Antibacterial Activity of Silver Nanoparticles (AgNPs) in *Staphylococcus aureus* and Cytotoxicity Effect in Mammalian Cells

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Staphylococcus aureus is a type of Gram-positive bacteria that can cause different healthcare-associated infections. Silver nanoparticles (AgNPs) are considered to be used in various applications against bacteria that are resistant to common antibiotics or even multiresistant bacteria as the *S. aureus.* This work evaluated the antimicrobial activity of AgNPs in strains of *S. aureus* resistant to a large number of antibiotics. Regarding antimicrobial susceptibility tests, the reference strain showed sensitivity for almost 75% of the evaluated antibiotics and also to AgNPs suspension. The clinical strains showed resistance to 80% of antibiotics tested but one of the clinical strains was more sensitive to AgNPs suspension. Silver nanoparticles were non-cytotoxicity at 0.156µg/ mL concentration in normal mouse fibroblasts 929 and tumoral HeLa and HepG2 cells.

Keywords: Silver nanoparticles; antimicrobial activity; *Staphylococcus aureus;* cytotoxicity

1. Introduction

Nanotechnology has attracted considerable attention in recent years. The impact of the nanostructured materials can bring improvement to the quality of life and preservation of the environment [1], and also represents a promising field for generating new types of nanomaterials with biomedical applications [2].

Staphylococcus aureus is one of the most important biofilm-forming pathogens that cause complications ranging from minor to life-threatening infections [3, 4]. Staphylococcal infections occur regularly in hospitalized patients in any organ system and have severe consequences, despite antibiotic therapy [5]. The success of *S. aureus* as a pathogen and its ability to cause such a wide range of infections are the result of its extensive virulence factors [6].

Although several new antibiotics were developed in the last few decades, none have improved activity against multidrug-resistant bacteria [7]. Therefore, it is important to develop alternative and more effective therapeutic strategies to treat Gram-negative and Gram-positive pathogens. Nanoparticles, which have been used successfully for the delivery of therapeutic agents [8], in diagnostics for chronic diseases [9], and treatment of bacterial infections in skin and burn wounds, are one option [10]. Silver is known for its antibacterial activity. Nanoparticles are now considered a viable alternative to antibiotics and seem to have a high potential to solve the problem of the emergence of bacterial multidrug resistance [11, 12]. In particular, silver nanoparticles (AgNPs) have attracted much attention in the scientific field [13].

1.1 Silver Nanoparticles (AgNPs, NanoAg, Nanosilver)

Since ancient times among various antimicrobial agents, silver has been most extensively studied and used to fight against infections and prevent spoilage [10, 14]. Silver nanoparticles are among the most widely commercialized engineered nanomaterials, because of their antimicrobial properties. They are already commonly used in medical devices, household products and industry [15].

Nanoparticles are defined as particulate dispersions or solid particles with a size in the range of 10-100 nm [16], which provides mechanical, optical, electrical and structural advanced, and an increased surface area than the original substance [17, 18].

The bactericidal activity of silver nanoparticles against the pathogenic, multidrug-resistant (MDR) as well as multidrug-susceptible strains of bacteria was studied by many scientists, and it was proved that the silver nanoparticles are the powerful weapons against the MDR bacteria such as *Pseudomonas aeruginosa*, ampicillin-resistant *Escherichia coli*, erythromycin-resistant *Streptococcus pyogenes*, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Staphylococcus aureus* (VRSA) [11].

The broad spectrum of silver nanoparticles includes microorganisms in general, as Gram-positive and Gram-negative bacteria, filamentous fungi, yeasts and viruses. Its most striking property is to have a large surface area. The antifungal activity of silver nanoparticles was also reported by several researchers [19, 20, 21 and 22]. However, despite its efficiency already well known, the mechanism of action of silver nanoparticles is not well understood yet. There are several proposed mechanisms of action (Figure 1), but due to the current dearth of knowledge on this subject, the exact basis for the activity of AgNPs is still uncharacterized [11, 23 and, 24].

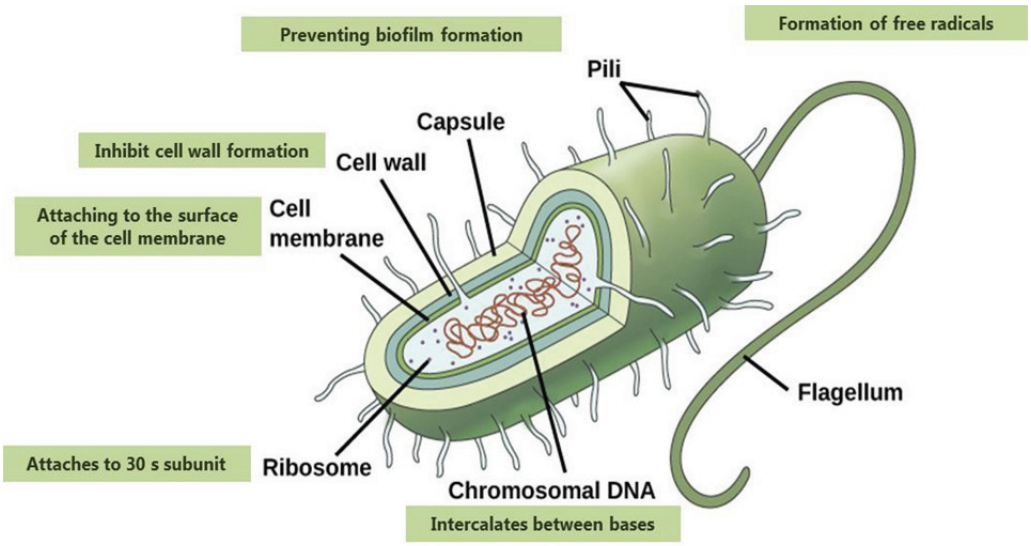


Fig. 1 Mechanism of action of Silver Nanoparticles (proposed by Marambio Jones & Hoek, 2010).

According Marambio Jones & Hoek [25] in submicromolar concentrations, silver ions are internalized and react with thiol groups of cellular proteins that lead to uncoupling of ATP synthesis from respiration, loss of proton motive force and interference with phosphate efflux system. At levels of milli-molar silver nanoparticles induce the detachment of the cell wall membrane from the cytoplasm, with the possible release of intracellular content, DNA condensation and loss of replicative capacity. Free radicals produce oxidative stress in reactive oxygen species (ROS) resulting in membrane damage and DNA. Finally, silver nanoparticles increase cell membrane permeability and subsequently penetrate into cells or whole inducing a cascade effect described above.

1.2 Cytotoxicity

Regarding toxicity to animal cells, silver nanoparticles are those with the lowest index [26]. *In vitro* studies have also showed silver nanoparticles effect [27]. In Marambio Jones and Hoek [25] review is suggested that eukaryotic cells could be impacted with the same mechanism already described in bacteria.

Cytotoxicity of nanoparticles has been a robust research area in recent years. Many medically relevant nanoparticles such as gold and silver were investigated for their cytotoxicity aspect. Gold nanoparticles and nanorods showed no significant toxicity in tumoral HeLa cells [28, 29] while significant size-dependent toxicity was observed in fibroblast, epithelial cells, and melanoma cells [29]. AgNPs showed different degrees of *in vitro* cytotoxicity [27, 31].

The neutral red uptake (NRU) assay provides a quantitative estimation of the number of viable cells in a culture. That is one of the most used cytotoxicity tests with many biomedical and environmental applications. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. NRU assay may be successfully used to most primary cells and cell lines from diverse origin [32].

2. Materials and Methods

2.1 Materials

Strain of *S. aureus* ATCC 27853 and two strains of *S.aureus* obtained from hospital-acquired infections and called S.a.1 and S.a.2.; Silver nanoparticles: AgNP 20 µg/mL solution (SIGMA), tested at dilutions: 5.0, 1.25 and 0.156 µg/ mL; Antibiotics: ceftazidime, meropenem, amikacin, ampicillin + sulbactam, levofloxacin, chloramphenicol, vancomycin, penicillin, oxacillin and cefoxitin; Mouse fibroblasts NCTC-929 was purchased from Adolfo Lutz Institute, HeLa and HepG2 were kindly provided by Hemocentro – USP, Ribeirão Preto; culture medium DMEM and trypsin-EDTA (Sigma – EUA) and fetal bovine serum (FBS, Invitrogen).

2.2 Methods

2.2.1 Inoculum of microorganisms

Fresh cultures with less than 24 hours incubation were prepared at a concentration of 0.5 McFarland (1.5 x 10^8 CFU / mL) and used at different dilutions in the designs proposed evaluation.

2.2.2 Antimicrobial susceptibility testing

From the bacterial exponential growth (~ 16 hours), colonies suspended in saline adjusted to 0.5 McFarland were inoculated in Muller Hinton Agar - MHA (SIGMA) using agar diffusion method [33, 34]. After 15 minutes standing, disc recommended by the Clinical Laboratory Standards Institute of - CLSI were placed on the plate and incubated at 35°C (± 2) 16 to 18 hours . The antibiotics tested were: ceftazidime, meropenem, amikacin, ampicillin + sulbactan, levofloxacin, chloramphenicol, vancomycin, penicillin, oxacillin and cefoxitin. Susceptibility was verified by reading the diameter of the halos formed and interpreted according to values set by the CLSI [35].

2.2.3 Growth inhibition test with AgNPs

To assess inhibition of bacterial growth by nanosilver, 16 hours growth bacteria suspensions were incubated at 35 °C (± 2), 150 rpm with different concentrations of silver nanoparticles (5.0, 1.25, and 0.156 µg/ml). Aliquots were removed every 1 hour during 12 hours to cell viability determination by the method of serial dilutions and counting of colonies on plates.

2.2.4 Cytotoxicity

Cytotoxicity evaluation was assessed by monitoring the neutral red uptake NRU assay using mouse fibroblasts cells NCTC-929, tumoral HeLa (cervix) and HepG2 (hepatoma) cells (100 µL; $1x10^5$ cells/ml) seeded into 96 well microliter plates and left to adhere for 24 h. The next day, the medium was removed from the wells and the cells were exposed to 0.156 µg/mL, 1.25 µg/mL and 5.0 µg/mL of nanosilver dispersed in complete medium with 5% FBS (100 µL/well). After 24 hours exposition, the medium was replaced with complete media 5% FBS containing neutral red dye (1 mg/mL) Plates were incubated for a further 3 h. Then the medium was removed and after dye extraction using ethanol/ acetic acid/water (50%/1%/49%) the absorbance was measured at 540 nm in spectrophotometer (Titertek Multiskan plate, EUA). Absorbance measurements of cells exposed only to medium were considered as 100 % cell viability (i.e the negative control). Inhibition of growth of cells was calculated from the relative absorbance of untreated control cells at 540 nm.

3. Results and Discussion

Since some years ago it has been known that silver nanoparticles have several properties which differ from those at the nanometer scale and have a wide field of applications. Many studies are being performed to verify the antimicrobial action of these nanoparticles, but there are few studies on the effects of these on human health, mainly regarding to the biological system and the possible toxic effects that they can cause [36].

The size and morphology of the silver nanoparticles can affect the biocide efficacy [37, 38 and 39]. The nano-scale size AgNPs affects the surface area of the particle which is in contact with the target bacteria. It has been recently suggested that AgNPs properties such as size, shape, surface finish and surface charge affect the rate, location and / or time of release of ionic silver [40].

3.1 Antimicrobial susceptibility testing with antibiotics

The literature reports that *S. aureus* is a strong producer of biofilm, which is a critical factor during infections with this strain. Regarding antimicrobial susceptibility tests, the reference strain showed more sensitivity to 75% of the evaluated antibiotics, while the clinical strain S.a.1 and S.a.2 strain showed resistance approximately to 80%. These results served as a reference for comparison with the results of antimicrobial susceptibility using silver nanoparticles.

3.2 Growth inhibition test with AgNPs

Silver nanoparticles with size ranging from 1 to10 nm have been reported to be most effective against bacteria through direct interaction with bacterial cells [41].

Although it is well known that silver, whether in an ionic or nanoparticle form, is highly toxic to microorganisms [42, 43, 44, 45and 46], the mechanism of its action has not been fully elucidated. In the case of Gram negative bacteria, a recent report demonstrated that AgNPs make breakages through of the outer membrane, affecting the permeability of it and these have been termed "pits", [47]. However, since literature surveys did not conclude about the mechanism of AgNPs in Gram-positive bacteria, it is important to study this effect [48]. Thus, this study aimed to evaluate the antimicrobial activity of silver nanoparticles in 3 strains *S. aureus* (reference strain and two clinical strains) that showed resistance to some types of antibiotics.

Control experiments without AgNPs run in parallel with experiments evaluating the three strains of *S. aureus* with AgNPs. All strains showed the same growth profile without AgNPs, allowing the building of an average for the three strains studied. On the other hand, concerning the growth in the presence of AgNPs the three strains showed different profiles.

According to results (Figure 2a, 2b, 2c) the reference strain ATCC 27853 and the clinical strain S.a.2 strains showed some sensitivity to the lowest concentration tested (0.156 µg/mL), while the clinical strain S.a.1 grew similar to the control without AgNPs. The clinical strain S.a.2 showed the highest sensitivity to the concentration of 1.25 µg/mL, although there was no total death at this concentration.

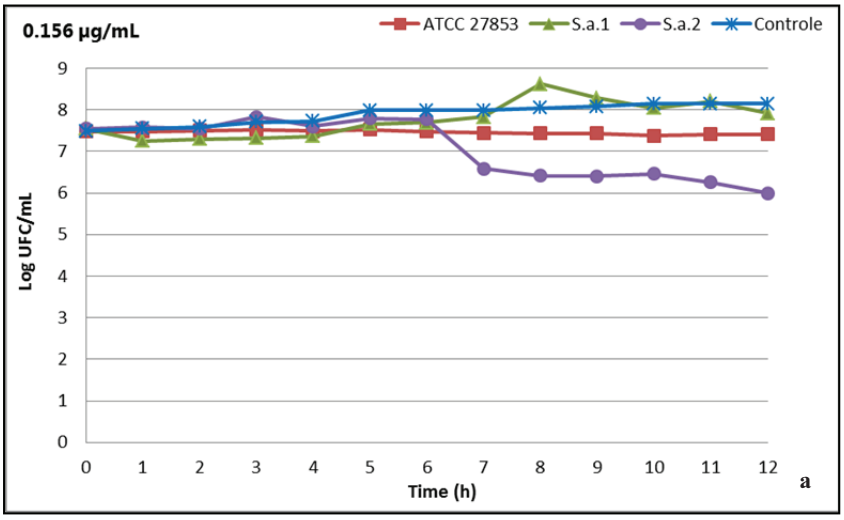
No growth of *S. aureus* ATCC 27853 was detected after 8 hours of exposition to 5.0 µg/mL AgNPs, showing to be the most sensible to the higher concentration of nanoparticles tested (5.0 µg/mL). The growth of clinical strain S.a.2 was not detected after 9 hours of exposition to 5.0 µg/mL of AgNPs and 11 hours of exposition to 1.25 µg/mL of AgNPs. This result indicates this strain is the most sensible among the three strains studied.

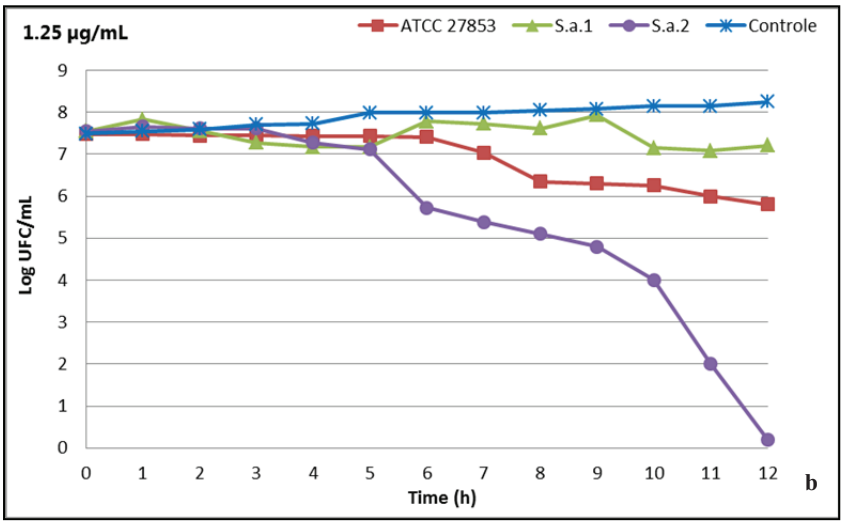
The clinical S.a.1 was resistant to 0.156 and 1.25 µg/mL AgNPs concentration. Non-growth was detected with 5.0 µg/mL after 12 hours silver nanoparticle exposition.

The results of the clinical strain S.a.1 corroborate the results of the tests with antibiotics, which showed extensive resistance, which did not happen with S.a.2 strain that showed great sensitivity to silver nanoparticles.

The results showed in this study demonstrate the importance of using silver nanoparticles as an alternative to conventional antimicrobial agents currently used. The use of nanosilver with antibiotics may promote enhance the antimicrobial action.

Further studies should investigate the combined action of AgNPs and antibiotics against resistant clinical strains.as an alternative to control infections.





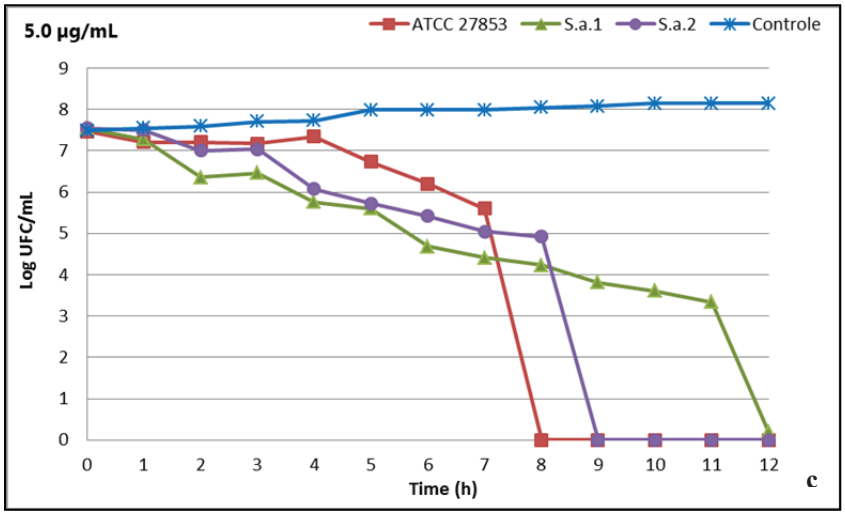


Fig. 2 Growth inhibition curve of the *S. aureus* strains tested at different concentrations of silver nanoparticles (AgNPs): a) 0.156 µg/mL; b) 1.25 µg/mL and c) 5.0 µg/mL.

3.3 Cytotoxicity

The results indicate no cytotoxicity potential at the 10 nm AgNP on normal murine cell lines NCTC 929 or HeLa and HepG2 tumoral cells using the AgNP concentration of 0.156 µg/mL. When cells were exposed to 1.25 µg/mL, the tumoral cell HepG2 showed more sensibility than others cells. Viability reduced was observed in both NCTC 929 and HepG2 cell lines using 5.0 µg/mL with more effect at HepG2 tumoral cells. These results contributed with the information that these bactericidal AgNP concentrations were non-cytotoxicity in normal cells but in tumoral HepG2 cells a decrease of the viability were observed with 5.0 µg/mL. HeLa cells were not affecting by any concentration AgNP used in this study (Figure 3). Cytotoxic effect and apoptosis induction by silver nanoparticles in HeLa cells were showed only at concentrations of 80 µg/mL or higher [49].

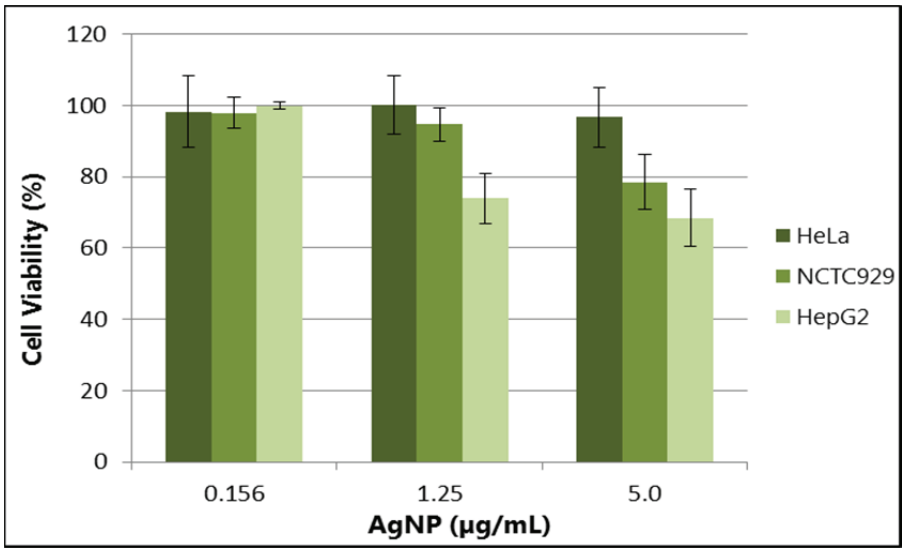


Fig. 3 Comparison of cytotoxicity effect by 10 nm AgNP treatment. Cell lines were pre-cultured for 24 hours, and then incubated with 0.156 µg/mL, 1.25 µg/mL and 5.0 µg/mL concentrations of AgNP (10 nm) for 24 hours. Cell viability was estimated by neutral red reagents. Each value indicates eight wells mean ±SD.

Concerns have been raised about potential adverse health effects due to increasing dispersion of AgNPs in the environment. Grosse [15] examined the cytotoxic effects of spherical, citrate-coated AgNPs (10, 50 and 100 nm) in rat brain endothelial (RBE4) cells. The results indicated that exposure of RBE4 cells to AgNPs lead to significant reduction in dye uptake as measured with the NRU assay. The effect was found to be related to particle size, surface area, dose and exposure time. Our results in compare with other studies highlight the importance of choosing different cells to evaluate cytotoxicity of nanoparticles, including AgNP, since the effects can be varied depending on the cell line tested. Researchers should harmonize the tests conditions to estimate and compare the toxicity of nanoparticles.

4. Conclusions

Silver is known for its antibacterial activity. This activity depends on the strain sensitivity and on the contact surface, wherein the silver can inhibit the respiratory chain enzyme systems of some bacteria and alter their DNA synthesis. The three evaluated strains, in the conditions tests, showed different profiles on sensitivity to AgNPs. The suspension of silver nanoparticles showed more antimicrobial activity on the reference strain and on one of the hospital strains. Concerning cytotoxic effects of silver nanoparticles in this study, 10 nm nanosilver showed no adverse effects on the fibroblast murine cells NCTC 929 and tumoral HeLa or HepG2 cells at 0.156 µg/mL revealing to be *in vitro* non-cytotoxicity in these cell lines. None concentration affect HeLa cells but HepG2 and NCTC 929 cells when exposed to AgNP 1.25 µg/mL and 5.0 µg/mL concentration had their viability reduced. Further studies should investigate the use of AgNPs combined with antibiotic against resistant clinical strains and also focus on the *in vitro* toxicity of nanoparticles in order to use them as new materials and substances in medical application.

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