8vo Congreso Latinoamericano de Órganos Artificiales, Biomateriales e Ingeniería de Tejidos

8VO COLAOB

20 - 23 Agosto de 2014
Rosario - Argentina

2E-04

SILVER NANOPARTICLES (AgNP) TOXICITY STUDY BY IN VITRO AND IN VIVO ASSAY

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Abstract. *Silver (Ag) is a metal with applications in medicine since the beginnings. Its antimicrobial potential has been long studied and recently has been a growing interest mainly in the nanoparticles form. Cytotoxic effects and induction of apoptosis by silver nanoparticles (AgNP) have also been described. The critical lethal concentrations can be identified and precautions can be taken to eliminate such concentrations in future applications, especially in the biomedical field. There is a great concern about AgNP toxicity mainly about nanoparticles exposure to living organism and their release waste to the environment. This work aimed to study the AgNP toxicity also in in vivo systems to point out the adverse effects on aquatic species. In vitro assay was cytotoxicity test by neutral red uptake method and the AgNP cytotoxicity index (CI_{50}) was 1.3 mg L^{-1}. In vivo assays were performed by acute ecotoxicity test utilizing micro invertebrates Daphnia similis (water flea), and the fish Danio rerio (zebrafish). AgNP median effective initial concentration ($CE(I)_{50}$) obtained for Daphnia similis was 6.92 μg L^{-1} and for Danio rerio 7.1 μg L^{-1}. The presented assays may help to further assess the applicability to screening by in vivo test the toxicity of new compounds.*

Keywords: *Silver nanoparticles, cytotoxicity, ecotoxicity, Daphnia similis, Danio rerio*

1. INTRODUCTION

Silver nanoparticles (AgNPs) are widely applied in many household products and medical uses. In nanotechnology, a nanoparticle is defined as a material with dimensions and tolerance limits of 0.1-100nm that behaves as a whole unit in terms of its transport, properties and unique characteristics. Metallic nanoparticles have unique optical, electrical and biological properties that have attracted significant attention due to their potential use in many applications, such as catalysis, biosensing, drug delivery and nanodevice fabrication [Park et al., 2010].

However, studies on the effects of AgNPs on human health and environmental implications are in the beginning stage. Furthermore, most data on the toxicity of AgNPs have been generated using nanoparticles modified with detergents to prevent agglomeration, which may alter their toxicities. Park et al. studied toxicity using AgNPs prepared by dispersing them in fetal bovine serum (FBS), biocompatible materials.

Recently, the monitoring of pharmaceuticals and personal care products in the environment has been gaining great interest in ecotoxicological studies because many of these substances are frequently found in environment matrices such as surface water and sediments, effluent of sewage water treatment plants in the world [Stackelberg et al., 2004]. The micro invertebrate *Daphnya similis, a* Zooplanktonic organisms are sensitive bioindicators and have been widely used in freshwater ecotoxicology studies.

A variety of alternative assays for developmental toxicity testing in animals has been developed over the years. *Danio rerio* fish species is known as zebrafish is used in the zebrafish embryotoxicity test (ZET). This test is gaining popularity, since it is a unique alternative that enables the study of the initial stages of a complete and well characterized

developmental period of a vertebrate embryo in a simple and fast culture system. Zebrafish embryos develop independently of the maternal fish, are simply kept in water and development until hatching takes only three days. All these advantages make the zebrafish embryo suitable for relatively high-throughput tests [Hermsen et al.].

The aim of this work was to study the AgNP toxicity in mammalian cell culture by *in vitro* assay and also in *in vivo* systems to point out the adverse effects on aquatic species, utilizing acute ecotoxicity assays with a microcrustacea *Daphnia similis* and fish species *Danio rerio* (zebrafish).

2. METHODOLOGY

In this work was utilized AgNP solution from Khemia with concentration of 22 ppm and nanoparticles have an average size of 89 nm.

2.1 Cytotoxicity assay

The cytotoxicity assay was carried out with the exposure of NCTC clone L929 cell culture from American Type Culture Collection (ATCC) bank to the AgNP solution in cell culture medium MEM (minimum Eagle's medium from Sigma Co). The cytotoxic effect was evaluated using the capacity of living cells uptake neutral red dye, according to International Standard Organization [ISO 10993-5, 2009].

0.2mL of serially diluted AgNP solution (50, 25, 12.5, 6.25%) with work MEM culture was dropped on each 96 microplate wells containing $7x10^4$ cells. The microplate was placed for 24h at 37° C in a CO_2 humidified incubator. Control of cell wells were replaced by fresh work-MEM. In the same microplate extracts of positive control (natural rubber latex) and negative control (HDPE) were used. Samples and controls were tested in triplicates.

After the incubation period, the medium was replaced by neutral red solution (50µg/mL) and left for 3h at 37° C. The dye medium was discarded and the microplate was washed twice with phosphate buffered solution pH 7.4 and one time with a solution of 1% $CaCl_2$ in 0.5% formaldehyde. The cells rupture and neutral red release was obtained by addition of 0.2mL/well of extractant solution (50% ethanol in 1% acetic acid). The optical density (OD) were read on an ELISA reader spectrophotometer Sunrise from Tecan at 540 nm filter. The viability percentage was calculated with the average of obtained OD compared with control cells, considered 100%.

2.2 Acute ecotoxicity assay

Acute ecotoxicity with Daphnia similis

The assay was carried out according to USEPA (2002) and NBR 12713 (ABNT, 2009). Immobility was adopted as the endpoint to determine the acute toxicity. Organisms that did not show movement in 15 seconds were considered immobile.

The *D. similis* specimen was reared in the laboratory. Neonates with ages between 0 to 6 hours were selected and exposed to different concentrations of AgNP as well as a control group. The freshwater used in the culture media was collected at the Ribeirão do Piraí Reservoir, Salto, São Paulo, Brazil, an area monitored by CETESB. The water was previously filtered, physical-chemical parameters fixed (pH 7.0, conductivity 120 µS cm^{-1} and hardness 44 mg L^{-1} of $CaCO_3$) and maintained under aeration. The organisms were cultured in the Ecotoxicology Laboratory at IPEN/CNEN-SP, Brazil and were maintained in natural freshwater as culture medium under controlled conditions of temperature (25 ± 2° C) with

light and dark cycle (12:12h) and this culture media was changed every day. The feeding was based on algal suspension of *Pseudokirchneriella subcapitata* at the concentration of 10^5 cells mL^{-1} and added to yeast and fish chow.

The assay conditions are presented in the Table 1.

Table 1. Acute ecotoxicity assay conditions of AgNP solution on *Daphnia similis*

PARAMETERS	CONDITIONS
Assay type	Static
Assay duration	48 h
Compound-test	AgNP 89nm
Temperature	23 ± 2° C (*D. similis*)
Photoperiod	16 h light; 8 h dark
Flask-test	Assay tubes
Volume solution-test	10 mL
Number concentrations-test	5 (2, 3, 4.5, 6.75, 10.13, 15.19 $\mu g\ L^{-1}$) + control
Number neonates/recipiente	5
Number replicates/concentrations	4
Feeding	No
Dilution water	Natural (Salto de Itu)
Effect evaluation	Immobility
Expression of results	Quantitative: CE(I)50
Assay validation	Control: Mortality <10%

***Danio rerio fishes* (zebrafish)**

The acute ecotoxicology test with zebrafish (*Danio rerio*) was carried out according to ABNT NBR 15088 (2011) and the assay conditions are shown in the Table 2.

The main advantages of zebrafish model for toxicological studies are: good correlation to mammal models, in comparison to rodents, faster development and higher genetic homology to human, small size and weight, high popularity of zebrafish system enables increasing data base and information flow.

This test with zebrafish is very suitable for environmental toxicity determination based on fast answers concerning pollution problems, toxic cyanobacterial blooms, etc.

Table 2. Acute ecotoxicology assay conditions of AgNP solution on zebrafish

PARAMETERS	CONDITIONS
Organism-test	Zebrafish (1-2 cm)
Assay type	Static
Assay time	48 h
Compound-test	AgNP 89nm
Temperature	25-27° C
Photoperiod	12-16 h dark
Volume solution-test	1 g fish/L solution
Number concentrations-test	5 (1.5, 2.25, 3.40, 5.10, 7.60 $\mu g\ L^{-1}$) + control
Number replicates/concentrations	1
Feeding	No
Dilution water	40-48 mg $CaCO_3$/L; pH 7.0-7.6
Effect evaluation	mortality
Expression of results	Quantitative: CL(I)50
Assay validation	Control: Mortality <10%

3. RESULTS AND DISCUSSION

3.1 Cytotoxicity assay

In the Table 3 are presented the obtained results in the cytotoxicity assay of silver nanoparticles (NPAg) by neutral red uptake method.

Table 3. % Cell viability results of Cytotoxicity assay

Extract concentration (%)	% Cellular viability			
	Negative control	Positive control	AgNP-1	AgNP-2
100	103±6	0±0	3±11	3±9
50	94±20	11±5	2±14	2±0
25	106±9	76±3	79±5	83±4
12.5	106±10	97±5	100±5	94±2
6.25	107±9	101±11	93±4	90±0

Plotting the percentage viability in relation to solution concentration was obtained the viabilities curves in the graphic, presented in the Figure 1.

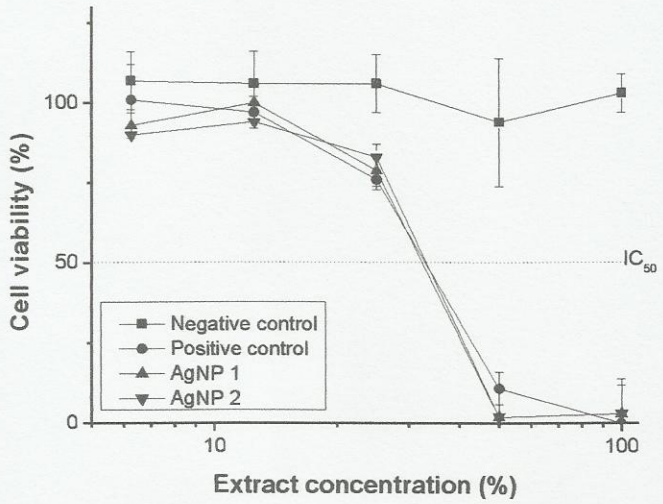

Figure 1. AgNP cytotoxicity assay: viability curves of AgNP toxicity on culture cell in vitro assay

The AgNP CI_{50} found in the cytotoxicity assay by neutral red uptake methodology was about 1.3 mg L^{-1}. CI_{50} is the solution concentration which injures or kills 50% of cell population in the assay.

Park et al. utilizing AgNPs (average size; 68.9 nm, concentrations; 0.2, 0.4, 0.8, and 1.6 ppm, exposure time; 24, 48, 72, and 96 h) observed cytotoxicity to cultured RAW264.7 cells by increasing sub G1 fraction, which indicates cellular apoptosis. When cells were treated with AgNPs, they were observed in the cytosol of the activated cells, but were not observed in the dead cells. They concluded that this observation seemed that AgNPs were ionized in the cells to cause cytotoxicity by a Trojan-horse type mechanism that was suggested by previous reported studies [Park et al., 2010].

3.2 Acute ecotoxicity assays

The AgNP EC(l)50 was calculated according to the Spearman-Karber method [Hamilton et al., 1977].

Acute toxicity on Daphnia similis

Table 4 and Fig.2 show the results of *D. similis* immobility when in contact with different concentrations of AgNP solutions.

Table 4. Acute Ecotoxicity assay of AgNP on *Daphnia similis*: results of organism immobility

AgNP Concentration ($\mu g\ L^{-1}$)	Organisms immobility											
	Assay 01						Assay 02					
	1	2	3	4	total	%	1	2	3	4	total	%
0.00	0	1	0	0	1	5	0	0	0	0	0	0
2.00	1	0	0	1	2	10	1	0	1	0	2	10
3.00	0	2	0	1	3	15	0	1	1	0	2	10
4.50	0	2	1	1	4	20	1	1	1	1	4	20
6.75	3	2	1	4	10	50	1	2	2	1	6	30
10.13	2	4	5	5	16	80	4	5	4	5	18	90
15.19	-	-	-	-	-	-	5	5	5	5	20	100

The AgNP CL50(I)48h to *Daphnia similis* was 6.9 $\mu g\ L^{-1}$. CL50 is the concentration of the compound that causes immobility in 50% of the organisms in the test.

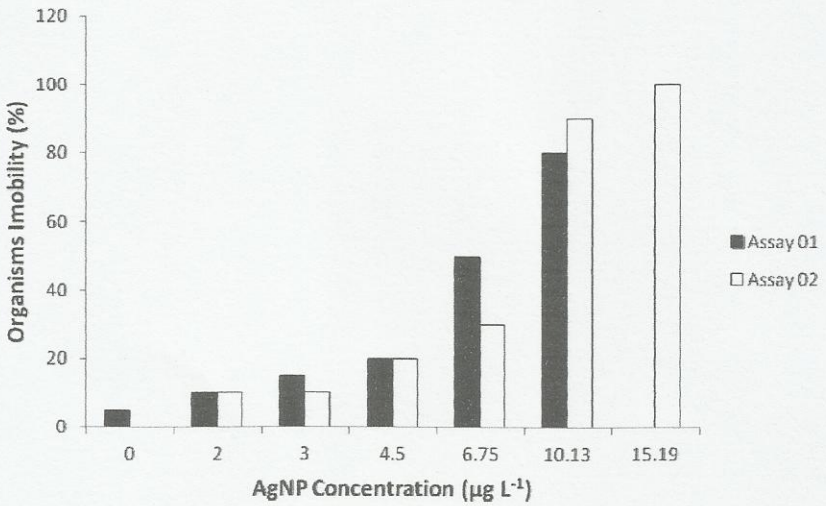

Figure 2. Acute ecotoxicity assay: *Daphnia similis* immobility under influence of AgNP

The AgNP CL50(I)48h to *Daphnia similis* was 6.9 $\mu g\ L^{-1}$. CL50 is the concentration of the compound that causes immobility in 50% of the organisms in the test.

Acute toxicity on Danio rerio (zebrafish)

Table 5 and Fig.3 present the AgNP toxicity results on zebrafish, in the acute ecotoxicity assay.

Table 5. Acute Ecotoxicity of AgNP on *Danio rerio (*zebrafish*)*: results of organism mortality

AgNP concentration ($\mu g\ L^{-1}$)	Assay 01		Assay 02	
	Mortality	% Mortality	Mortality	% Mortality
0.00	0	0	0	0
1.50	0	0	0	0
2.25	0	0	0	0
3.40	1	10	3	30
5.10	3	30	4	40
7.60	6	60	5	50

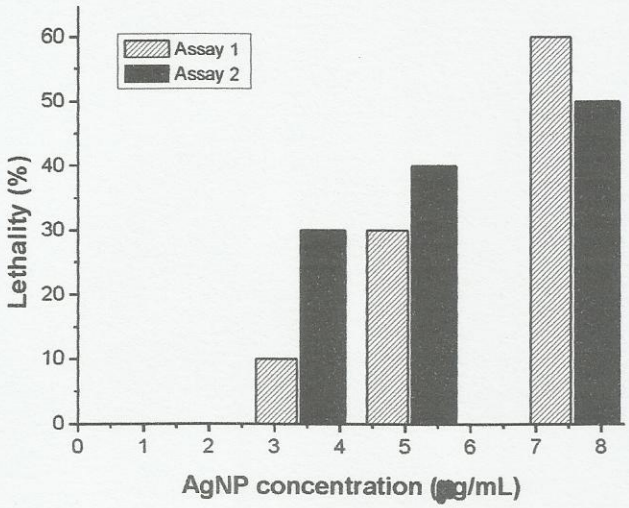

Figure 3. Acute Ecotoxicity assay: *Danio rerio* lethality graphic in the assay

The AgNP CL50(I)48h to zebrafish was 7.1 $\mu g\ L^{-1}$, very similar to that found for *D.similis*. Analizing the results of acute ecotoxicity assays of AgNP with average size of 89 nm was verified that this nanoparticles showed about a thousand times higher in the aquatic organisms than in vitro test with mammalian cell culture (cytooxicity assay).

The zebrafish embryotoxicity test (ZET) is a fast and simple method to study chemical toxicity after exposure of the complete vertebrate embryo during embryogenesis *in ovo*. Asharani et al. developed a novel quantitative evaluation method to assess the development of the zebrafish embryo based on specific endpoints in time, the general morphology score (GMS) system.

In general, the potency ranking of the compounds within their class in the ZET was comparable to their *in vivo* ranking. Asharani et al. concluded that the ZET with the GMS

system appears an efficient and useful test system for screening embryotoxic properties of chemicals within the classes of compounds tested. This alternative test method may also be useful for the detection of embryotoxic properties of other classes of chemicals [Asharani et al., 2008].

4. CONCLUSION

AgNP ecotoxicity test results showed that the CL(I)50 obtained in the assays of both organisms-test, *Daphnia similis* and zebrafish were similar.

The AgNP toxicity in aquatic organisms was about 1.000 times higher than in mammalian cell culture. This fact allows alerting of metal nanoparticles use in biomaterial area and taking care of effluents discharge in the aquatic environment.

The in vivo tests could be applied as *screening* test for toxicity of new compounds.

AKNOWLEDGMENTS

We are grateful to Jonny Ros from Khemia for AgNP solution sample, to CNPq for PIBIC fellowships and to Núcleo de Cultura Celular of Instituto Adolfo Lutz.

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