

Toxicity effect of colloidal silver nanoparticles on fertilization capacity and reproduction success of rainbow trout (*oncorhynchus mykiss*)

Abstract

Due to the increasing use of nanomaterials in human daily life and probable release of these substances into the environment, the present study investigated the effect of the presence of colloidal silver nanoparticles (cAgNPs) in the aquatic environment on the reproduction of rainbow trout as a model aquatic organism. Chemically produced cAgNP colloid was purchased from Pars Nano-Nasb (Iran) and contained 4000mg/l silver nanoparticles in deionised water. According to the transmission electron microscopy (TEM) results, the Count median diameter of nanoparticles in the colloid was 6.47 nm. Rainbow trout eggs and sperm were obtained by stripping the fish. For each treatment 500 eggs were completely mixed with 1µl of milt. In the control group (0 mg/l cAgNPs), water was added to activate sperm motility and leading to fertilization. To evaluate effect of AgNPs on fertilization, water containing three concentrations of cAgNPs including 0.1, 0.01 and 0.001 mg/L were added for sperm activation. Resulted eggs were incubated in a recirculation incubator system at a constant temperature of 10°C. Survival rate from 24 hours post-fertilization to the eyed-stage (eyeing rate), survival from eyed-stage to hatching (hatching rate), and survival from hatching to the end of yolk sac absorption were monitored. The results showed that the eyeing rate of all cAgNPs exposed treatments were same as the control; while the hatching rates of all cAgNPs exposed treatments was significantly lower than the control ($0 > 0.001 > 0.01 > 0.1$ mg/l). A significant decrease was observed in the survival at the end of yolk-sac absorption only in the 0.1 mg/l cAgNPs group. Also significant and dose-dependent decrease in duration of sperm motility was observed after *in vitro* sperm exposure to cAgNPs ($0 > 0.001 > 0.01 > 0.1$ mg/l). According to these results, presence of 0.1 mg/l cAgNPs in the water environment can affect the fertilization and reproduction success of rainbow trout. Thus, the presence of silver nanoparticles in the aquatic environment can interfere with fish reproduction and thereby fish populations will be at risk.

Keywords: Aquatic Nanotoxicology, Colloid, Rainbow trout, Silver nanoparticles, Sperm Motility, Eyeing Rate, Hatching Rate

Abbreviations: cAgNPs, Colloidal Silver Nanoparticles; CASA, Computer Assisted Sperm Analyzer; NTA, Nanoparticle Tracking Analysis; TEM, Transmission Electron Microscopy

Introduction

Although the development of modern technologies will provide better opportunities for human life and health, on the other hand, these technologies are usually causing damage to the environment. Aquatic ecosystems as a part of the environment are constantly exposed to a variety of physical and chemical pollutants, which amount of these contaminants is increasing with the development of technology. In this respect, aquatic toxicology is responsible for studying the effects of organic and inorganic pollutants on aquatic organisms. Although nanotechnology has been used for many years in human life, but its scientific understanding and development has greatly expanded in recent decades; and it is anticipated that many human needs to be answered through this technology over the coming years. Based on the statistics obtained from thirty countries of the world, the number of consuming products by human being in which the nanomaterials are used, has increased from 54 in 2005 to 1628 in 2013 and it is predicted that this number would be rapidly increasing in future years too.¹ According to forecasts, the market of products containing nanomaterials will be worth \$ 1 trillion by 2015.²

Silver nanoparticles due to the unique features such as antimicrobial

properties, are considered as the most important and most widely used man-made nanomaterials and are used in the different types of products such as cosmetics, detergents, clothes, shoes, food additives, as well as coating on respiratory masks, water filters, phones, laptops, toys, and home water treatment systems (such as Aquapure, Kinetico and QSI-Nano). One of the types of products that contain silver nanoparticles today as antimicrobial disinfectant and are widely available on the market, colloid silver nanoparticles. The colloidal silver nanoparticles (cAgNPs), is one of the antimicrobial products which is widely available on the market. For example, in Iran a variety of silver nanoparticle colloids are produced with brand names such as "NanoCid", "NanoNip", "NanoFarm", "NanoClean" NanoSilveral" etc. According to estimates 63 tons of silver nanoparticles released into the aquatic ecosystems annually.³ Moreover, the concentration range of silver nanoparticles in aquatic biomes has been estimated as 0.03 to 0.32µg/l.⁴ Thus, understanding the potential effects of this nanomaterial on aquatic organisms is very important. Because of the external fertilization in most of fish, gametes may be exposed to contaminants in the water, which could affect their reproductive success. Because the duration of sperm motility in fish is very short (for example, between 30 and 60 seconds and sometimes a little more in rainbow trout), fertilization should occur as soon as possible after the release of gametes in the aquatic environment; therefore if external factors affect this process, fertilization success changes subsequently. In a study it showed that concentrations higher than 1 mg/l of methyl

mercury reduce the viability of trout sperm.⁵ Another study also showed reduction of the fertilization ability of rainbow trout sperm after exposure to 10 mg/l of mercury or cadmium.⁶ Several indices are used to measure the strength of sperm fertilization, for example: the speed and duration of sperm motility, fertilization rate and hatchability.⁷ In many studies, the sperm motility have been used as an accurate and sensitive biological indicator to assess the impact of pollutants on sperm cells and computer software such as CASA is used to analyze motility data.⁸ Although this is a very quick and simple method for measuring the toxicity, it could not explicitly show the success rate of fertilization by sperm.⁹ Therefore, the use of other indicators (fertilization rate, hatching rate, etc.) is more efficient to assess the impact of environmental pollutants on fish reproduction capability.^{10, 11}

While several studies in recent years have focused on the toxic effects of silver nanoparticles in different aquatic organisms (e.g. fish,¹² daphnia,¹³ algae,¹⁴ mussels,¹⁵ etc.), however, to our knowledge there is no report on the effects of nano-silver on fish reproduction. Accordingly, this study examined the effect of colloidal silver nanoparticles on fertilization capacity and reproduction success of rainbow trout (*Oncorhynchus Mykiss*).

Materials and methods

Nanomaterial and characterization

This study used colloidal silver nanoparticles (cAgNPs) which are commercially available in the Iranian Market. This commercial nanomaterial was chosen because we wanted to study on a material that is currently marketed and most likely is included in consumer products, and therefore can be relevant for future risk assessments. Detailed characterizations of this product can be found in our previous study,¹² and are briefly shown in (Table 1). Figure 1 shows TEM image of the particles (prepared by Hitachi H-7100FA). Also the hydrodynamic diameter distribution of silver particles in colloid was measured by using NTA system (model LM10-HS) it was 63 ± 24 nm.

Table 1 Characterizations of silver nanoparticles used (derived from Johari et al., 2013 [12])

Brand	Nanocid L2000	
Manufacturer	Nano Nasb Pars Co., Ltd (Tehran, Iran)	
Appearance	Yellowish-brown Colloid	
Manufacturer	Size	16.6
Information	Concentration (mg/l)	4000
Information from TEM*	Max. Diameter (nm)	129
	CMD* (nm)	6.47
	GMD* (nm)	12.65
	GSD*	1.46
Information from DLS*	Shape	Spherical
	Zeta potential (mV)	-53.33 \pm 7.86
Information from ICP-AES*	Hydrodynamic diameter (nm)	54.8
	Concentration (mg/l)	3980
	pH	2.4

* CMD: Count Median Diameter; GMD: Geometric Mean Diameter; GSD: Geometric Standard Deviation; TEM: Transmission Electron Microscope; DLS: Dynamic Light Scattering; ICP-AES: Inductively Coupled Plasma-Atomic Emission Spectroscopy

In vivo experiments

The ova and milt of five male and five female rainbow trout were used for this study. To avoid the influence of genetic differences between selected females on test results, all ova were pooled right prior to fertilization and then about 500 ova were taken for each

treatments. The coelomic fluid was separated by a mesh prior to mixing the ova and milt. For fertilization of each batch of ova, 1 μ l of milt of each male was added and mixed well with ova (a total of 5 μ l milt for each 500 ova per treatment). To activate sperm motility and fertilization in the control group, normal water (without any chemicals) with a temperature of 10°C was used. In order to evaluate the effect of cAgNPs on the fertilization capacity, in the other treatments of this study water containing different concentrations of cAgNPs (0.001, 0.01, 0.1 mg/l) was used to induce sperm motility. After the end of fertilization, the eggs were washed several times with normal water to remove any extra sperm. Then the eggs were allowed to absorb water for 60 min. Obtained eggs from different treatments were incubated in a recirculation incubator to pass embryonic stages. The water temperature of this incubator was kept constant at 10°C by an automatic cooling system. In order to reduce the concentration of waste products in water (especially ammonia compounds), 10 percent of water were replaced daily. Required oxygen for the eggs was supplied by air stones connected to an air pump. Various indices were calculated during the incubation period, including survival rate from 24 hours post-fertilization to the eyed-stage (eyeing rate), survival from eyed-stage to hatching (hatching rate), and survival from hatching to the end of yolk sac absorption.

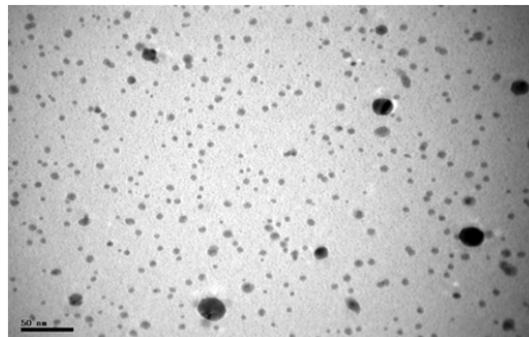


Figure 1 TEM micrographs of colloidal silver nanoparticles.

In vitro experiments

After performing all fertilization processes related to *in vivo* experiments, in a complementary experiment, to evaluate the effect of silver nanoparticles on rainbow trout sperm cells *in vitro*, duration of sperm motility was evaluated following exposure to normal water (control group) or water containing 0.001, 0.01 and 0.1 mg/l cAgNPs. For this purpose, for each of the above treatments the milt sample of three male was used (each one as a replicate). First, 1 μ l of semen was dropped onto a clean-dry slide and after putting a cover slip on it and putting it under a preset light microscope, 1 μ l of activator water (from control or treatments) was poured to activate spermatozoa. Then the duration of spermatozoa motility was assessed from the moment of contact with the water until the end of mobility of about 90% of spermatozoa.¹⁶

Statistical analysis

Statistical analysis of data was performed using SPSS and normality of the data was evaluated by Kolmogorov-Smirnov test. Statistical analyses were carried out using standard ANOVA techniques, followed by Duncan's significant difference test. Differences were statistically significant when $p < 0.05$.

Results

The results of reproductive success indices of rainbow trout following fertilization the sperm and ova in water containing different concentrations of silver nanoparticles are briefly shown in (Table

2). Based on these results, no significant difference was observed in the case of eyeing rate of eggs between different concentrations of cAgNPs and the control group (eyeing rate of $0=0.001 = 0.01=0.1$ mg/l). On the other hand, the hatching rate in the control group was significantly higher than that of the exposed treatments to cAgNPs and as shown in (Table 2), with increasing the concentrations of silver nanoparticles, the percentage of hatching eggs showed a significant decrease (hatching rate of $0 > 0.001 > 0.01 > 0.1$ mg/l). Although survival rate from hatching to the end of yolk sac absorption in the treatments containing low concentrations of silver nanoparticles was similar to the control group, however this amount was significantly lower in the treatments containing 0.1 mg/l. Measurement results of duration of spermatozoa motility of rainbow trout after activation with water containing different concentrations of silver nanoparticles are shown in (Table 3). According to these results, the duration of sperm motility in the control group was significantly longer than that of spermatozoa cells that had been activated with water containing silver nanoparticles. Also, with increasing concentration of silver nanoparticles, duration of sperm motility was reduced ($0 > 0.001 > 0.01 > 0.1$ mg/l).

Table 2 The indices of reproductive success of rainbow trout, following fertilization of sperm and ova in water containing different concentrations of colloidal silver nanoparticles

Concentrations of silver nanoparticles in water (mg/l)				
0.1	0.01	0.001	0 (control)	
55.91 ± 0.29^a	54.79 ± 1.43^a	55.26 ± 1.08^a	56.72 ± 0.54^a	Eyeing rate of eggs
29.38 ± 2.63^d	33.91 ± 0.86^c	35.50 ± 0.58^b	40.56 ± 1.38^a	Hatching rate of eyed eggs
54.39 ± 0.81^b	56.66 ± 1.33^a	57.69 ± 0.51^a	57.18 ± 0.64^a	Survival rate from hatching to the end of yolk sac absorption

Table 3 Duration of spermatozoa motility of rainbow trout after activation with water containing different concentrations of colloidal silver nanoparticles

Concentrations of silver nanoparticles in water (mg/l)				
0.1	0.01	0.001	0 (control)	
56.33 ± 12.09	69.00 ± 28.16	71.00 ± 24.82	82.33 ± 26.57	Duration of spermatozoa motility (seconds)

Discussion

According to the results of *in vitro* experiments, the toxic effects of silver nanoparticles on rainbow trout sperm cells is evident clearly as a concentration-dependent decrease in duration of sperm motility. In this regard, also the results from *in vivo* experiments were often concentration-dependent and the only exception was the percentage of eyeing rate of eggs. Therefore when testing the effects of toxic chemicals on fish fertilization success, although the sperm motility assay is a rapid, simple and striking method, but the use of other indicators, such as examination of fertilization rate, eyeing rate, hatching rate and survival rate of larvae, can better show prediction of the effects of pollutants on fertilization capacity of the egg by the sperm. More studies are needed to understand the mechanism by which the silver nanoparticles affect the fertilization of the egg and sperm of fish. Although no information is available about the effect of silver nanoparticles on fertilization and reproduction of fishes, but

in a study about aquatic organisms, it has been shown that although fertilization capability of sea urchin sperm is not influenced by silver nanoparticles, their offspring exhibit morphological abnormalities.¹⁷ In few studies which have been conducted on mammalian sperm, signs of toxic effect of silver nanoparticles are observed. For example in a study by Moretti et al.¹⁸ it has been shown that silver nanoparticles exerted a significant dose-dependent effect on motility and viability of human spermatozoa.¹⁹ Also Asare et al.²⁰ indicated toxic effect of silver nanoparticles against human testicular embryonic carcinoma cell line as well as primary testicular cells of mice.²⁰ In another study by Gromadzka-Ostrowska et al.¹⁹ it have been shown that injection of rats with nano silver cause a size-dependent, dose-dependent and time-dependent decrease of the epididymal sperm count as well as increase in the level of DNA damage in germ cells.²⁰

Conclusion

Totally, the results of this study indicate that silver nanoparticles influence on trout fertilization and also on survival of larvae in stages after fertilization. Therefore more attentions are needed in relation to the prevention of waste containing silver nanoparticles to aquatic ecosystems and developing environmental standards for determining permissible levels of nanomaterials in aquatic ecosystems.

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Conflicts of interest

None.

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