IN VITRO AND IN VIVO TOXICITY EVALUATION OF RESVERATROL ASSISTED GOLD NANOPARTICLES

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Abstract. *Gold nanoparticles (AuNP) are being investigated for diagnostic and therapeutic nanomedicines considering their low toxicity and stability against oxidation, among other features. The increasing production and use of AuNP can result in release of them into aquatic environment and the impacts on the aquatic organisms are not clear and the safety of AuNPs are still under investigation. This work aimed analyze the toxicity of resveratrol assisted AuNP synthesized in buffer phosphate pH 7.0 with approximately 47 nm in DLS and 15 nm in TEM analysis and gold ionic solution (Au^{+3}). Cytotoxicity by neutral red uptake method and acute ecotoxicological assay on Daphnia similis were used. AuNP presented no cytotoxicity up to 246 mg L^{-1} while Au^{+3} showed IC50=7.95 mg L^{-1}. AuNP CE50 was 113.15 mg L^{-1} and for Au^{+3} 0.05 mg L^{-1}. More studies can be conducted for the determination of safety ionic Au^{+3} and AuNP concentrations in aquatic environment.*

Key-words:*Gold nanoparticles, Resveratrol, Cytotoxicity, Acute ecotoxicity*

1. INTRODUCTION

Gold nanoparticles (AuNP) are being investigated for diagnostic and therapeutic nanomedicines considering their low toxicity and stability against oxidation, among other features. AuNP may be administered site-specifically or intravenously for diagnostic imaging by computed tomography or for therapy. One of the arguments to use gold nanoparticles (AuNPs) *in vivo* is that gold in bulk form is nontoxic. Gold cannot be dissolved in any single acid, is resistant to rusting, tarnishing, corroding, decomposing and is considered as biocompatible. Bulk gold is century-long accepted as a safe-to-use metal and has well functioned in anti-rheumatoid arthritis medication and as dental prosthesis [Kattumuri, 2007 and Viator, 2010].

In comparison to the gold in bulk form, AuNPs have distinct physical-chemical properties and the large amount of surface atoms makes AuNPs reactive. Moreover, AuNPs can potentially access many cellular or sub cellular structures, which are unreachable by the larger compound and may induce toxic effects.

Synthesis of gold nanoparticle commonly involve noxious chemicals reducing agents to Au ions reduction, generally this strategy poses toxicity-related problems. Thus, eco-friendly reducing agents, such resveratrol, have been used for the synthesis of AuNPs as a green agent to convert gold ions to AuNPs [Sanna, 2014].

Resveratrol is a polyphenol found in some sources as grapes, wine, peanuts and showing beneficial effects on human health due to strong antioxidant property and was used in this work as reducing agent.

In the aquatic environment the toxicity of chemical agents is evaluated by means of ecotoxicological tests with organism representative of the water column or sediments of fresh, estuarine or marine environments. The knowledge of the toxicity of these agents to different aquatic organisms allows ascertaining the temporary impact that these pollutants cause the biota of the water bodies, besides the determination of the permissible limits of several chemical substances to protect the aquatic life [Zagatto, 2006].

Daphnia similis (Crustacea, Cladocera) is a planktonic microscrustacean with a maximum length of 3.5 mm. They act as primary consumers in the aquatic food chain and feed by filtration of particulate organic material in suspension. The organisms of this genus are commonly known as water fleas and have wide distribution in the northern hemisphere. Reproduction by parthenogenesis (asexual) in *Daphnia similis* ensures the production of genetically identical individuals, thus allowing the collection of test organisms with constant sensitivity. In addition, they are widely distributed in freshwater bodies, are a significant source of fish feed and are important in many food chains. These organisms are easily grown in the laboratory, sensitive to various contaminants in the aquatic environment and because of their reduced size, require smaller volumes of test samples and dilution water [ABNT - Associação Brasileira de Normas Técnicas, 2009].

However, despite the great potential, the safety of AuNP is highly controversial and important concerns have been considered. Many factors such as shape, size, surface charge, surface coating and surface functionalization are expected to influence interactions with biological systems as far as gold nanoparticle potentiality in biomedical applications is concerned. The toxicity of AuNPs is already known to depend on their size. However, the impact of the AuNPs on the environment, organism and embryonic development are not clear and the critical parameters determining the safety of AuNPs are still under investigation. This work provides a contribution about toxicity of AuNPs synthesized using resveratrol as reducing agent, on mammalian cells and aquatic test organisms compared with ionic gold (Au^{3+}).

2. METHODOLOGY

2.1 Gold nanoparticles

Sodium tetrachloroaurate (III) dehydrate (Sigma Aldrich - USA), Resveratrol (PharmaNostra - Brazil). All acquired reagents were of analytical grade.

Resveratrol assisted AuNP and Au^{3+}synthesis

Gold nanoparticles were synthesized according to Sanna et al. [2014] using 5,0 mM sodium tetrachloroaurate (III) dehydrate and 1.67 mM resveratrol in buffer phosphate (50mM) pH 7,0. The mixture was allowed to stand for 12 hours at room temperature under stirring.

Ionic gold sample was obtained by sodium tetrachloroaurate dissolution in water.

AuNPs characterization

Particle Size Determination. Particle size was determined by Dynamic Light Scattering on a Zeta Pals (Brookheaven, USA) device at scattering angle of 90º. Results were reported by an average hydrodynamic diameter obtained by number, using 10 sets of 5 runs.

Particle Morphology: Gold nanoparticle size and dimensions were determined by Negative-Staining Transmission Electron Microscopy (NS-TEM) in a 120 kV JEM 1400 Plus electron microscope (Jeol, Japan). The ultrathin carbon film 400-mesh Cu grids (Ted Pella. Inc, USA) were subjected to glow discharge treatment a Pelco easiGlow^{TM} Discharge system (Ted Pella, Inc, USA) at 15 mA negative current for 25 s at atmospheric conditions. The samples of 3 µL (40 µg.mL^{-1}) were then transferred to the grids for 1 minute and negative staining was performed using 3 µL of 2% uranyl acetate (Sigma-Aldrich, USA). Specimen

preparation was performed at room temperature and the images were analyzed with a defocus range of −1 to −2 μm.

2.2 Cytotoxicity *in vitro* assay

The cytotoxicity assay was carried out with the exposure of NCTC clone L929 (CCIAL 020) cell culture from Núcleo de Culturas Celulares of Instituto Adolfo Lutz, São Paulo, SP, Brazil to AuNP-RES and $Au3^{+}$solutions.The used culture medium MEM (minimum Eagle's medium from Sigma Co) was supplemented by 5% bovine fetal serum (work-MEM).The cytotoxic effect was evaluated using the neutral red uptake method according to International Standard Organization [ISO 10993-5, 2009].

0.2mL of serially diluted AuNP-resv and Au^{3+}solutions with work-MEM 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 ($50\mu g.mL^{-1}$) and left for 3h at 37 °C.The dye medium was discarded and the microplate was washed twice with phosphate saline buffered solution pH 7.4 and one time with a 1% $CaCl_2$ in 0.5% formaldehydesolution. The cells rupture and neutral red release was obtained by addition of 0.2mL perwell of extracting solution (50% ethanol in 1% acetic acid). The optical densities (OD) were read on an ELISA reader spectrophotometer Sunrise from Tecan at 540 nm filter and 600 nm as reference. The viability percentage was calculated with the average of obtained OD compared with control cells, considered 100%.

2.3 Acute ecotoxicity *in vivo* assay

The assays were carried out according to USEPA (2002) and NBR 12713 (2011). *Daphnia similis* was the test organism in the acute ecotoxicological assay,cultured in the Laboratório de Ecotoxicologiaof Instituto de Pesquisas Energéticas e Nucleares (IPEN), São Paulo, Brazil and they were maintained in MS solution as culture medium under controlled conditions of temperature (20±2 ºC), light and dark cycle (12:12h) and the culture medium was changed three times a week. The feeding was an algal suspension of *Pseudokirchneriella subcapitata*, 10^5 cells mL^{-1}, added to yeast and fish chow.

The culture medium was maintained under aeration after the physical-chemical parameters fixed (pH 7.0, conductivity 120 μS cm^{-1} and hardness 44 mg L^{-1} of $CaCO_3$) and these parameters were measured at the beginning and in the end of the tests.

Neonates with ages between 6 to 24 hours were selected and exposed to different concentrations of AuNP-res and Au^{3+} as well as a control group, for 48 hours. Immobility was adopted as the endpoint to determine the acute toxicity.

The obtained results were analyzed by Trimmed Spearman-Karber statistical program [HAMILTON, 1977].

3. RESULTS AND DISCUSSION

Due to the large volume specific surface areas with high diverse surface activities, gold nanoparticles technology holds great promise vehicle for a wide range of biomedical applications. However, the huge impact arising from the physiochemical properties has given rise to new concerns for future health status. Currently, there is a lack of information on

AuNPs health and environmental effects and no regulatory safety and guidelines relating their properties about toxicities.

Characterization

The reaction between resveratrol and sodium tetrachloroaurate (yellow solution) turned into a deepruby-red solution after 60 min, as shown in Fig. 1 (a), indicating the completion of the reaction. The UV–Vis absorption spectrum of the AuNP showed a distinct surface plasmon resonance band at 531 nm, as shown in Fig. 1(b), confirming the formation of gold nanoparticles.

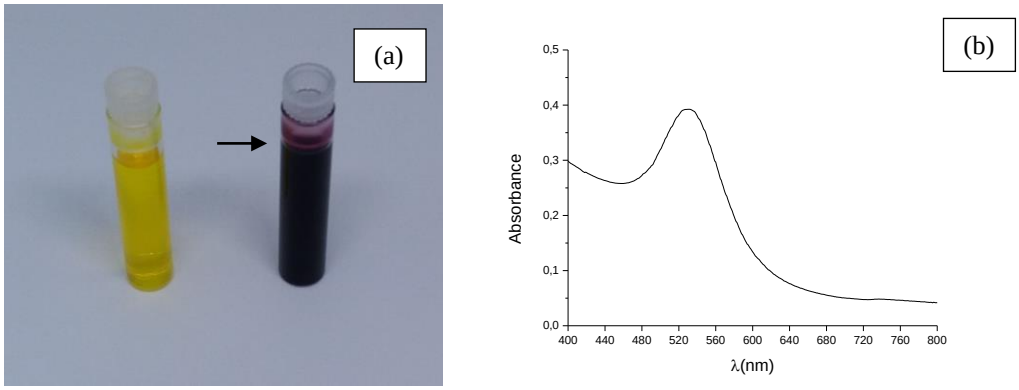

Figure 1. (a) Unassisted vision observation of the formation of AuNP from $NaAuCl_4$ solution and with resveratrol. (b) The UV–Vis spectra of AuNP-resv in phosphate buffer solution.

The size and shape of the nanoparticles were investigated with TEM. The images reported in Fig. 2 show that AuNP-resv are most of them spherical in nature, followed by occasionally hexagonal and rarely other morphology and the gold nanoparticles size was in the range of 10–15 nm

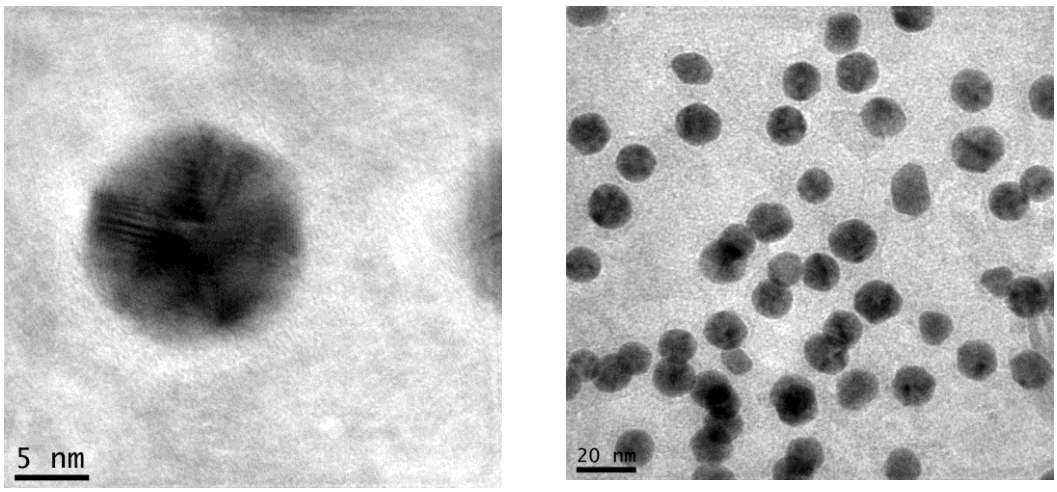

Figure 2. Transmission electron microscopy (TEM) micrographs of AuNP-resv.

Detection limit of DLS varies from instrument to instrument significantly, because the detected scattering light intensity is related to the wavelength and power of the incident light, the type of detector, and the detection angle. The average diameter of gold nanoparticles by DLS show dimensions of 47 ± 12.3 nm maybe influenced bysaline of buffer solution

Comparing the gold nanoparticle toxicity results from different work groups is difficult. One of the reasons is that the describing units of AuNPs concentrations in the literature are not identical. Some groups use nanoparticle molarity without exact knowing the atom number, the molecular weight and polydispersity. In this work it was used the gold atom concentration to compare AuNP-resv and $Au3^{+}$ toxicity.

The viability curves of the cytotoxicity assay was obtained in a graphic of viability percentage against the extract concentrations and in this curve it was possible to obtain the cytotoxicity index IC_{50}, as shown in the Fig. 3. IC_{50} is the extract concentration which causes injury or death on 50% of the cell population in the assay.

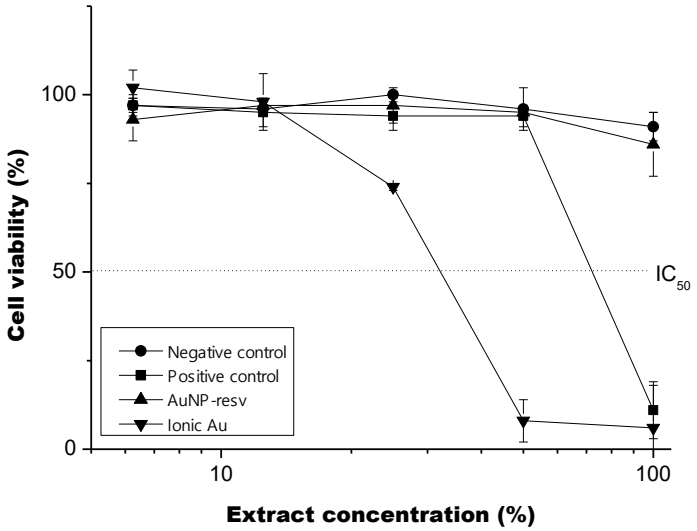

Figure 3. Viability curves of AuNP-resv and Au^{+3} in the cytotoxicity assay by neutral red uptake methodology

In this graphic all the curves above the IC_{50} line are considered no presenting cytotoxicity as the negative control and AuNP-resv up to 492.5 mg L^{-1}. The curves under or crossing the IC_{50} line are considered cytotoxic, as positive control presenting IC_{50}=72% and ionic Au IC_{50}= 32. 26 %. The Au^{+3} 100 % solution was 24.63 mg L^{-1} so the IC_{50} was 7.95 mg L^{-1}.

The cytotoxicity assay showed that the toxicity effect of ionic gold was about more than 50 times higher than AuNP-RES.

The study carried out by Vijayakumar&Ganesan (2012) to investigate i*n vitro* cytotoxicity of three types of gold nanoparticles: citrate-AuNPs, starch-AuNPs, and gum arabic-AuNPs using MTT assay on PC-3 and MCF-7 cells with 20, 50, 80, 110, and 140 mg L^{-1} AuNPs concentrations showed that these AuNPs showed no cytotoxicity up to 140 mg L^{-1}.

Fig. 4 shows the illustrative morphology of *Daphnia similis* and a control organism in the acute ecotoxicity assay.

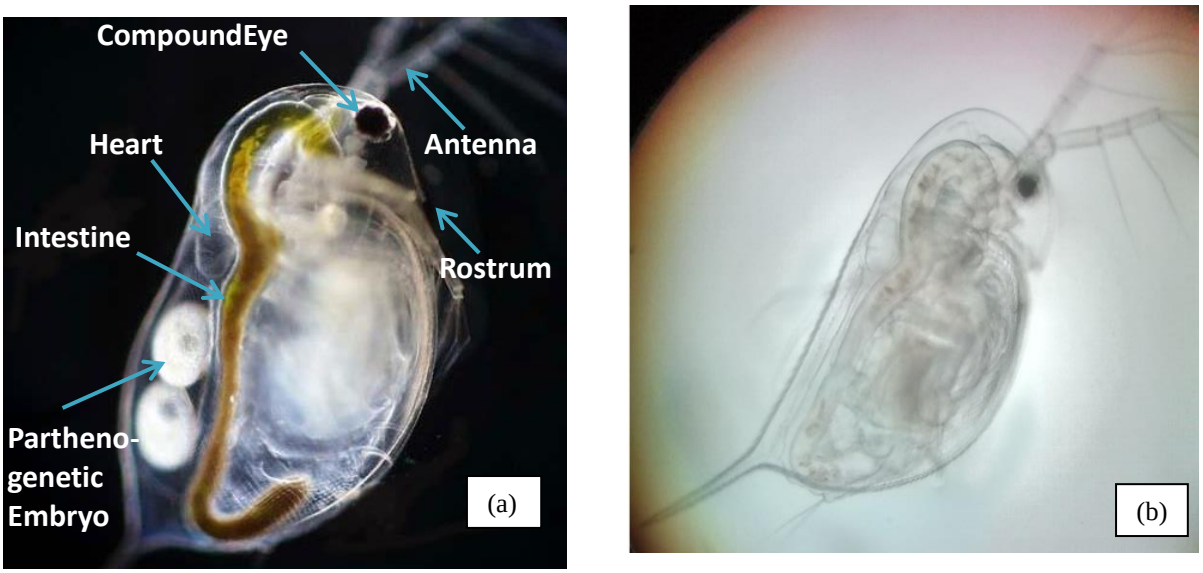

Figure 4. *Daphnia similis* morphology: (a) Illustrative form - Reference: adapted from http://petecologiaufrpe.blogspot.com.br/2013/02/artigo-o-uso-do-bioindicador-daphnia.html. (b) Control organism in the acute ecotoxicity assay

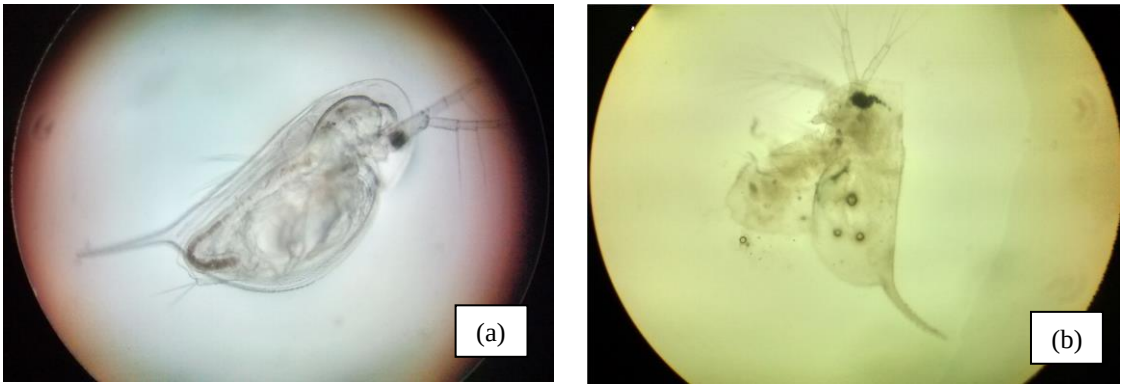

Figure 5. *Daphnia similis* in the acute ecotoxicity of Au^{+3} assay: (a) Au^{+3} 0.01 mg L^{-1} and (b) Au^{+3} 0.16 mg L^{-1} concentrations.

The Fig. 5 presents *Daphnia similis* in Au^{+3} solutions: in the 0.01 mg L^{-1} the organism was without signs of toxicity and in the higher concentration of 0.16 mg L^{-1} we can observe that the organism was completely damaged. In the acute ecotoxicity of ionic Au assay the EC50 (effective concentration causing immobility at 50% of exposed organisms) was 0.05 mg L^{-1}.

In the Fig. 6 it can be observed that the gold nanoparticles were ingested and were visible by the reddish coloration in the gastrointestinal tract of the organism. In high concentrations, it was noticed that the gold nanoparticles showed toxicity to the organism, leading it to the death. According to Palem et al, 2009 it is known that the fate and transport of AuNPs in surface waters depends significantly on their interactions with organic materials.

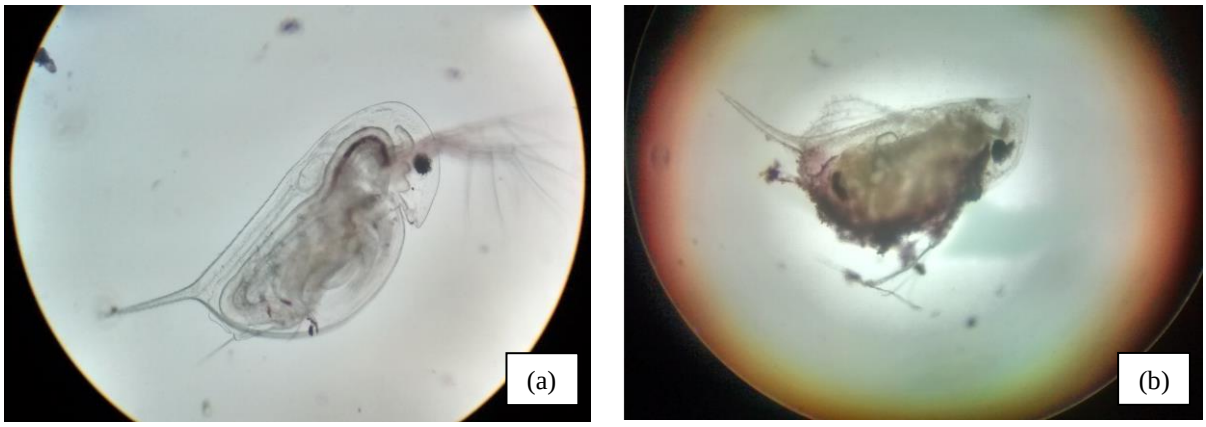

Figure 6. *Daphnia similis* in the acute ecotoxicity with AuNP-resv assay: (a) presence of AuNPs (49.25 mg L^{-1}) in the gastrointestinal tract of the organism and (b) organism in the AuNPs concentration of 197 mg L^{-1}.

The AuNP-resv EC50 was 113.15 mg L^{-1}. It can be inferred that the toxicity of gold in the ionic form is greater than gold nanoparticle form.

The nanoparticles are different from traditional chemical substances, since they do not have standard behavior for the same product, although they have the same composition, they are different due to particle size, its metabolism and interaction with the site of action.

It was verified that in each size, the same material can present very different characteristics. According to Asharani (2008), studies have shown that ultrafine particles can cause more damage than larger particles in the same concentration and the toxicity of AuNPs is already known to depend on their size.

AuNPs are intensively investigated and large numbers of novel AuNPs are designed for *in vivo* applications. However, the impact of the AuNPs on the environment, organism and embryonic development are not clear and the critical parameters that determine the safety of AuNPs are still under investigation. The AuNP toxicity in aquatic organisms was 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.

There is still much to study about human health toxicity and impacts to the aquatic environment. The in vivo tests could be applied as *screening* test for toxicity of new compounds.

4. CONCLUSION

AuNPs are intensively investigated and large numbers of novel AuNPs are designed for *in vivo* applications. The impact of the AuNPs on the environment, organism and embryonic development about the safety of AuNPs are still under investigation.

The AuNP-resv toxicity in aquatic organisms was 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* test of acute ecotoxicity could be applied as screening test for toxicity of new compounds.

This study has to be continued with concern of human health toxicity and impacts to the aquatic environment.

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