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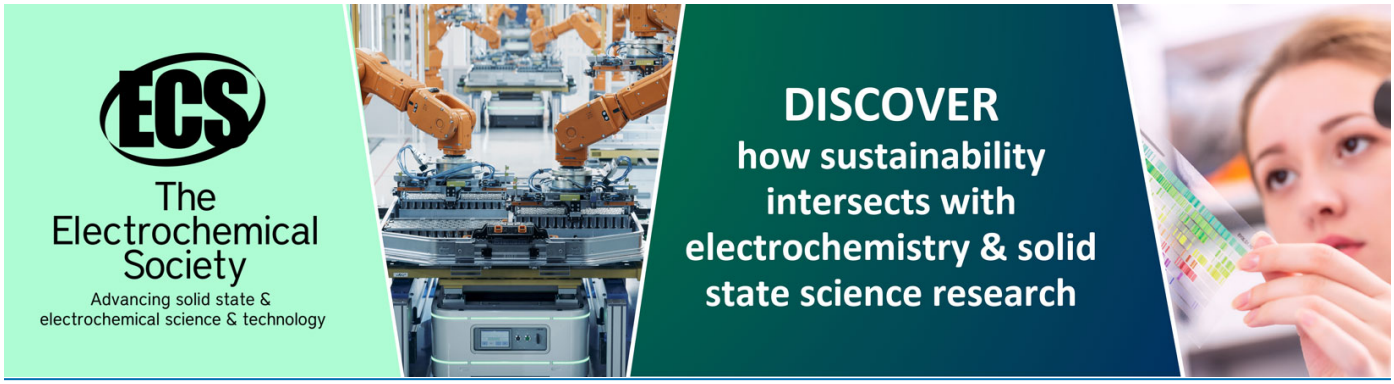

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Cytotoxicity and genotoxicity of biogenic silver nanoparticles

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Abstract. Biogenic silver nanoparticles with 40.3 ± 3.5 nm size and negative surface charge (- 40 mV) were prepared with *Fusarium oxysporum*. The cytotoxicity of 3T3 cell and human lymphocyte were studied by a TaliTM image-based cytometer and the genotoxicity through *Allium cepa* and comet assay. The results of BioAg-w (washed) and BioAg-nw (unwashed) biogenic silver nanoparticles showed cytotoxicity exceeding 50 μg/mL with no significant differences of response in 5 and 10 μg/mL regarding viability. Results of genotoxicity at concentrations 5.0 and 10.0 ug/mL show some response, but at concentrations 0.5 and 1.0 μg/mL the washed and unwashed silver nanoparticles did not present any effect. This in an important result since in tests with different bacteria species and strains, including resistant, MIC (minimal inhibitory concentration) had good answers at concentrations less than 1.9 μg/mL. This work concludes that biogenic silver nanoparticles may be a promising option for antimicrobial use in the range where no cyto or genotoxic effect were observed. Furthermore, human cells were found to have a greater resistance to the toxic effects of silver nanoparticles in comparison with other cells.

1. Introduction

Metal nanoparticles (NP) have been increasingly recognized for their important industrial applications, especially in the fields of medicine, food, and agriculture. Most metals can be used to produce metal nanoparticles; however, the most common are nickel, copper, palladium, platinum, gold and silver [1-10].

Silver nanoparticles (silver NP) are employed in textile engineering and water treatment, as well as in medicine, where their small size and high surface area enhances their antimicrobial activity. The particles can be synthesized by different techniques, including biological methods using fungi, bacteria, and plants, as well as by chemical methods involving the reduction of organic or inorganic salts, and by physical and photochemical procedures [11-17].

In addition to their bactericidal action, silver NP can also be used to combat viruses and fungi [18-19]. Its high antimicrobial activity can be attributed to their affinity for chemical groups containing the elements phosphorus and sulfur present in cell membrane, causing damage and interrupting cellular respiration, and interacting with DNA and its interference in cell division [20].

In recent years, there has been increased interest in silver NP and their applications, while at the same time there are concerns regarding the possible impacts of using NP on a large scale, including problems of toxicity and risks to the environment and human health. In response, there have been a

number of published *in vivo* studies concerning potential inflammatory and other effects of silver NP [22-27]. However, little is known concerning the genotoxicity of silver nanoparticles to different organisms and cell types [28]. This study is very relevant due to broad application of silver NP and to high industrial interest in these particles. The importance of these silver NP in the area of health and materials is due to their antimicrobial activity. More than 400 products are already on the market. This large investment reflects the efficacy of silver in combating resistant microorganisms [29], in enhancing the effects of antibiotics such as gentamicin [30], and in producing antimicrobial fabrics [2]. Furthermore, this great investment and the number of products indicate the importance of study of genotoxic potential of these particles [28].

2. Material And Methods

2.1 Silver nanoparticle production

Fungal inoculates were prepared at 28°C in Petri dishes with a medium containing malt extract (2%) and yeast extract (0.5%). Silver reduction was carried out by placing approximately 10 g of *F. oxysporum* biomass in a conical flask containing 100 mL of distilled water, and leaving for it 72 h at 28°C. The aqueous solution components were then separated by filtration, which was followed by addition of $AgNO_3$ (10^{-2} mol/L) to the solution and leaving for several hours at 28°C [31].

2.2 Characterization of the biogenic silver nanoparticles

2.2.1 Transmission electron microscopy (TEM)

The AgNP were characterized using a Carl Zeiss Libra transmission electron microscope, operated at 120 keV. One drop of the particle dispersion was deposited onto carbon-coated Parlodion films supported on 300 mesh copper grids (Ted Pella).

2.2.2 Particle size and zeta potential

The mean diameter (z-average diameter) and size distribution of the nanoparticles were measured by photon correlation spectroscopy (PCS) with a Zetasizer Nano ZS (Malvern Instruments, UK), at 25 ºC, using polystyrene cuvettes with a path length of 10 mm. The zeta potential was measured with the same instrument using capillary cells with a path length of 10 mm.

2.3 Cell culture and *Allium cepa* test conditions

Analyses were performed using human lymphocytes and 3T3 cell line fibroblasts. The lymphocyte tests employed RPMI medium supplemented with 10% fetal bovine serum, and the 3T3 cell analyses used DMEN medium supplemented with 15% fetal bovine serum. Both media were fortified with antibiotics, and the cultures were kept at 37 °C in a 5% CO_2 humidified atmosphere. Contact with the nanoparticles was maintained for 1 h in both the cytotoxicity analyses (evaluated by Tali scan) and the comet tests. For the *Allium cepa* analyses, the seeds were germinated in ultrapure water at room temperature. When the roots reached a length of 2 cm, the seedlings were removed from the germination box and placed into contact with the test materials. The analyses performed are summarized in Figure 1.

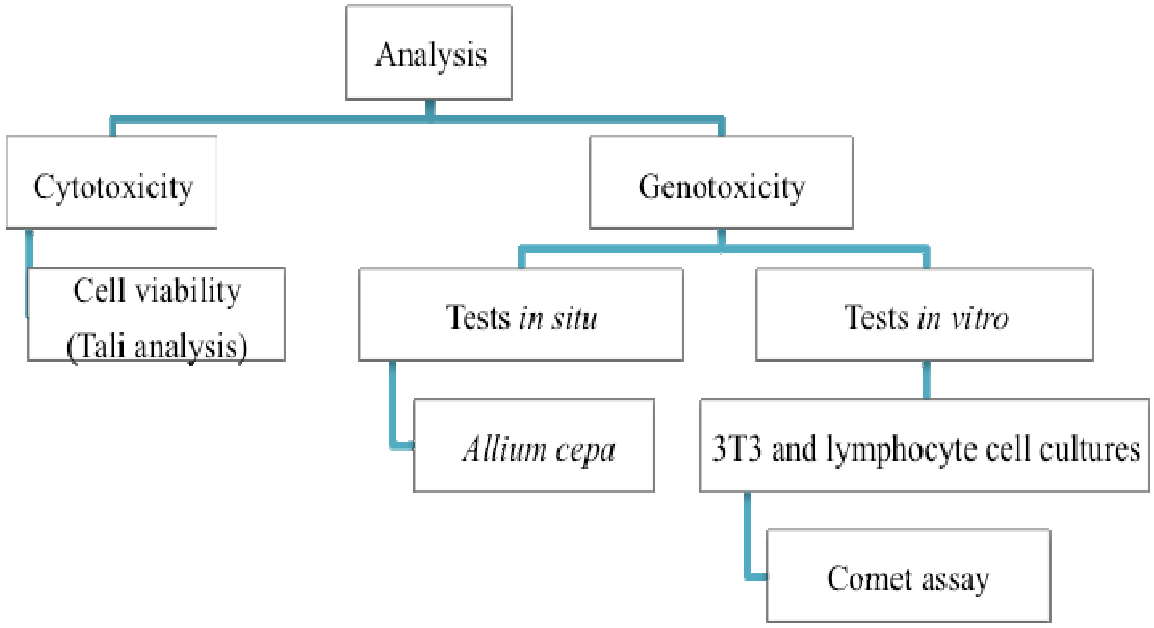

Figure 1 – Scheme of the experimental protocol adopted.

2.4 Tali analysis

Cell viability was measured using a Tali™ Apoptosis Kit consisting of Annexin V Alexa Fluor® 488 and Propidium Iodide (Invitrogen), and a Tali™ image-based cytometer, which enabled the numbers of viable, apoptotic, and dead cells to be counted. The cells that had been treated with nanoparticles were centrifuged and concentrated to 1 x 10^6 cells per mL. 100 µL aliquots of sample were prepared according to the specifications of the kit, and the tests were performed in triplicate.

2.5 *Allium cepa* assays

In the *Allium cepa* assays, ultrapure water was used as a negative control and trifluoraline (0.075 g/L) as the positive control. After 24 hours of contact with the silver nanoparticles, the roots were fixed with Carnoy's reagent and subjected to acid hydrolysis. They were then washed in distilled water and submitted to a Schiff-base reaction. The meristematic region of the roots was cut, placed on a slide together with a drop of 2% acetocarmine, and covered with a cover slip, which was used to gently press and spread the cells. The slides were examined under an optical microscope. The results of the *Allium cepa* tests were used to calculate the MI, RMI (treatment mitotic index / negative mitotic index), CAS (number of damaged cells / number of cells in division), and RCAS (treatment chromosomal aberration index / negative treatment chromosomal aberration index), as described by Parida et al. [32].

2.6 Comet assays

The lymphocytes and 3T3 cells were exposed to the silver nanoparticles for a period of 1 hour. Negative and positive controls employed phosphate buffered saline (PBS) and H_2O_2 (200 µmol/L), respectively. The comet assay was performed as described by Azqueta et al. [33]. Each treatment used 10 µL of lymphocytes in 110 µL of low melting point agarose (0.8%), and the mixture was placed onto microscope slides that had been pre-coated with normal melting point agarose (1.5%). After polymerization, the cover slips were removed and the slides were treated with an ice-cold lysis

solution for 20-30 minutes. All treatments were then incubated in an electrophoresis buffer for 20 minutes, followed by electrophoresis for 20 minutes at 1.3 V/cm.

After electrophoresis, the slides were stained and analyzed by optical microscopy. Scores of between 0 and 4 were assigned according to the quantity of DNA in the tail and the length of the tail: Score 0 corresponded to intact cells, with no damage caused by the exposure; Score 1 corresponded to cells with minimal damage; Score 2 to average damage; Score 3 to severe damage; and Score 4 to cells with maximum damage (Figure 2). In this visual method, the number of cells found for each score was multiplied by the value of the score and the values were summed at the end of the analysis of each slide. Since the score depended on the number of cells observed, an index of tail damage (TD) was created by dividing the score given for the slide by the number of cells analyzed on the slide [33-36].

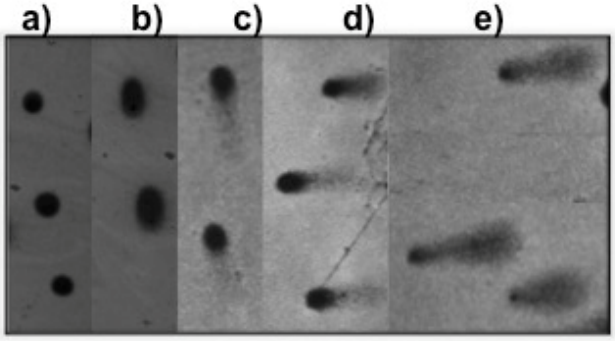

Figure 2 - Representative images (at x40 magnification) illustrating the tail intensity for the fibroblast comet test scores: a) score = 0 (negative control); b) score = 1; c) score = 2; d) score = 3; e) score = 4.

3. Results and Discussion

3.1. Biogenic silver nanoparticle characterizations

The spherical biogenic silver NP prepared using *Fusarium oxysporum* was obtained with average size of 40.3 ± 3.5 nm as shown Figure 3.

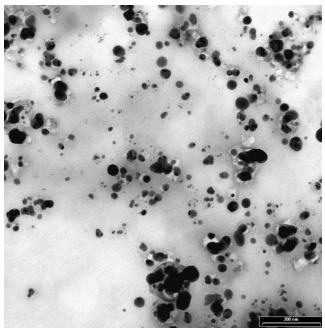

Figure 3 - TEM image of unwashed biogenic silver nanoparticles.

The particles dispersion was stable for several months (6 months) due to protein (from fungi) capping around the particles as described by Durán et al. [31]. To separate the protein bound with silver NP from protein present in the dispersion, the particles were washed (3 times) and centrifuged at 5000 rpm for 30 minutes. The particle size, polidispersity index (PdI) and zeta potential of these washed particles (BioAg-w) was analyzed and compared with unwashed silver NP (BioAg-uw) as shown in Table 1. The BioAg-w exhibited larger sizes than BioAg-uw, indicating particle agglomeration in the centrifugation process. The PdI for both particles was around 0.2, showing low polydispersity. In relation to the zeta potential, an increase in modulus was observed when the

particles were washed. This difference can be attributed the protein lost (i.e. not bound to the NP) from the dispersion in the washing processes.

Table 1 – Size, polydispersivity, and zeta potential of washed and unwashed silver nanoparticles.

Particle type	Size (nm)	Polydispersivity	Zeta potential (mV)
BioAg-uw (unwashed)	106.2 ± 13.0	0.231	-37.1 ± 2.6
BioAg-w (washed)	145.1 ± 4.5	0.206	-47.8 ± 1.1

4. Cytotoxicity and genotoxicity

4.1. Cytotoxicity

The cytotoxicity analyses using 3T3 cells and lymphocytes were performed by the Tali method for concentrations of 5, 10, and 50 µg/mL of the washed (BioAg-w) and unwashed (BioAg-uw) biogenic silver nanoparticles. For both nanoparticles, the results revealed cytotoxicity at concentrations exceeding 50 µg/mL (not shown), while for concentrations of 5 and 10 µg/mL neither of the cell cultures showed any significant differences in response in terms of viability (Figure 4).

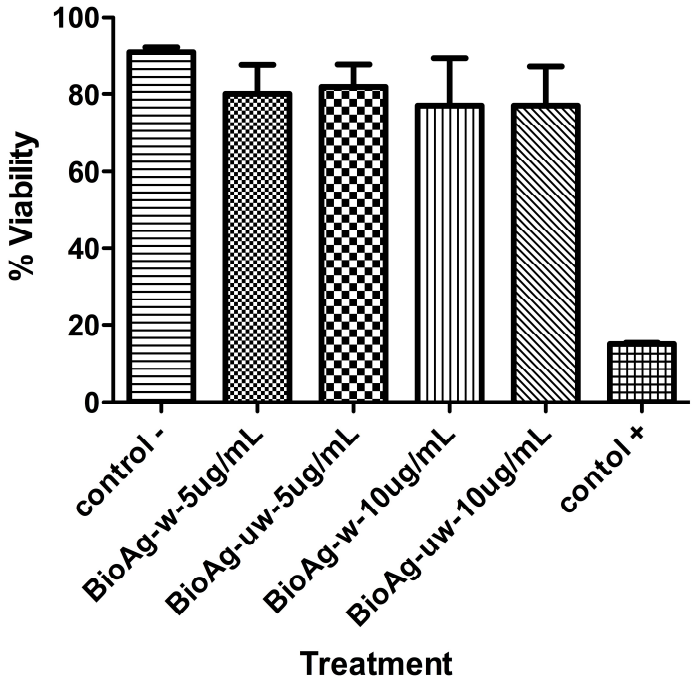

Figure 4 – Results of the Tali analyses using lymphocyte cells.

4.2 Genotoxicity

4.2.1 Allium cepa assays

The results of the *Allium cepa* cytogenetic tests revealed no significant differences in mitotic indices between the treated groups and the negative control (Figure 5). However, in the case of the alteration indices, significantly higher values were obtained for the treatments with BioAg-uw, compared to the negative control and BioAg-w (Figure 6). This effect can be related to nanoparticle

aggregation in the washing processes, as verified by PCS analysis. Silver nanoparticles with 20 nm were more toxic in all endpoints evaluated than larger silver nanoparticles (80 and 113 nm). Park et al. (2011) [37] verified a correlation between silver nanoparticle sizes and cyto and genotoxicities.

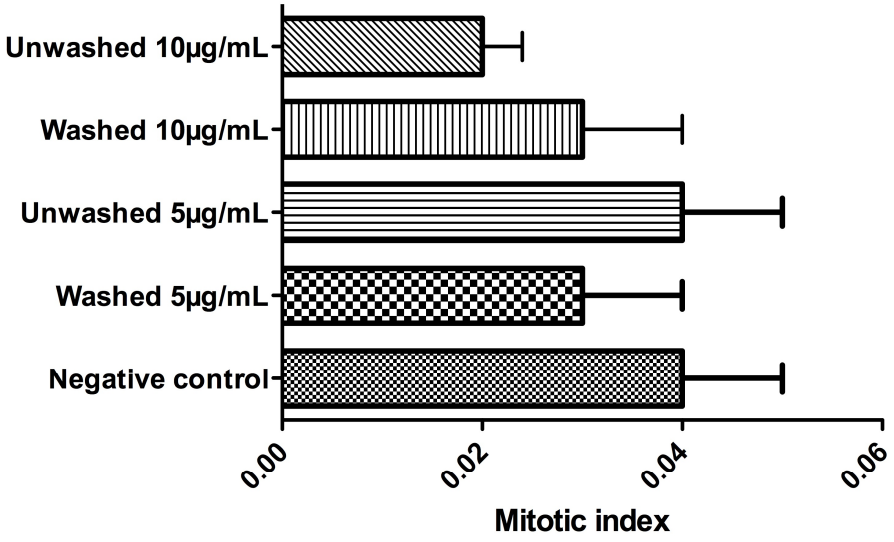

Figure 5 - Results of the mitotic index analyses.

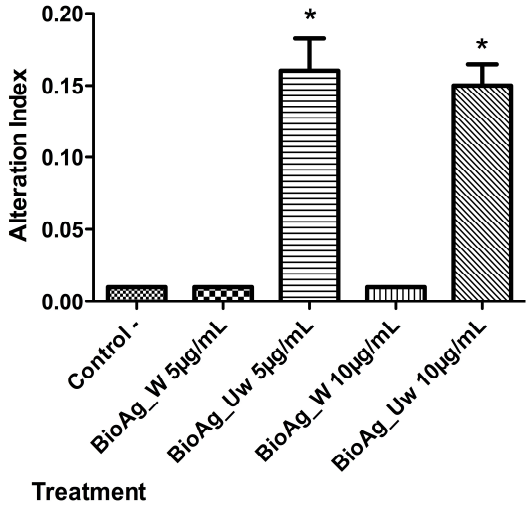

Figure 6 – Results of the alteration analyses using the *Allium cepa* test.

4.2.2 Comet assays

The comet assays identified a genotoxic response at nanoparticle concentrations of 5 and 10 µg/mL. However, at concentrations of 0.5 and 1 µg/mL, neither washed (BioAg-w) nor unwashed silver nanoparticles (BioAg-uw) exhibited any genotoxicity (Figure 7). This is an important finding, since tests with different bacteria species and strains, including resistant strains, have found MIC (minimum inhibitory concentration) values of less than 1.9 µg/mL (data not shown).

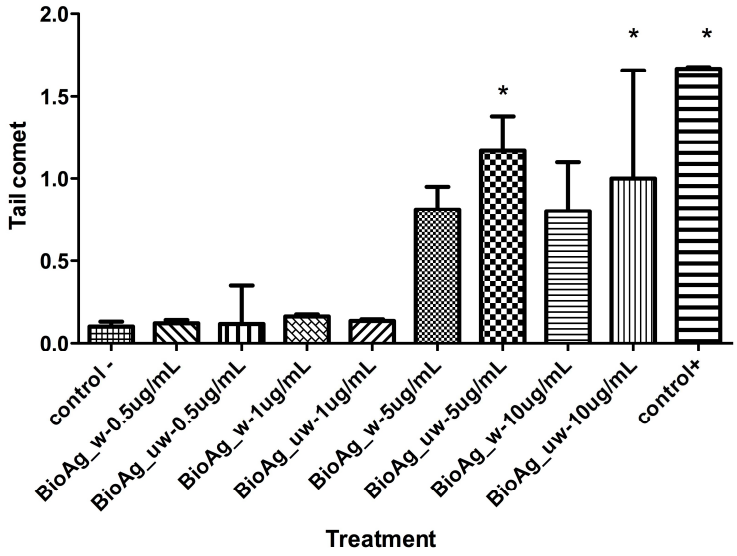

Figure 7 – Results of the comet assays.

The results suggest that a concentration of 5 µg/mL could be a critical level for genotoxicity for biogenic silver NP. As verified in the *Allium cepa* assays (alteration indices), BioAg-w showed lower genotoxic effects than BioAg-uw at concentrations of 5 and 10 µg/mL. Further studies will be needed to investigate the relation between nanoparticle aggregation and alteration in genotoxicity.

5. Conclusions

Silver biogenic nanoparticles showed similar response to cytotoxicity tests in which both (washed and unwashed) were not cytotoxic up to the concentration of 10ug/mL. In relation to genotoxicity the washed and unwashed silver nanoparticles did not show significant differences related to the mitotic index at the *Allium cepa* test; but at the damage analysis unwashed nanoparticles showed significant damage index. The same was observed for the comet assay in which unwashed nanoparticles were genotoxic while the washed silver nanoparticles were not. Deeper studies are necessary in relation to biogenic nanoparticles, mainly regarding the substances that cap the nanoparticles.Despite nanoparticles show genotoxic in concentration of 5µg/mL we remind that their MIC is between 0.9-3.8 µg/mL.

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