of variance (ANOVA) and Tukey's multiple range test at the significant level of 5%.

RESULTS

Determination of silver nanoparticles lethal levels

The number of losses at 12.5, 25, 50 and 100 μg L^{-1} Ag NPs is shown in Table 2. Based on the results, at 12.5 μg L^{-1}, no mortality was observed after 96 h and the fish did not exhibit any physical and behavioral abnormality. In the treatment with 25 μg L^{-1} Ag NPs after 96 h, no mortality was observed but the fish displayed abnormal behavior. At 50 μg L^{-1}, abnormal behaviors were observed after 24 h and, followed by beginning mortality after 48 hours. At 100 μg L^{-1}, 100% mortality occurred at the first 24 h. Therefore, the lethal concentration range of Ag NPs in striped catfish is from 25 μg L^{-1} with no mortality to 50 μg L^{-1} with 100% mortality after 48 h.

Table 2. Determination of Ag NPs lethal concentration range in *Pangasianodon hypophthalmus*.

Ag NPs concentrations (μg L^{-1})	Mortality rate (%) after 24 h	Mortality rate (%) after 48 h	Mortality rate (%) after 72 h	Mortality rate (%) after 96 h
12.5	0	0	0	0
25	0	0	0	0
50	0	100	-	-
100	100	-	-	-

Determining Median lethal concentration (LC_{50})

In order to determine the LC_{50}, the fish were exposed to 21, 25, 30, 36, 43 and 52 μg L^{-1} Ag NPs, then behavioral changes and mortalities were recorded at 24, 48, 72 and 96 h after exposure. The exposed fish exhibited a significant increased mortality rate by elevating in the Ag NPs concentration during 24 to 96 h (Table 3). Based on the observations, LC_{50} of Ag NPs in 94 h was 37.32 μg L^{-1} (Table 4).

Hepatic enzymes
Aspartate aminotransferase (AST)

The results demonstrated that by the increased Ag NPs concentrations, the AST levels raised significantly. In $T_{10\%}$, $T_{20\%}$ and $T_{50\%}$, the AST levels elevated significantly compared to the control group. The AST level in $T_{50\%}$ was also significantly higher compared to other treatments. There was not any significant difference between $T_{20\%}$ and $T_{10\%}$ (Fig. 3).

Hepatic enzymes
Aspartate aminotransferase (AST)

The results demonstrated that by elevating the silver nanoparticle concentrations, the AST levels also increased significantly. In $T_{10\%}$, $T_{20\%}$ and $T_{50\%}$, the AST levels elevated significantly compared to the control group. The AST level in $T_{50\%}$ was also significantly higher compared to other treatments. There was not any significant difference between $T_{20\%}$ and $T_{10\%}$ (Fig. 3).

Table 3. determination of median lethal concentration (LC_{50}) of the Ag NPs in *Pangasianodon hypophthalmus* in 96 h.

Ag NPs concentrations ($\mu g\ L^{-1}$)	Number of losses after 24 h	Number of losses after 48 h	Number of losses after 72 h	Number of losses after 96 h	Total number of losses after 96 h
21	0	0	0	0	0
25	0	0	7	0	7
30	0	0	1	0	1
36	0	1	7	0	8
43	0	1	3	0	4
52	0	5	7	2	14

Table 4. LC_5, LC_{10}, LC_{50} and LC_{90} of Ag NPs *Pangasianodon hypophthalmus*.

LC_5 ($\mu g\ L^{-1}$)	LC_{10} ($\mu g\ L^{-1}$)	LC_{50} ($\mu g\ L^{-1}$)	LC_{90} ($\mu g\ L^{-1}$)	p-value (significant)	95% confidence interval
18.34	21.45	37.32	64.91	<0.0001	2.896-7.765
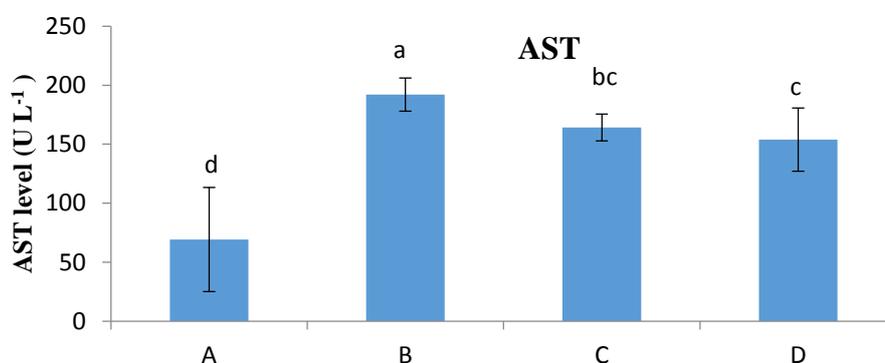
Fig. 3. Alterations in AST activity level ($U\ L^{-1}$) in the Striped catfish treated with Ag NPs (lower case letters on each bar indicate a significant difference between Treatments).

Alanine aminotransferase (ALT)

There were no differences in the alanine aminotransferase levels between $T_{20\%}$ and $T_{10\%}$, but these treatments exhibited significantly higher levels of ALT compared to the control and $T_{50\%}$. There was no difference between $T_{50\%}$ and the control group (Fig. 4).

Alkaline phosphatase (ALP)

Alkaline phosphatase level in $T_{50\%}$ was significantly higher compared to all other treatments and the control group. There was a significant difference between $T_{20\%}$ and $T_{10\%}$ but both of these treatments were not significantly different from the control group (Fig. 5).

Lactate dehydrogenase (LDH)

LDH level in $T_{50\%}$ was significantly higher compared to all other treatments and the control group. There was not a significant difference between $T_{20\%}$ and $T_{10\%}$ but these both treatments were significantly different from the control group (Fig. 6).

Thyroid hormones
Triiodothyronine

T3 level in $T_{20\%}$ was significantly lower compared to $T_{10\%}$ and the control group but there was not any difference between $T_{10\%}$ and the control group (Fig. 7).

Thyroxine (T4)

There was not any significant difference in T4 levels between the groups (Fig. 8).
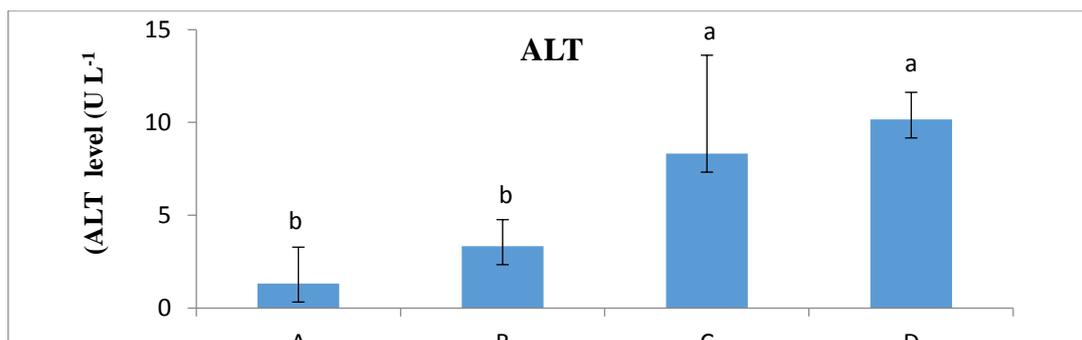
Fig. 4. Alterations in ALT activity level (U L^{-1}) in the striped catfish treated with Ag NPs (lower case letters on each bar indicate a significant difference between Treatments).
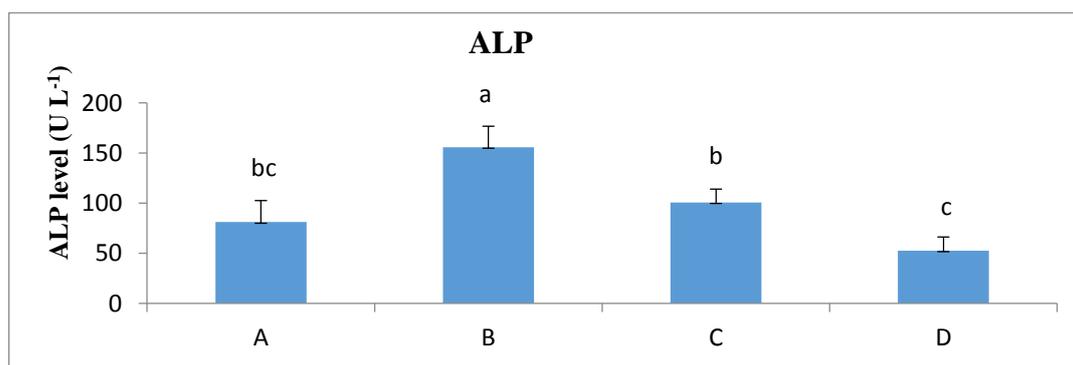
Fig. 5. Alterations in ALP activity level (U L^{-1}) in the striped catfish treated with Ag NPs (lower case letters on each bar indicate a significant difference between Treatments).
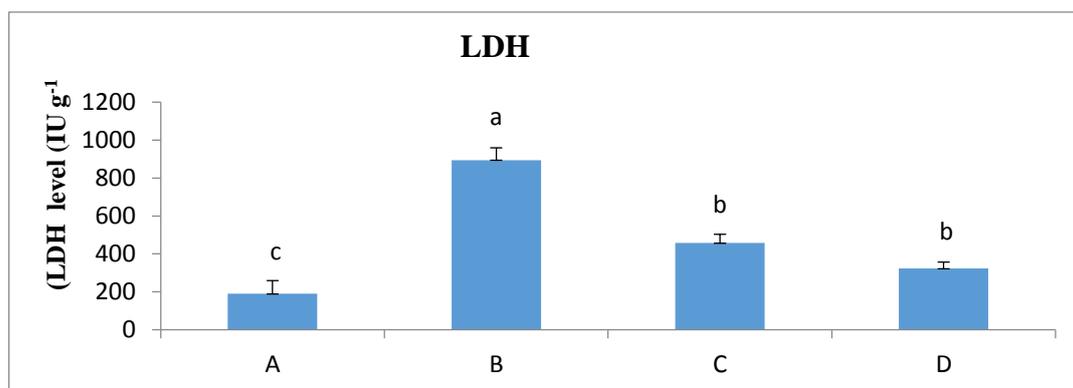
Fig. 6. Changes in LHD activity level in the striped catfish treated with Ag NPs (IU g^{-1}) (lower case letters on each bar indicate a significant difference between Treatments).
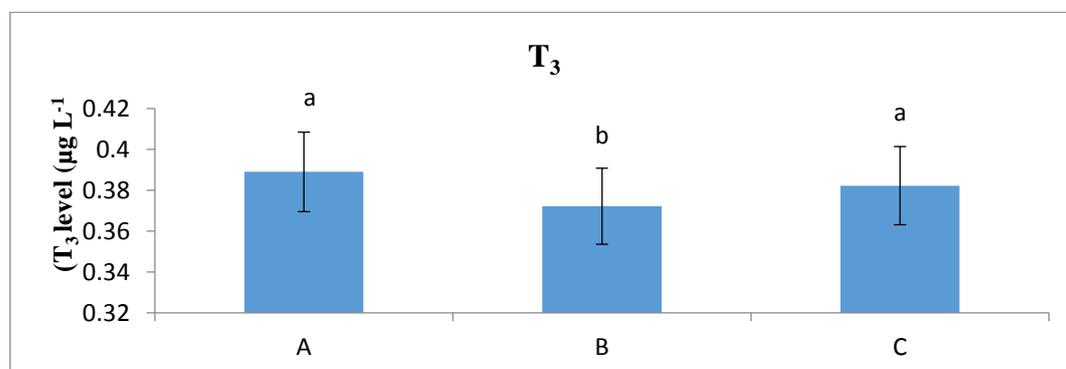
Fig. 7. Alterations in T3 activity level (µg L^{-1}) in striped catfish treated with Ag NPs (lower case letters on each bar indicate a significant difference between Treatments).
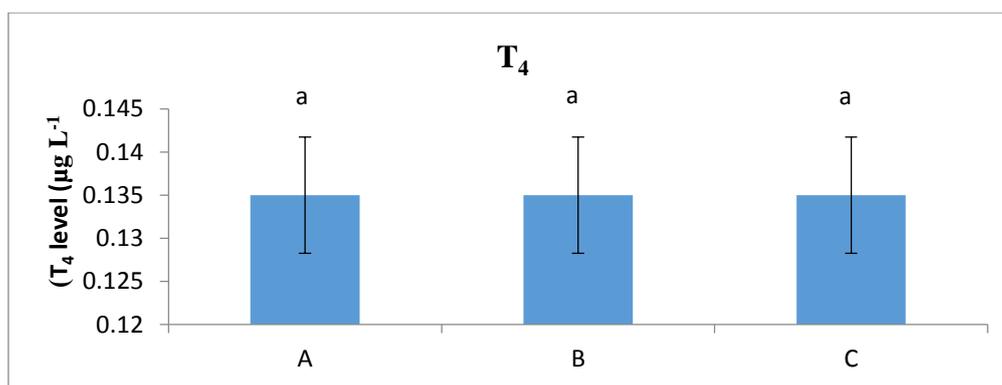
Fig. 8. Alterations in T4 activity level ($\mu g\ L^{-1}$) in striped catfish treated with Ag NPs (lower case letters on each bar indicate a significant difference between Treatments).

DISCUSSION

The expansion of nanotechnology applications in recent years has led to various environmental issues (Bar-Ilan *et al.* 2009). Despite the increasing use of nanoparticles in various industries, there are still limited reports about the toxicity of Ag NPs in the aquaculture industry (Griffitt *et al.* 2008). On the other hand, the excessive use of antibacterial agents such as antibiotics in aquaculture is a growing problem and finding suitable alternatives is one of the most challenging issues. Ag NPs are the most commonly known for their antibacterial effects, and due to these effects, it is possible to use them for aquatic health issues (Reynolds 2001). Therefore, it is necessary to find the lethal and the maximum permissible concentrations of these materials in different fish species. According to the European Union (UNION 2008) and Guide No. 67/548 / EEC of the Council of this Union, adopted on June 27, 1967 (Directive 1967), chemicals based on the average lethal concentration (LC_{50}) obtained from 96-h toxicological tests on fish, are categorized as follows: If the LC_{50} is less than 1 mg L^{-1}, the chemical is very toxic to aquatic organisms and has adverse effects on aquatic ecosystems. If the LC_{50} value is between 1 and 10 mg L^{-1}, the chemical is toxic to aquatic organisms and may have adverse effects on aquatic ecosystems, and eventually, if the LC_{50} is between 10 and 100 mg L^{-1}, the chemical is harmful to aquatic organisms and may have adverse effects on aquatic ecosystems. According to the results of the present study, the 96-h LC_{50} value for the Striped catfish was 37.32 $\mu g\ L^{-1}$ (0.03732 mg L^{-1}) (Table 4), which is toxic, and has adverse effects on this organism.

In the present study, the mean lethal concentration (LC_{50}) values obtained for the juvenile Striped catfish were lower than the values reported by Alishahi & Misbah (2010) on *Astronotus ocellatus* and *Heros severus* exhibiting the higher resistance of the Striped catfish to Ag NPs compared to these species. Numerous studies have revealed that cytotoxicity of nanoparticles is highly related to their size, structure, shape and properties of their surface (Park *et al.* 2006; Gill *et al.* 2007). Nanoparticles can pass through the physiological barriers and get to the different organs by the means of circulation system and cause physiological problems by interfering some cellular processes. The occurrence of disorders such as tissue necrosis, allergic sensitization, as well as impaired function of organs such as heart, liver and kidneys are among the main consequences of the exposure to nanoparticles in living organisms (Borm *et al.* 2006; Zahr *et al.* 2006; Singh *et al.* 2009). Some nanoparticles increase free radical production and lipid membrane oxidation leading to the structure damage and improper function of the liver (Rastogi 2012). Lasagna-Reeves *et al.* (2010) have also reported that a large amount of absorbed nanoparticle is accumulated in liver and spleen, causing tissue disorders and cell damage. There are numerous reports on the effects of nanoparticles, as well as various chemicals or drugs, on increased hepatic enzymes in organisms, especially fish species. It is demonstrated that administration of Ag NPs in rainbow trout, induced oxidative stress and oxidation of membrane lipids which is a sign of severe damage of the cell membrane. Cell damage with membrane degradation causes leakage and release of cytosolic contents, including enzymes into the bloodstream, as well as the increase in hepatic enzymes such as ALT, ALP, AST in blood serum in examined fish, which can be related to hepatic damage induced by oxidative stress. ALT, AST and ALP activity are often used to diagnose fish diseases and detect tissue damage caused by environmental pollution (Fırat *et al.* 2011). In rainbow trout, administration of nanoparticles led to their accumulation in the liver, decreasing the size and diameter of the hepatocytes, total protein decrease, hepatocyte necrosis, and consequently significant increase in hepatic enzymes (Monfared & Soltani 2013; Imani *et al.* 2015). Our findings on Striped catfish also in line with the results obtained

from rainbow trout and common carp on hepatic damages along with ALT and AST elevation levels caused by exposure to Ag NPs (Nemcsok & Benedeczky 1990; Monfared & Soltani 2013; Imani *et al*. 2015). Disorders in skeletal muscle, heart failure and cardiac disorders, branchial epithelium hyperplasia, branchial lamella adhesion, significant reductions in glomerular diameter and biochemical changes are some of other severe damages of Ag NPs mentioned in the literature (Monfared & Soltani 2013; Imani *et al*. 2015). As it is reported in some other species, in the present study, significant increases in LDH levels in fish exposed to Ag NPs may indicate an upraised conversion of lactate to pyrovate and consequently to sugars (Hori *et al.* 2006). Elevated ALP and other hepatic enzymes may be due to the cytotoxicity properties of Ag NPs, obstruction of the bile ducts inside or out of the liver, hepatic cirrhosis and disorders. Alterations in the permeability of the plasma membrane in hepatocytes may be the other reason for the upraised hepatic enzymes in serum levels. The increased concentrations of AST and ALP also may be due to elevated anabolism or reduced catabolism (Christ-Crain *et al*. 2004). The elevation of LDH concentrations in treated fish serum in the present study also is in accordance with results of Lee *et al*. (2012) about nanoparticle effects on *Cyprinus carpio.* Thyroid hormones (T3 and T4) are derived from the amino acid tyrosine synthetized by the thyroid gland in relation to iodide absorption. T3 is a metabolic hormone and its concentration in the plasma depends on the conversion of T4 to T3 affected by environmental conditions, especially the temperature. Conversion of T4 to T3 mostly happens in tissues such as the liver, kidneys, brain and pituitary. Thyroid hormones are essential for the proper and complete metabolism of cells in the body and the ultimate guarantee of the normal growth in the body (Guyton & Hall 2012). It has been shown that Ag NPs can be responsible for upraised thyroxin (despite our findings in this study) and decrease in TSH levels in rats. Moreover, Chronic sublethal exposure to Ag NPs disturbs thyroid hormone signaling during the *Xenopus laevis* metamorphosis (Carew *et al*. 2015). These particles likely affect the endocrine system by inhibiting the pituitary-hypothalamus axis (Afkhami-Ardakani *et al*. 2013). Figs. 7 and 8 illustrate alterations in T3 and T4 in the Striped catfish exposed to Ag NPs. Statistical analyses exhibited no significant decrease neither in T3 nor in T4. It has been shown that in fishes, pollutants reduce the rate of metabolism both directly by reducing the T3 levels and indirectly by elevating the cortisol concentrations in plasma (Carletta *et al*. 2002; Thangavel *et al*. 2005). There are three possible mechanisms in which Ag NPs defect T3 levels in the Striped catfish including: alterations in the activity of monodeiodinase, preventing the transcription of the genes which induce active forms of T3 and elevating the transcription of the genes which suppress T3. In addition, the decreased T3 levels in this fish may be due to an alteration in homeostasis or the function of this hormone when exposed to Ag NPs. In the present study, we observed a significant decrease in T3 but no significant alteration in the plasma T4 levels which is in line with results of Du *et al*. (2016) on the toxic effects of zinc oxide nanoparticles in zebra fish. Furthermore, the significant decreased plasma T3 levels due to the exposure of the striped catfish to Ag NPs can be considered as a distinction index between anabolic and metabolic energy supply. Since thyroid hormones are present in anabolic processes, the alterations in these hormones' levels may affect the physiological and metabolic status of this fish during the stress period. Finally, significant changes in thyroid hormone levels in short periods after exposure to Ag NPs exhibited that these indices could be suitable for assessing the environmental pollution and health status of the aquatic animals.

CONCOLUSION

In general, based on our results, it can be concluded that due to the lower LC_{50} of Ag NPs in Striped catfish, this species is more susceptible compared to various other examined fish species. In addition, Ag NPs at different concentrations significantly increased the hepatic enzymes (ALT, AST, LDH and ALP) in Ag NPs treatment compared to control group. Furthermore, these nanoparticles significantly decreased T3 level but had no effect on the plasma T4 concentration.

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اثرات نانو ذرات نقره بر آنزیم‌های کبدی و هورمون‌های تیروئیدی در ماهی پنگوسی گیاهخوار (*Pangasianodon hypophthalmus*)

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چکیده

فناوری نانو بهره‌برداری از ویژگی‌های فیزیکی، شیمیایی و زیستی مواد با اندازه‌های کمتر از ۱۰۰ نانومتر در علوم و صنایع مختلف هست. استفاده از فناوری نانو در حال گسترش است. قسمت بیشتر نانو ذرات تولیدی (۵۶٪) را نانو ذرات نقره تشکیل می‌دهند. با توجه به استفاده از این مواد در مصارف صنعتی و خانگی و نیز رهایش این مواد در بوم سازگان آبی در این پژوهش به بررسی غلظت کشنده میانی (LC₅₀) و تأثیر نانو ذرات نقره بر آنزیم‌های کبدی (ALT، AST، LDH و ALP) و هورمون‌های تیروئیدی (T3 و T4) در ماهی پنگوسی گیاهخوار (*Pangasianodon hypophthalmus*) پرداخته شد. بدین منظور ۸۴ عدد ماهی به‌مدت ۹۶ ساعت در معرض غلظت‌های ۲۱، ۲۵، ۳۰، ۳۶، ۴۳ و ۵۲ میکروگرم در لیتر نانو ذرات نقره قرار گرفتند و تلفات روزانه ثبت شد. نتایج توسط آزمون رگرسیون پروبیت بررسی و ثبت شد و غلظت نیمه کشندگی (LC₅₀) در این ماهی ۳۷/۳۲ میکروگرم بر لیتر به‌دست آمد. در مرحله بعد ۱۶۸ عدد ماهی در ۴ تیمار (۰، ۳٫۳۷، ۷٫۴۶، ۱۸٫۶۶ میکروگرم در لیتر) در سه تکرار قرار داده شدند. بعد از ۱۴ روز از هر تکرار ۶ عدد ماهی به‌صورت کاملاً تصادفی انتخاب شد. آنزیم‌های کبدی و هورمون‌های تیروئیدی سنجش شدند. نتایج نشان داد که نانو ذرات نقره در غلظت‌های مختلف باعث افزایش معنی‌دار آنزیم‌های کبدی (ALT، AST، LDH و ALP) و همچنین کاهش معنی‌دار غلظت هورمون T3 در ماهی پنگوسی می‌شود، اما تأثیری بر روی هورمون T4 ندارد.

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