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ORIGINAL ARTICLE

The effect of silver nanoparticles and silver ions on zebrafish embryos (*Danio rerio*)

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Abstract

OBJECTIVES: The aim of this study was to establish and evaluate the mortality rate, hatching rate and observe the presence of sublethal changes in zebrafish embryos after exposure to silver ions and nanoparticles.
METHODS: Tests were conducted on newly fertilized zebrafish embryos, according to the modified OECD guideline 236, using a semistatic method and 96 hour incubation time. Silver nitrate and two different silver nanoparticles, stabilized with 0.01% solution of maltose and gelatine in the first case, and stabilized with polyvinylpyrrolidone, in the latter, were tested.
RESULTS: Significant differences in toxicity of tested substances were recorded. The value of 96hLC50 for silver nitrate was 58.44 μg/L. The value of 96hLC50, calculated for silver nanoparticles stabilized with 0.01% solution of maltose and gelatine, was nearly 100 times higher, 4.31 mg/L. The value 96hLC50 for silver nanoparticles stabilized with polyvinylpyrrolidone exceeded 100mg/L, occurrence of sublethal effects caused by silver nanoparticles stabilized with polyvinylpyrrolidone was insignificant in most of the exposition groups, but only in this substance caused decreased hatching rate.
CONCLUSION: Properties of different silver nanoparticles play an important role in levels of their toxicity and predominant mechanisms of action. In general, silver nanoparticles are less toxic for *Danio rerio* embryos than silver ions.

Abbreviations:

$AgNO_3$	- silver nitrate
AgNPs	- silver nanoparticles
AgNP M&G	- silver nanoparticles stabilized with 0.01% solution of maltose and gelatine
AgNP PVP	- silver nanoparticles stabilized with polyvinylpyrrolidone
ROS	- reactive oxygen species
OECD	- Organisation for Economic Co-operation and Development
96h LC50	- 96-hour lethal concentration 50

INTRODUCTION

The field of nanotechnology has been dynamically developing since the 1980's, when the first nanoparticle, fullerene was synthesized (Shampo *et al.* 2010). Nowadays, significant advancement in the field of nanotechnologies is noted. There is a great demand for nanotechnologies, which is already multi-billion dollar market and is expected to grow rapidly (Sabourin & Ayande, 2015). Silver nanoparticles (AgNPs) belong amongst the most frequently used nanoparticles. For example, they find their use in medicine, agriculture, construction and production of wide variety of consumer goods, such as cosmetic products, packaging, electronics and textiles. (European Commission, 2014). The annual worldwide production is estimated to 320 tons per year, thanks to their characteristics, such as antibacterial, antivirotic and antimycotic activity, good electrical and thermal conductivity and nonlinear optical properties (Nowack *et al.* 2011; Shaalan *et al.* 2016). Nanoparticles can also arise from natural synthesis, Hou *et al.* (2013) reported their formation by reduction of silver ions by natural organic matter, driven by sunlight.

With their growing usage, a rise of concentrations of AgNPs in the environment is expected. Their predicted environmental concentrations in European rivers are 0.03–0.08 μg/L (Mueller & Nowack, 2008). There is a concern, that the presence of AgNPs in the ecosystem might have a negative impact on nontarget species, such as fish. The effects of AgNPs are frequently compared to the effects of silver ions, which are one of the most toxic ions for fish, causing disruption of osmoregulation, due to the inhibition of gill Na^+/K^+-ATPase (Hogstrand & Wood, 1998). In case of AgNPs, the exact toxicological mechanisms are not completely understood yet. There is also a wide range of reported LC50 values – ranging from tens of micrograms to tens of milligrams per liter (Bilberg *et al.* 2012; Katuli *et al.* 2014).

Thus, we conducted Fish Embryo Acute Toxicity Tests by the modified OECD guideline 236 (OECD, 2013). This test is a suitable alternative to the Acute Fish Toxicity Test by guideline OECD 203 (Belanger *et al.* 2013). The substitution of animal testing with an alternative method is in line with requirements of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. An embryo in its early stages of development is unable to perceive pain and suffering, therefore is not protected by the legislation of European Union (Embry *et al.* 2010).

The aim of our study was to establish the mortality rate, hatching rate and observe the presence of sublethal changes in zebrafish embryos. We tested three different solutions for impact evaluation of silver ions and silver nanoparticles.

MATERIAL AND METHODS

Embryos and experimental design

We carried out three experiments, using two kinds of AgNPs and silver nitrate, on zebrafish embryos (*Danio rerio*), purchased from the certified breeding facility – Mendel University in Brno, Czech Republic. We followed modified methods, described in OECD guideline 236 (OECD, 2013).

First, we selected fertilized, properly developing fish eggs in the blastula stage (up to 4 hours post fertilization), using a stereomicroscope. Mortality, sublethal effects (such as the presence of heart and yolk sac oedema, head, spine and tail deformities and a lack of pigmentation) and hatching were recorded daily. We used the semi-static method with the replacement of solutions every 24 hours. Embryos were incubated for 96 hours, with the photoperiod of 12 hours of light and 12 hours of darkness. The temperature was maintained at 26±1 °C. Each group consisted of 20 embryos in separate wells, exposed to 1 ml of the test solution per embryo, or, in case of control, dilution water. Each test was performed as a triplicate.

Chemicals

Tests were performed with silver nitrate, as a source of free silver ions, in concentration range 1–120 μg/L. Commercially available AgNPs stabilized with polyvinylpyrrolidone (AgNP PVP) were purchased at Sigma-Aldrich (Czech Republic), with size distribution 58.4±8.9 nm (manufacturer declared sizes up to 100 nm). Tests were conducted in concentration range 1–100 mg/L. AgNPs stabilized with 0.01% solution of maltose and gelatine (AgNP M&G) with size distribution 30.7±0.6 nm, were synthesized at the Regional Centre of Advanced Technologies and Materials (Olomouc, Czech Republic), experiments were conducted in concentration range 0.1–10 mg/L. Those AgNPs are characterized by yellow colour with the absorption maximum of 408 nm, which is specific for this size of nanoparticles optical spectrometry (Variskan Flash Reader) to confirm the size of nanoparticles during the experiment.

Transmission electron microscopy

We placed the suspensions of AgNPs on copper mesh – formvar coated mesh stabilized with evaporated carbon film (300 Old Mesh, Agar Scientific, Austria) and observed at 80 kV under Philips EM 208 Morgagni (FEI, Czech Republic) transmission electron microscope.

Statistical analysis and LC50 calculation

For statistical analysis, Unistat 5.6 for Excel software (Czech Republic) was used. Data on mortality, sublethal effects and hatching rate were tested using χ^2 test. The differences between control and exposure groups were considered significant at $p<0.05$. 96h LC50 (96-hour

lethal concentration 50) values were calculated with the Ekotox 5.2 software.

RESULTS

This study brings the clear data on the comparison of AgNP and silver ions on the mortality rate, hatching rate and observe the presence of sublethal changes in zebrafish embryos.

AgNPs characterization

The average size of nanoparticles was 30.7±0.6 nm for the AgNP M&G and 58.4±8.9 nm for the AgNP PVP. AgNP M&G were sperically shaped and more stable in solution, as shown in Figure 1. In contrast, AgNP PVP were also predominantly spherically shaped, but they were much more prone to aggregation, as seen in Figure 2.

Mortality

96h LC50 for silver nitrate ($AgNO_3$) was calculated as 58.44 μg/L. In contrast, we established the 96h LC50 for the AgNP M&G as nearly 100 times higher, in particular as 4.31 mg/L. The AgNP PVP caused the lowest mortality rate with 96h LC50 higher than 100 mg/L, proving its lower acute toxicity for zebrafish embryos. According to the embryo mortality rate, we observed significant changes, caused by $AgNO_3$ in comparison to the control group for all of the concentrations above the 30 μg/L. Moreover, in concentration 120 μg/L the mortality was 100%. The AgNP M&G induced significant mortality in concentrations above 5 mg/L, in concentration 25 mg/L caused 100% mortality. For the AgNP PVP, we observed significant changes in the two highest concentrations, the 75 and 100 mg/L. However, mortality rate in none of the tested concentrations exceeded 22.2%. Mortality rates are displayed in Tables 1 and 2.

Sublethal effects

We examined the embryos under the stereomicroscope for the presence of sublethal effects (Figure 3). Concentration-dependent deposition of silver on the surface of chorion was observed in embryos, both with and without developmental changes. This effect was observed in concentrations above 1 mg/L in case of AgNP M&G, and above 25 mg/L, in case of AgNP PVP. In group exposed to 100 mg/L AgNP PVP, the chorion was heavily covered and nearly opaque. In $AgNO_3$ groups, silver deposition was observable in concentrations above 30 μg/L, when were also able to see a silver mass floating inside the perivitelline space.

Pigmentation of the embryos was not affected by any of tested substances. Mostly, we observed the heart and yolk sac oedemas and spine deformations in tested embryos. Usually, the observed effects were combined thus we are not evaluating them separately, but as a complex. These complex sublethal changes were also coupled with lagging behind the physiological development of the embryos. Physiologically, we can observe the chest position of the larvae in 96h post fertilization, but embryos with sublethal changes stayed laying on their side and did not try to escape, when gently taped with a needle, as a control larvae did.

The $AgNO_3$ sublethal changes were statistically significant in all of the concentrations above 30 μg/L. The percentage of sublethal changes reached 100% at a concentration of concentration 90 μg/L. In the AgNP M&G expositions, we observed significant changes in all of the tested groups above 1 mg/L. Moreover, we observed sublethal changes for 100% of embryos in concentration 10 and 15 mg/L. The AgNP PVP showed a significant change only in 25 mg/L group and, in this case, the sublethal changes were observed in 14.7% of the embryos in total. Sublethal changes in exposition groups are listed in Tables 1 and 2.

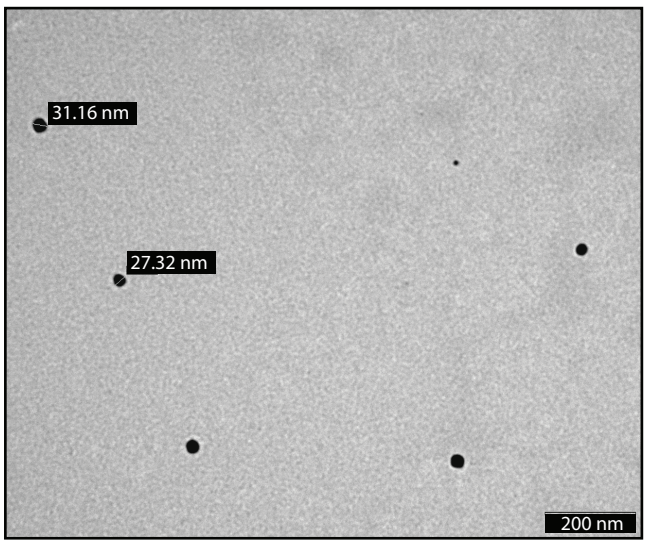

Fig. 1. Electron-microscopic image of silver nanoparticles stabilized with 0.01% solution of maltose and gelatine.

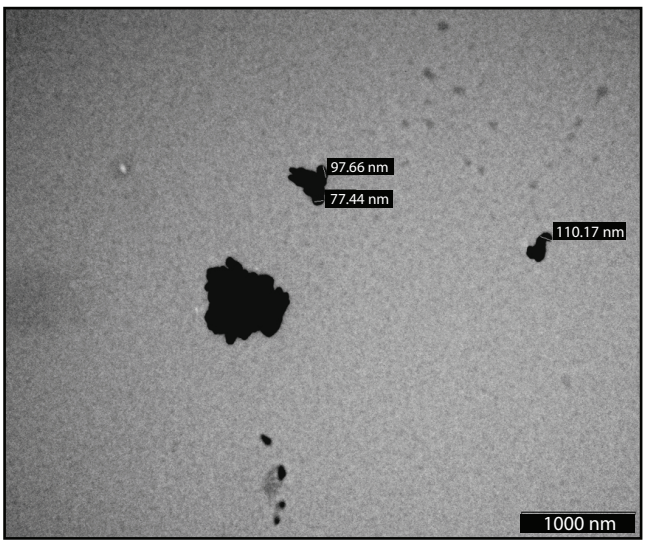

Fig. 2. Electron-microscopic image of silver nanoparticles stabilized with polyvinylpyrrolidone.

Tab. 1. Results of mortality, non-hatched embryos and sublethal changes in embryos after exposure to the different concentrations of silver nitrate ($AgNO_3$) in µg/L.

	$AgNO_3$					
Concentration [µg/L]	**Control**	**1**	**30**	**60**	**90**	**120**
Mortality	0.0[a]	0.0[b]	22.2[b]	46.8[b]	90.0[b]	100.0[b]
Non-hatched	0.0[a]	0.0[a]	2.1[a]	0.0[a]	40.0[a]	–
Sublethal changes	0.0[a]	1.7[a]	6.4[b]	48.5[b]	100.0[b]	–

Data are expressed as percentage part from 100% of embryos (n=60). The results are comparation between control and every concentration group and lacking a common letter of superscript (a,b) when differ significantly ($p< 0.05$).

Tab. 2. Results of mortality, non-hatched embryos and sublethal changes in embryos after exposure to the different concentrations of silver nanoparticles (AgNP M&G, AgNP PVP) in mg/L.

	AgNP M&G						
Concentration [mg/L]	**Control**	**0.1**	**1**	**5**	**10**	**15**	**25**
Mortality	0.0[a]	0.0[a]	2.8[a]	47.2[b]	93.1[b]	98.6[b]	100.0[b]
Non-hatched	0.0[a]	1.4[a]	1.4[a]	0.0[a]	20.0[b]	0.0[a]	–
Sublethal changes	0.0[a]	1.4[a]	4.2[b]	35.3[b]	100.0[b]	100.0[b]	–
	AgNP PVP						
Concentration [mg/L]	**Control**	**1**	**25**	**50**	**75**	**100**	
Mortality	5.6[a]	5.6[a]	5.6[a]	2.8[a]	16.7[b]	22.2[b]	
Non-hatched	0.0[a]	0.0[a]	11.8[b]	28.6[b]	40.0[b]	50.0[b]	
Sublethal changes	5.9[a]	5.6[a]	14.7[b]	11.4[b]	10.0[b]	3.6[b]	

Data are expressed as percentage part from 100% of embryos (n=60). The results are comparation between control and every concentration group and lacking a common letter of superscript (a,b) when differ significantly ($p<0.05$).

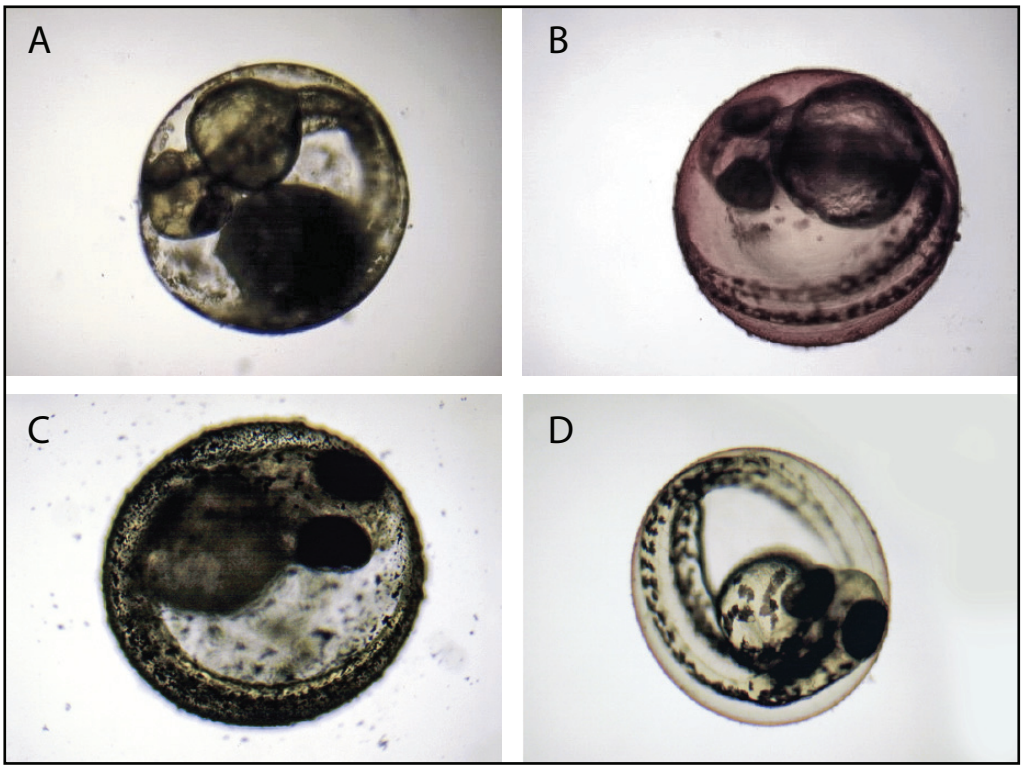

Fig. 3. Embryos at 48h exposed to: (A) silver nitrate (60 µg/L), (B) silver nanoparticles stabilized with 0.01% solution of maltose and gelatine (10 mg/L), (C) silver nanoparticles stabilized with polyvinylpyrrolidone (75 mg/L), (D) dillution water (control).

Hatching rate

Regarding the hatching rate, we revealed no statistically significant changes for the $AgNO_3$. In case of the AgNP M&G, we observed a statistically significant decrease in hatching rate (to 80%) only in 10 mg/L concentration. For the AgNP PVP, all the concentrations except for the lowest (1 mg/L) caused significant decrease in the ability to hatch. The hatching rate for AgNP PVP was the lowest at the highest tested concentration – only 50% of embryos successfully hatched in concentration 100 mg/L. The hatching incompetence caused by AgNP PVP was higher than the mortality in these groups, as shown in Tables 1 and 2.

DISCUSSION

Our results are in agreement with the available literature, silver ions are usually more toxic, than AgNPs (Griffitt *et al.* 2008; Ribeiro *et al.* 2014). Silver ions likely play a part in the AgNP toxicity, but it appears, that AgNPs have an inherent toxicity. In a study with adult zebrafish exposed to the AgNPs and silver nitrate, pathological findings such as blood extravasations were only observed in fish exposed to AgNPs (Bilberg *et al.* 2012). In case of silver ions, well-documented mechanism of toxicity is previously mentioned osmotic failure caused by the inhibition of gill Na^+/K^+-ATPase (Hogstrand & Wood 1998). Cytotoxic action of silver ions is inactivation of enzymes by binding sulfhydryl groups. Sulfhydryl groups are also present in enzymes responsible for dealing with oxidative stress, such as lactate dehydrogenase and in glutathione (Gordon *et al.* 2010). The generation of ROS (reactive oxygen species) occurs after exposition to the silver ions, but it is not the main mode of action.

In case of AgNPs, production of ROS plays an important role in the mechanism of AgNPs toxicity. There are two distinct ways – the first and more prevalent is the generation of ROS via surface plasmon enhancement, that occurs, when free electrons at the surface of AgNPs interact with oxygen, creating the superoxide radical, that can be converted to other ROS. The other way is a Fenton-like reaction at acidic pH, catalyzed by zerovalent iron, that leads to the dissolution of AgNPs. (Massarsky *et al.* 2014). The similarity of outcomes of silver ion and AgNP toxicity makes the distinction between the modes of action complicated.

The difference in hatching rate might have been caused by the fact, that AgNP PVP had a greater tendency to aggregate and caused mechanical difficulty for the embryo to hatch, by blocking the chorion pore canals and causing hypoxia. This effect was observed by Shih *et al.* (2016), who studied the adsorption of nano titanium oxide onto zebrafish embryos. In this case, excessive nano titanium oxide particles obturated chorion pore canals completely and reduced the hatching rate. Silver ions and non-aggregated AgNP M&G had greater chance to pass through the chorion pores and affect the embryonic cells directly, thus causing severer effects. This passage of AgNPs into the chorionic space and inner mass of the embryo was observed *in vivo* by Lee *et al.* (2007). This study shows, that the chorion pore canals are approximately 0.5–0.7 μm in diameter, which provides enough space for a single nanoparticle to pass through with Brownian diffusion. Böhme *et al.* (2017) confirmed bioconcentration in the inner mass of the embryo by applying laser ablation inductively coupled plasma mass spectrometry and electron microscopy. This study showed greater bioconcentration of silver nanoparticles, than ions, in the embryos. A greater share of silver mass after exposure to AgNPs was deposed in the chorion structures, than in the perivitelline space. In both silver species, the smallest share of silver mass was localized inside the embryo itself. In case of Ag^+ dissociated from $AgNO_3$, Most of the silver mass was found in the perivitelline space. This is in line with our observations – in $AgNO_3$ experiments, we were able to observe a silver mass not only on the surface of chorion but also floating inside the perivitelline space.

Similarly to our study, there is a wide range of LC50 values in the literature – from tens of micrograms to tens of milligrams per liter (Bilberg *et al.* 2012; Katuli *et al.* 2014). There are many physicochemical characteristics influencing AgNPs behaviour, including size, shape, surface area, surface chemistry, the presence of coatings, purity of the material, rate of aggregation, agglomeration and ionization. Interactions with other ions and ligands contained in the solution also play an important role (Nel *et al.* 2006; Wijnhoven *et al.* 2009). Larger particles tend to be less toxic, they have a smaller surface area, thus at the same mass, they have fewer atoms in the surface layer available for interaction with other compounds, the rate of ionization is also lower (Angel *et al.* 2013).

CONCLUSION

Our results clearly show great differences in the embryotoxicity levels of different AGNPs and Ag ions. It appears, that there were different mechanisms involved in causing the observed effects in embryos exposed in AgNP PVP and AgNP M&G. Namely, mechanical blockage and hypoxia in case of aggregation-prone AgNP PVP, causing reduced hatching rate, that was not observed in AgNP M&G exposed groups. The production of ROS probably played more important role in case of AgNP M&G, which were more stable in solution. Those findings also emphasize the need for detailed characterization of nanoparticles in every study. We can conclude, that AgNPs should be viewed rather as a group of chemical substances, than just one substance, like for example silver nitrate.

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