Effect of silver nanoparticles on human primary keratinocytes

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Introduction

Nanoparticles are gaining much importance for biological application in biomedicine, biotechnology and other life sciences. For their unique physicochemical properties they are used in molecular diagnostics, cancer therapy, as well as drug and gene delivery systems. Silver nanoparticles (AgNPs) have the highest degree of commercialization. For their strong antimicrobial, antiviral and antifungal activity, they have been used extensively in a range of medical settings especially in wound dressings. Nanoparticles are defines as structures that have at least one dimension in 1-100 nm range. Their ultra-small size comparison to enormous surface are, makes AgNPs very reactive forms. Small size also confers grater particles mobility and has impact on their cellular distribution. Depending on the size, shape and the type of a carrier, AgNPs demonstrate different physicochemical properties. It was observed that smaller nanoparticles enter the cells more easily than the larger ones and have more significant effect on membrane integrity, the level of reactive oxygen species (ROS), induction of apoptosis and cell cycle arrest. This study was undertaken to examine the potential toxic effects of 15 nm polyvinylpyrrolidone-coated (PVP) AgNPs on Normal Human Primary Keratinocytes (NHEK). Cells were treated with different concentrations of AgNPs and then cell morphology, viability and metabolic activity were studied. Furthermore we analysed the effect of AgNPs on integrity of keratinocytes genome, cellular stress as well as activation of apoptotic processes. To determined a role of AgNPs in signal transduction pathway, we examined an activation of MAPKs, like p38, ERK1/2 and JNK, as well as kinase Akt and p53 protein.

Aim of study

This study was undertaken to examine the potential toxic effects of 15 nm PVP-coated AgNPs on NHEK cells.

Methods

NHEK cells from three individuals were obtained from Lonza (Switzerland).

The following study was carried out:

1. Detection of AgNPs in cells using light microscope Nikon eclipse Ti-S.
2. Study of cell viability by measurement of ATP content.
3. Verification of cell viability by MTT assay.
4. Determination of proliferation rate by BrdU assay.
5. Determination of apoptosis induction by measurement of caspase 3/7 activity.
6. Verification of cells migration by time-lapse microscopy.
7. Measurement of DNA damages using comet assay.
8. Protein activation analysed by western blot technique.
9. Analysis of genes expression by real time PCR reaction.

Results

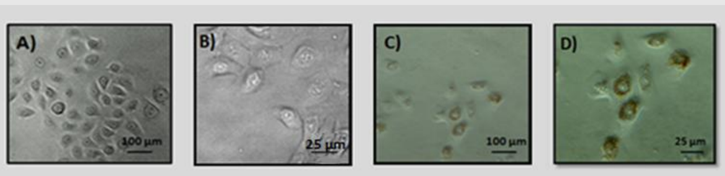

Fig. 1. Optical micrographs of untreated NHEK cells (A,B) and cells treated with 25 µg/µl AgNPs (C,D). The pictures were made by using light microscope Nikon eclipse Ti-S.

Results

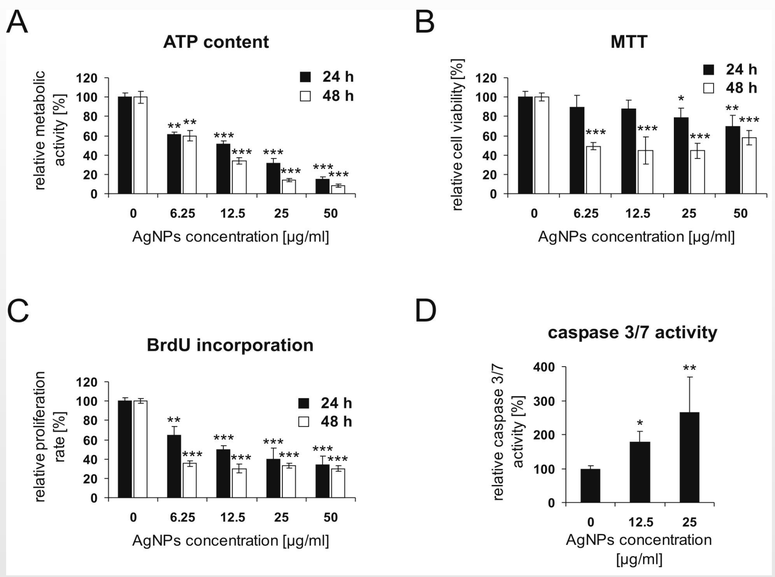

Fig. 2. Effects of AgNPs on primary keratinocytes biology.
A) Metabolic activity of NHEKs exposed to AgNPs. Intracellular ATP content was measured to determine metabolic cell condition. NHEKs (plated at the density of 1.25×10^4 cells/cm^2) were treated with different concentrations of AgNPs for 24h and 48h. Untreated cells served as a control. For statistics Student's t-test was performed (*p<0.05; **p<0.01; ***p<0.001). This graph represents the mean ±SD of five independent experiments.
B) Viability of NHEKs exposed to AgNPs. Cell viability was analysed using MTT assay. NHEKs (plated at the density of 1.25×10^4 cells/cm^2) were treated with different concentrations of AgNPs for 24h and 48h. Untreated cells served as a control. For statistics Student's t-test was performed (*p<0.05; **p<0.01; ***p<0.001). This graph represents the mean ±SD of five independent experiments.
C) Proliferation rate (BrdU assay) of NHEKs exposed to AgNPs. NHEKs (plated at the density of 9.4×10^3 cells/cm^2) were treated with indicated concentrations of AgNPs for 24h and 48h. Cells were incubated with BrdU labeling solution for 12h. Untreated cells served as a control. For statistics Student's t-test was performed (*p<0.05; **p<0.01; ***p<0.001). This graph represents the mean ±SD of five independent experiments.
D) Apoptotic cell death measured by the determination of caspase 3/7 activity. The luminescent assay for caspase 3/7 activity was performed using 3 µg of total protein isolated from NHEKs after incubation with 12.5 and 25 µg/ml AgNPs for 48h. For statistics Student's t-test was performed (*p<0.05; **p<0.01; ***p<0.001). Graph represents the mean ±SD from three independent experiments.

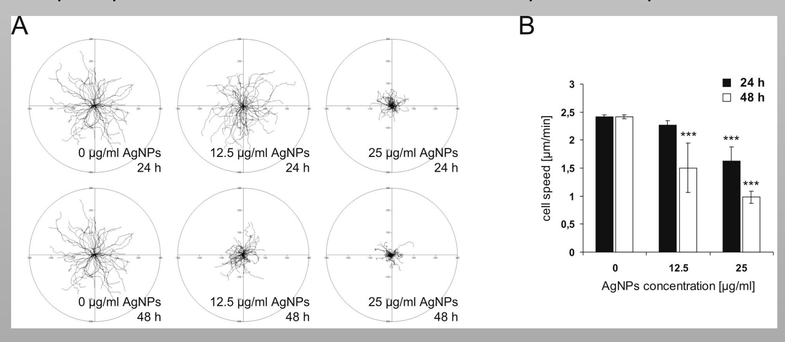

Fig.3. Effect of AgNPs on cell migration. Cell migration was examined with a Leica DMI6000B microscope equipped with LAS AF software. NHEKs were plated in 6 wells culture plates at the density of $6.4\text{x}10^3$cells/cm^2 and treated with 12.5 and 25 µg/ml AgNPs. Cells movements were recorded 24h and 48h after AgNPs addition for 90 minutes with time-lapse 1.5 minutes (A). The tracks of individual cells were determined from series of changes in the cell central positions, pooled and analyzed to determinate the total determinate the velocity of cell movement (VCM)(B). All analyses were done using Hiro software v 1.0.0.4. Student's t-test was carried out for statistics (*p<0.05; **p<0.01; ***p<0.001).

Results

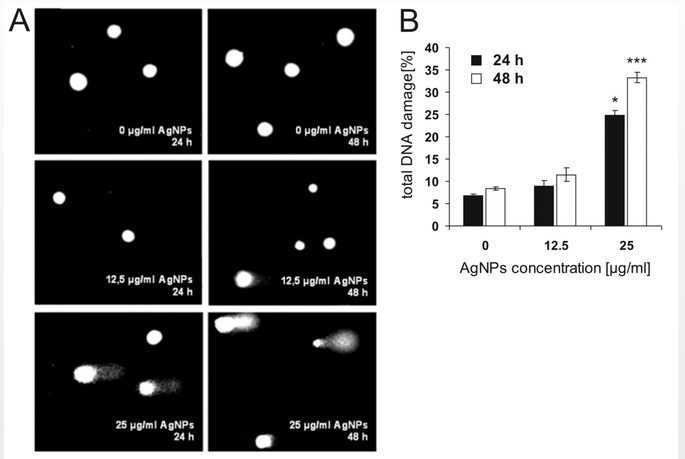

Fig. 4. DNA damage in NHEKs verified by comet assay. NHEKs were plated at the density of $2.1\text{x}10^3$ cells/cm^2. After 24h cells were treated with 12.5 and 25 µg/ml AgNPs for 24h and 48h. Untreated cells served as a control (A). DNA damage presented as the mean value of the percentage of DNA in the comet tail (%DNA). Images were made using the computer program Comet Plus, Theta System Gmbh, Germany (B). Graph represents the mean ±SD of three independent experiments. Statistically significant differences were calculated by RIR Tukey test (*p<0.05; ***p<0.001).

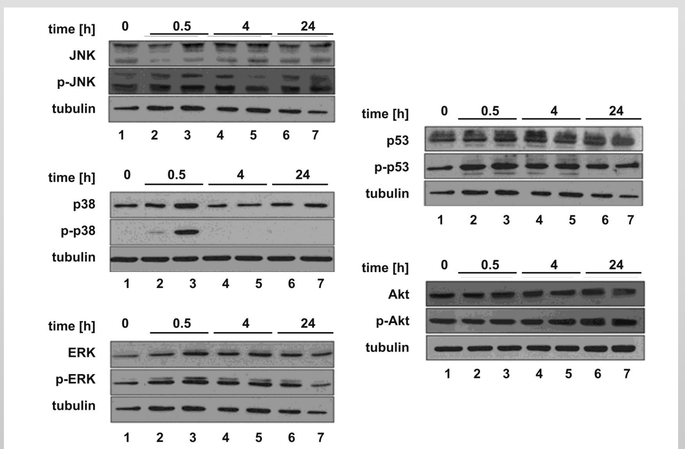

Fig. 5. The activation of ERK, p38, and JNK MAPKs, as well as Akt and p53 protein was verified by western blot analysis. NHEKs were cultured in 60 mm tissue culture dishes at density of 9.5×10^3 cells/cm^2. The cells were exposed to 12.5 µg/ml (lines 2, 4 and 6) or 25 µg/ml (lines 3, 5 and 7) AgNPs for the indicated periods of time. Untreated cells were used as a control (line 1). Tubulin was used as an internal control to monitor for equal loading. These blots are representative of three independent experiments.

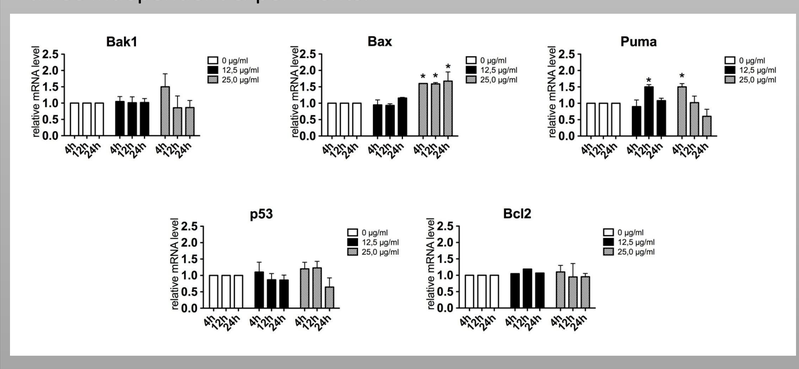

Fig. 6. Expression of selected pro-apoptotic and anti-apoptotic genes in NHEKs. Graphs show expression of Bak1, Bax, Bbc (PUMA), p53 and Bcl2 mRNAs in NHEKs treated with various concentrations of AgNPs (0; 12,5; 25 µg/ml) for different time (4, 12 and 24 h). Specific mRNAs were normalized to B2M level and presented as relative units compared to the control. Data represent the mean ± SE (n = 3-4). The asterisk indicates significant difference between the treated sample and untreated control ($p < 0.05$).

Conclusions

For their unique properties AgNPs have recently received much more attention in their medical application, especially in the treatment of wounds and burns. A skin is the biggest organ which is directly liable to AgNPs and in this case keratinocytes are probably the cells most frequently exposed to these nanoparticles. In our study we observed that 15 nm PVP-coated AgNPs decreased cell viability and metabolic activity, as well as, they inhibited proliferation and migration of keratinocytes. Moreover AgNPs caused DNA damages and induced apoptosis via increase in caspase 3/7 activity. Western blot analysis showed that AgNPs significant activated p38 MAPK, as well as they caused weaker activation of ERK 1/2 kinase and p53 protein, which is directly engaged in regulation of DNA repair processes and apoptosis. We observed also changes in expression level of selected transcripts from Bcl-2 family coding for pro-apoptotic proteins. Our study for the first time shows that depending on concentration and time of stimulation, AgNPs may present possible danger for NHEK cells, concerning activation of genotoxic and cytotoxic processes.

Literature

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3. AshaRani PV et al. ***Cytotoxicity and genotoxicity of silver nanoparticle in human cells.***
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