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Chronic effect of waterborne colloidal silver nanoparticles on plasma biochemistry and hematology of rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Objective: To investigate the possible effects of silver nanoparticles (AgNPs) on some blood and plasma indices of rainbow trout (*Oncorhynchus mykiss*).
Methods: Hence, fish were exposed for 21 days to sub-lethal concentrations of colloidal AgNPs and blood parameters including erythrocyte size and hematocrit, plasma parameters including cholinesterase, cortisol, sodium, chloride, and potassium, and also silver concentration in plasma were measured following the 11th and 21st days of exposure.
Results: According to the results of present study, higher concentrations of AgNPs had more significant effects on plasma biochemistry and hematology of trout. The greatest impacts were decline of chloride ions and increase of cortisol and cholinesterase. Also fish exposed to AgNPs significantly increased silver concentration in the plasma.
Conclusions: Further studies are needed to identify appropriate blood biomarkers following fish exposed to nanomaterials, especially AgNPs.

1. Introduction

The rapid growth of nanotechnology in various areas of human life in recent decade created a global concern about the possible impacts of nanomaterials on environment and human health. To date, nano silver materials including Ag nanoparticles (AgNPs), Ag nanowires, Ag nanorods, *etc*. are the most important nanomaterials listed in consumer product inventories[1]. Keller *et al.* estimated annual entering of about 63 tons of silver nanomaterials into the water bodies[2]. Also Batley *et al.* estimated the presence of 0.03 to 0.32 μg/L silver nanomaterials in the aquatic environment[3]. Accordingly several researchers around the world are studying the possible effects of silver nanomaterials on terrestrial and aquatic organisms[4-19].

Several studies investigated the effect of AgNPs on hematological indices of fish. Vignesh *et al.* showed that the amount of hemoglobin and total count of blood cells considerably increased following exposure of *Labeo rohita* to AgNPs[20]. Johari *et al.* showed that acute exposure of rainbow trout [*Oncorhynchus mykiss* (*O. mykiss*)] to AgNPs reduced chloride and potassium, but increased cortisol and cholinesterase of blood plasma in a concentration-dependent manner[21]. Shaluei *et al.* showed that sub-acute exposure of silver carp (*Hypophthalmichthys molitrix*) to AgNPs decreased the amounts of red blood cell (RBC) count, hematocrit, and hemoglobin, but increased the amounts of white blood cell (WBC) count, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and cortisol[22]. Rajkumar *et al.* showed that a 7 days sub-acute exposure to AgNPs can impact on RBC and WBC of *Labeo rohita*[23]. Imani *et al.* showed that short term exposure of rainbow trout to AgNPs increased the values of RBCs, WBCs, and mean corpuscular hemoglobin concentration, but decreased the values of hematocrit, mean corpuscular volume, and mean corpuscular hemoglobin[24].

Accordingly, in the present study we evaluated the blood parameters including erythrocyte size and hematocrit, plasma

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parameters including cholinesterase, cortisol, sodium, chloride, and potassium, and also silver concentration in plasma of rainbow trout after a period of chronic exposure to sub-lethal concentrations of colloidal AgNPs.

2. Materials and methods

The AgNPs used in this study are in colloidal form and detailed characterizations of this commercial available nano product can be found in our previous studies[12,21].

Juvenile rainbow trout (*O. mykiss*) with a mean total body weight of (23.06 ± 1.66) g was used for the toxicity tests. Before the toxicity tests began, the fish were kept in fiberglass tanks filled with 1 000 L fully aerated, dechlorinated tap water with a daily water change and 12/12 h light/dark, and were fed with pellet feed (Chineh, Iran) at 1% of their body weight.

After one week of acclimation, the fish were transported to separate 90 L tanks, and allowed to adapt for one more day prior to beginning the toxicity tests. The chronic toxicity tests (21-day) were performed according to standard Organization for Economic Cooperation and Development TG 204 (fish, prolonged toxicity test) [25]. To evaluate the toxicity of AgNPs and determine the desirable concentrations for the chronic experiments, some preliminary tests were conducted (data not shown). Accordingly, three nominal concentrations, including 0.10, 0.25, and 0.50 mg/L of AgNPs, were selected for the chronic toxicity experiments. The fish were exposed to AgNPs in triplicate and based on a semi-static exposure regime and complete water change and re-dosing performed quartan. For each replicate, 10 healthy fish were directly transferred to exposure concentration (thirty fish per treatment). Control groups were also included (10 fish in 3 replicates in freshwater only).

Fifty milliliters of water sampled from the central part of the water column in each test vessel 48 h after dosing and actual concentrations of silver was measured after nitric acid digestion using a graphite furnace atomic absorption spectroscopy (PerkinElmer Analyst Model AA800).

Since feeding of fish during toxicity tests usually caused overestimating of the results (due to absorption of tested materials in the fecal material or food as well as increasing the dissolved organic carbon in water), fish were not fed until 48 h before starting and during the toxicity experiments[26]. However, to consider ethical issues and to avoid fish mortality due to starvation, 10 pellets per fish were given to all the fish on Days 12 and 16, one hour before the water change. All experimental procedures involving animals were conducted in accordance to Animal Welfare Act and Interagency Research Animal Committee guidelines[27], and approved by Research Committee of Marina Sciences Faculty of Tarbiat Modares University.

On Days 11 and 21, nine fish were randomly sampled from each concentration and control (three fish from each replicate). At concentration of 0.5 mg/L AgNPs, all fish died between 11th and 21st day and so the fishes of this treatment were not sampled at 21st day. Each fish individually was anesthetized with clove powder (100 mg/L) and whole blood (about 1 mL) collected via the caudal vein into heparinised syringes (heparin sodium, Rotexmedica, Germany). A very small drop was used for the preparation of blood smears on the glass slides. The smears were fixed using 95% methanol for 3 min, left to air-dry, stained with 10% Giemsa solution for 10 min; RBCs were observed and photographed using light microscopy. In each treatment, a total of 90 RBCs were selected randomly and their small (a) and large (b) axes were measured at magnification of 400 using AxioVision digital image processing software (Release 4.8.2.0, Carl Zeiss Micro Imaging GmbH, Germany). It should be noted that only the individual cells were selected so that they didn't overlap with any cell and also were completely elliptical in shape and appearance of their nucleus and cytoplasmic membrane was intact. The volume of RBCs (V) was calculated using ($V = 4/3\ л \times a^2 \times b$) formula.

The hematocrit was determined by centrifuging heparinized blood in microhematocrit capillary tubes at 10 000 r/min for 5 min. The remaining blood was centrifuged at 2 000 r/min for 10 min, and the plasma was removed and stored at –20 ˚C until analysis. The plasma Na^+ and K^+ were analyzed using a Jenway flame photometer (UK). The chloride and cholinesterase were analyzed using a Technicon AutoAnalyzer (USA), where the chloride was measured using a colorimetric technique and the Chemenzyme chloride reagent (Iran), while the cholinesterase was measured using an enzymatic technique and Parsazmun kits (Iran). The cortisol was measured using a radioimmunoassay technique with an LKB gamma counter (Finland), plus cortisol RIA kits from Immunotech (France).

Some of the blood plasmas were frozen at –20 ˚C to assess the silver accumulated in the plasma. For this purpose, before measuring the amount of silver, at a rate of 10% of the total volume of plasma, concentrated nitric acid was added to it. The silver concentrations in plasma were measured using a graphite furnace atomic absorption spectroscopy (PerkinElmer Analyst Model AA800).

The statistical analyses of the blood and plasma parameters were performed using SPSS statistics 17.0 software. All the data were tested for normality (Kolmogorov-Smirnov test) and analyzed using One-way ANOVA. The significant means were compared using Tukey's test and $P < 0.05$ was considered statistically significant.

3. Results

During the study, the mean water temperature, pH, and dissolved oxygen were (12 ± 2) ˚C, 8.20 ± 0.14, and (8.00 ± 0.24) mg/L, respectively. The measured silver concentrations in the test vessels containing nominal AgNPs concentration of 0.50, 0.25, and 0.10 mg/L were (0.67 ± 0.05), (0.30 ± 0.07), and (0.10 ± 0.03) mg/L, respectively.

The results of the hematological and plasma analyses were summarized in Table 1. On different days, the concentration of AgNPs showed no significant effect on hematocrit levels. Only on Day 21, at 0.25 mg/L AgNPs the hematocrit was significantly higher than 0.10 mg/L AgNPs and the control.

No significant difference of volume of RBCs was observable in different days and at different AgNPs concentrations. The only exception was the concentration of 0.25 mg/L AgNPs in the 21st day where volume of RBCs was significantly lower than other treatments. The amounts of potassium in blood plasmas were similar in all days and at different AgNPs concentrations. Plasma cortisol levels were similar in all AgNPs treatments and control. Only the plasma cortisol of 0.50 mg/L AgNPs in the 11th day was strongly higher than other treatments. In the control group, the plasma levels of sodium showed a significant reduction on Day 11 compared to the beginning day, but on the 21st day it was increased again by the same amount of the beginning day. On Day 11, with the increasing of AgNPs concentration, first the amount of sodium decreased, then increased, and then decreased again at the concentration of 0.5 mg/L AgNPs. On Day 21, the amount of sodium was not significantly different between

Table 1
Plasma biochemical and hematological parameters of rainbow trout (*O. mykiss*) chronically exposed to colloidal AgNPs.

Parameters	Sampling day							
	0	11				21		
NAC (mg/L)	0.00	0.00	0.10	0.25	0.50	0.00	0.10	0.25
RBCs volume (μm^3)	5 819 ± 1 334^{bcd}	6 225 ± 1 114^{d}	6 138 ± 1 226^{cd}	5 778 ± 1 461^{bcd}	5 668 ± 1 156^{bcd}	5 598 ± 1 301^{bc}	5 332 ± 1 172^{b}	4 745 ± 1 051^{a}
Hematocrit (%)	50.6 ± 2.1^{a}	49.5 ± 2.2^{a}	53.8 ± 3.5^{ab}	53.6 ± 3.3^{ab}	54.0 ± 3.4^{ab}	46.4 ± 2.6^{a}	49.4 ± 3.9^{a}	59.5 ± 5.2^{b}
Cholinesterase (IU/L)	262.00 ± 5.35^{ab}	258.00 ± 3.20^{abc}	264.00 ± 6.23^{c}	266.00 ± 2.94^{c}	352.00 ± 6.95^{e}	245.00 ± 2.05^{a}	248.00 ± 1.20^{ab}	319.00 ± 3.74^{d}
Chloride (mmol/L)	123.00 ± 0.81^{def}	120.00 ± 0.40^{de}	119.00 ± 0.36^{d}	112.00 ± 0.81^{c}	100.00 ± 3.25^{b}	125.00 ± 1.63^{ef}	127.00 ± 1.25^{f}	95.00 ± 0.47^{a}
Cortisol (ng/mL)	22.00 ± 2.62^{a}	27.00 ± 1.24^{a}	23.00 ± 2.05^{a}	17.00 ± 4.32^{a}	157.00 ± 3.10^{b}	18.00 ± 2.45^{a}	23.00 ± 1.63^{a}	18.00 ± 6.80^{a}
Sodium (mmol/L)	151.00 ± 1.63^{c}	140.00 ± 0.84^{b}	129.00 ± 0.82^{a}	138.00 ± 0.80^{b}	132.00 ± 0.76^{a}	149.00 ± 0.71^{c}	148.00 ± 0.56^{c}	151.00 ± 0.81^{c}
Potassium (mmol/L)	0.97 ± 0.04^{a}	0.90 ± 0.08^{a}	0.90 ± 0.15^{a}	0.90 ± 0.07^{a}	0.94 ± 0.05^{a}	0.90 ± 0.08^{a}	0.96 ± 0.12^{a}	0.94 ± 0.04^{a}

In each column, the numbers with different superscript letters differ significantly ($P < 0.05$). NAC: Nominal AgNPs concentration.

the treatments containing AgNPs and the control group. On Day 11, the amount of chloride at 0.10 mg/L AgNPs had no significant differences compared to the control group, but significantly reduced in 0.25 and 0.50 mg/L AgNPs. On Day 21, the amount of chloride at 0.10 mg/L AgNPs was significantly similar to the control group, but significantly reduced in 0.25 mg/L AgNPs. The amounts of cholinesterase in 0.10 and 0.25 mg/L AgNPs on the 11th day were significantly similar to the control group, but significantly increased in 0.50 mg/L AgNPs. Also on the 21st day, the amount of cholinesterase in 0.25 mg/L AgNPs was significantly higher than 0.1 mg/L AgNPs and the control group.

Silver levels measured in the plasma of exposed fish to all concentrations of AgNPs were about 32–68 times higher than to the controls (beginning day) (Table 2). On Day 11, accumulated silver in the 0.10 and 0.50 was significantly higher than 0.25 mg/L AgNPs (0.50 = 0.10 > 0.25 > 0.00). Also on Day 21, silver concentration in 0.25 was significantly higher than 0.10 mg/L AgNPs (0.25 > 0.10 > 0.00).

Table 2
Silver bioconcentration in the plasmas of rainbow trout (*O. mykiss*) chronically exposed to colloidal AgNPs.

Sampling day	Nominal AgNPs concentration (mg/L)	Silver concentration in blood plasma (μg/mL)
0	0.00	0.004 5 ± 0.000 5^{a}
11	0.10	0.202 0 ± 0.017 3^{d}
	0.25	0.145 5 ± 0.000 0^{b}
	0.50	0.202 4 ± 0.003 6^{d}
21	0.10	0.170 6 ± 0.011 1^{c}
	0.25	0.305 7 ± 0.003 9^{e}

The numbers with different superscript letters differ significantly ($P < 0.05$).

4. Discussion

Primary mechanism of ionic silver toxicity in fish is disruption of ion regulation in the gill surface which causes reduction of some of the plasma ions such as sodium and chloride[28]. Also increase of plasma glucose and cortisol by increasing the concentration of silver ions in water has been reported by different researchers[29,30]. Also increase of cortisol and calcium as well as decrease of sodium and chloride have been reported in other stress conditions[31].

In the present study, significant increase of hematocrit was only in the last day of sampling at 0.25 mg/L AgNPs. Principally the increase in hematocrit in terms of stress occurred due to the migration of RBCs from the spleen into the blood or swelling of RBCs[31]. Since the increase in hematocrit in this study was associated with a significant reduction in volume of RBCs, it can be concluded that the reason for this increase, has been increasing the number of RBCs in order to better cope with stress. Also the amount of plasma cortisol just showed a sharp increase in 0.50 mg/L AgNPs (11th day), which can be a sign of the fish response to the stress due to exposure to AgNPs. Increase of plasma cortisol in the short term is a good response to stress and leads to the release of RBCs into the circulatory system and stimulates the absorption of required ions by the gills[32].

In the present study, the reduction of plasma chloride that was observed in higher concentrations of AgNPs, on the one hand may be due to the dysfunction of ion regulation by the gills, and on the other hand it is possible that due to the reaction of silver ions and chloride ions in the water, chloride ions are removed from the water columns and thus go out of the reach of fish and as a result, chloride ions are reduced in the plasma. Proving this hypothesis requires more detailed studies and measurements of possible compounds made in the water (such as AgCl). Although the results of the present study showed fluctuations of plasma sodium in response to different concentrations of AgNPs, Schultz *et al.* reported the inhibition of sodium uptake in rainbow trout following exposure to AgNPs[33].

In the highest concentrations of nanoparticles in this study, plasma cholinesterase levels showed a significant increase both on the 11th and 21st days. Acetylcholinesterase is an enzyme that hydrolyzes the neurotransmitter acetylcholine at synapses. The importance of this enzyme is reversibility of reactions that occur in the central nervous system. Certain pesticides, such as carbamates and organophosphorus, are known to selectively inhibit cholinesterase activity[34]. Increase of brain cholinesterase has been reported in rainbow trout[35] and gilthead bream[36] following exposure to sub-lethal copper concentrations. Also Johari *et al.* reported the sharp rise of cholinesterase in rainbow trout following 3 h exposure to lethal concentrations of colloidal AgNPs (1–8 mg/L) and proposed the potential use of cholinesterase as a reliable biomarker for toxicity assessments of AgNPs[21].

The results of this study demonstrated a high bioconcentration capacity of silver in the circulatory system of rainbow trout following exposure to AgNPs which could then be transferred to other tissues and organs. In general and based on the results of this study, the effects of AgNPs on examined blood and plasma indices have been observed mostly in higher concentrations of this substance. More studies are needed to find the most important and sensitive biomarkers in response to effect of AgNPs on fish.

Conflict of interest statement

We declare that we have no conflict of interest.

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