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Research paper

Comparison of silver nanoparticles stored under air or argon with respect to the induction of intracellular free radicals and toxic effects toward keratinocytes

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ABSTRACT

Bacterial infections decreased considerably after the discovery of antibiotics. Nevertheless, because of the rising rate of infections caused by antibiotic-resistant bacteria strains, the search for new bactericidal agents has again become a crucial topic in clinical medicine. Silver nanoparticles (AgNP) have a huge potential in dermatology and wound care management because of their ability to release silver ions (Ag^+ ions) in a prolonged and sustained way. However, negative effects of silver on the patient's cells should not be underestimated. Furthermore, it has been controversially discussed whether AgNP are responsible for nanoparticle-specific outcomes or not. In this study, we investigated the effects of AgNP on human skin keratinocytes (HaCaT) in order to better understand the mechanisms of cytotoxicity and to improve the use of this highly reactive biocide in wound healing. We found that most of the cells with internalized AgNP displayed the typical morphological signs of apoptosis. The cell viability assay (XTT) showed concentration-dependent toxic effects of the AgNP toward HaCaT cells. The generation of reactive oxygen species (ROS) induced by AgNP was investigated in cell suspensions by means of electron paramagnetic resonance (EPR) spectroscopy. In order to distinguish between the effects of Ag^+ ions released during AgNP storage and those of Ag^+ ions released after nanoparticle application, we compared AgNP stored under air (O_2) with AgNP stored under argon (Ar). Dispersions of AgNP stored under Ar have a low content of Ag^+ ions because of the absence of oxygen which is needed for oxidative dissolution. The results show that Ag^+ ions released during particle storage are responsible for most of the ROS produced during 1 h incubation with the cells. AgNP (Ar) also induced intracellular ROS but to a much smaller extent compared to AgNP (O_2). These findings highlight the complexity of experiments to assess the toxicity of AgNP and suggest the possibility of reducing AgNP toxic effects by storing AgNP formulations and even silver-containing wound dressing under an inert gas atmosphere.

1. Introduction

Silver nanoparticles (AgNP) and related products are one of the first achievements of biomedical engineering applied to nano-scaled materials. Medical applications such as silver-containing wound dressings are based on the antibacterial potential of silver ions (Ag^+ ions) [1–3]. AgNP, which release Ag^+ ions in a slow and sustained manner, can improve the outcomes of antibacterial dermatotherapies and wound care management. Several publications have described AgNP as potent and efficient antimicrobials, although the toxic effects of this agent cannot be denied [4,5]. A number of studies have described particle-specific as well as Ag^+

Abbreviations: AgNP, silver nanoparticles; HaCaT, Human adult low calcium high temperature; XTT, (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide); EPR, electron paramagnetic resonance; ROS, reactive oxygen species; TEM, transmission electron microscopy; FCS, fetal calf serum; PVP, polyvinylpyrrolidone; SEM, scanning electron microscopy; RPMI, Roswell Park Memorial Institute; PBS, phosphate buffered saline; UV, ultraviolet; OD, optical density; TEMPO, 2,2,6,6-tetramethylpiperidinyloxyl.

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ion cytotoxic effects [6]. Numerous other experiments indicated the release of Ag^+ ions in the presence of oxygen as the main mechanism of cytotoxicity [7]. It was shown that an anaerobic atmosphere reduces the process of AgNP oxidation to Ag^+ ions so that AgNP were much less noxious to bacteria [8]. Ag^+ ions exert toxic effects on both bacteria and eukaryotic cells [6,9], such as fibroblasts [10]. Damages to DNA, cell structures or membranes can lead to a dysfunction of the metabolism or of the cell cycle, resulting in apoptosis and necrosis [11]. However, it is still discussed controversially, whether nanoparticle-specific effects are responsible for noxious outcomes or not [12].

In this study, based on the broad application of AgNP in dermatology and wound healing, we investigated the toxic effects of AgNP (spherical, diameter of 70 ± 20 nm) on the human keratinocyte cell line (HaCaT). For the characterization of the AgNP see Table 1 and Ref. [13].

Silver nitrate is well soluble and leads to Ag^+ ion concentrations of up to 3200 ppm [14]. However, because silver reacts rapidly with biomolecules (forming complexes) as well as chloride (forming AgCl) and is thereby inactivated, in order to maintain the concentration of active Ag^+ ions, silver sulfadiazine is commonly applied twice a day and silver nitrate up to 12 times a day. For this reason, the most efficient silver releasing formulations and dressings provide a sustained release of the active ions in concentrations of 70–100 ppm [15]. The bactericidal concentrations of AgNP vary depending on the bacteria strains, nanoparticle size, culture media and other experimental conditions. In the review of Chernousova and Epple, toxic and inhibitory concentrations for AgNP in the range of 0.1–20 μg/mL were reported [4]. As reviewed by Chopra et al., the MIC level for *Staphylococcus aureus* and *Pseudomonas aeruginosa* was in the ranges of 8–80 μg/mL and 8–70 μg/mL, respectively, when silver nitrate ($AgNO_3$) is used as a delivery system for Ag^+ ions [15]. Thus, in this study we used concentrations of AgNP between 10 and 50 μg/mL, which is in the range of the effective bactericidal concentration for most commonly used silver formulations [14]. The internalization of particles by cells, their interaction with cell structures and cell morphological changes were investigated by means of transmission electron microscopy (TEM). Cell viability was monitored at different AgNP concentrations using the XTT test. Additionally, we investigated the influence of fetal calf serum (FCS) content in the cell media on AgNP cytotoxicity. FCS is widely used as a supplement for cellular culture media and is essential for cell growth and viability [16–19]. By diluting the FCS concentration, changes in the cellular viability may occur and the defense ability of cells against an exogenous stress may be weakened, resulting in an overestimation of AgNP cytotoxicity.

The generation of intracellular reactive oxygen species (ROS) induced by AgNP was investigated by means of electron paramagnetic resonance (EPR) spectroscopy. EPR spectroscopy is a reliable, non-colorimetric method used to detect free radicals in cell suspensions. We have already used this technique to measure free radicals induced by UVB radiation and nanoparticles in HaCaT cells or in *in vivo* measurements [20,21]. In the presence of oxygen, Ag^+ ions are continuously released, also during synthesis and storage. To differentiate between the toxic effects of Ag^+ ions released during particle synthesis and storage and that of ions released only during incubation with cells, two different batches of AgNP were tested. One was synthesized and stored under air in the presence of oxygen (O_2) with a high Ag^+ ion concentration [7] and one under argon (Ar) with a low Ag^+ ion content [13]. The two particle batches differ in that AgNP (Ar) started releasing silver ions after addition to the cells, whereas AgNP (O_2) already released silver ions during storage (until a steady state was reached) and released additional Ag^+ ions once they were diluted and added to the cells.

Table 1
Characterization of the investigated AgNP.

	AgNP (O_2)	AgNP (Ar)
Size (by SEM)	70 ± 20 nm	70 ± 20 nm
Dispersion	Ultrapure water under air	Argon saturated ultrapure water under argon atmosphere
Functionalization	PVP	PVP
Zeta potential	−25 mV	−25 mV

2. Methods and materials

2.1. Preparation and characterization of the silver nanoparticles

Polyvinylpyrrolidone (PVP)-coated AgNP were synthesized by reduction of silver nitrate with glucose in the presence of PVP according to the method of Wang [22]. Briefly, 2 g of glucose and 1 g of PVP were dissolved in 40 g of water and heated to 90 °C. Then, 1 mL of $AgNO_3$ (0.5 g/mL) aqueous solution was quickly added. The dispersion was kept at 90 °C for 1 h and then cooled to room temperature. The particles were purified by repeated ultracentrifugation (3×10^4 rpm; 30 min) and re-dispersion in pure water. The silver nanoparticles were then redispersed in water [23]. The silver concentration was determined by means of atomic absorption spectroscopy. The particles were negatively charged (zeta potential = −25 mV; Malvern Zetasizer Nano ZS ZEN 3600, Malvern Instruments, Worcestershire, UK) and stored at 4 °C in the dark. Scanning electron microscopy (SEM) was performed on the particles with a FEI Quanta 400 ESEM instrument (FEI, Hillsboro, Oregon, USA) under a high vacuum after sputtering with Au:Pd. The spherical particles had a diameter of 70 nm ± 20 nm as determined by SEM. Further characterization parameters can be found in Table 1. The particle characterization in RPMI-1640 (RPMI) medium has been reported in Loza et al. [13]. The batches produced under argon atmosphere were aliquoted in vials and filled with argon.

2.2. Cell culture and irradiation

For the investigations the human keratinocyte line (HaCaT) was used (Deutsches Krebsforschungszentrum, Heidelberg, Germany) [24]. This immortalized, non-tumorigenic cell line was derived from primary human keratinocytes [25]. HaCaT cells were grown in 75 cm^2 flasks in 10 mL RPMI with phenol red, 10% FCS, 4 mM glutamine, 10 μg/mL streptomycin and 100 IE/mL penicillin. Cells were cultivated in an incubator at 37 °C with 100% humidity and 5% CO_2. The cells were split every 2–3 days at a ratio of 1:10. When the cells reached a confluency of 70–80%, the RPMI medium was removed and the cells were washed with phosphate buffered saline (PBS). After removing the PBS, 5 mL of the digesting enzyme trypsin (0.2%) and EDTA (0.02%) was added. After 5 min, the cells were detached from the flask. The reaction was stopped with the same volume of RPMI medium supplemented with 10% FCS. The cells were centrifuged (300 g, 10 min) and, after resuspending them in RPMI medium, were counted with a Neubauer chamber and seeded in new flasks or used for further investigations. For irradiation, a UV lamp (280–400 nm; 3.5 mW/cm^2; TH-1E, Cosmedico Medizintechnik, Villingen-Schwenningen, Germany) was used. The lamp was placed at a distance of 1 cm and the cells were irradiated for 1 min, which corresponds to a UV radiation dose of 210 mJ/cm^2.

2.3. Incubation conditions

The AgNP in the aqueous dispersion were stored in aliquots under argon or air atmosphere. Shortly before incubation with