Effects of Short-Term Silver Nanoparticle Exposure on Proliferative Signaling Pathway in Human Skin Keratinocyte

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Abstract

The growing usage of silver nanoparticles in healthcare products, household appliances, textile and agriculture in recent years has increased skin exposure to these particles. However, there is limited number of studies available on the safety and effects of silver nanoparticles on human skin in short time of contact. Human skin keratinocytes (HaCaT) cell line were used in the present experiments because they traditionally represent normal keratinocyte function.

Objective: This study aimed to determine the effects of silver nanoparticles on proliferation of human skin keratinocytes after 24-hour exposure.

Methods: Silver nanoparticles were synthesized by chemical reduction method to get average particle size of about 20 nm. Human skin keratinocytes cells were treated with various concentrations of silver nanoparticles (1, 3, 5 and 10 µg/ml) for 24 hours. Then the cells were grown in DMEM culture medium without silver nanoparticles. The cell number was compared between silver nanoparticles-treated keratinocytes and untreated cells. Changes in expression and phosphorylation of proteins involved in cell proliferation signaling pathway (ERK1/2 and p38 MAPK) were determined by immunoblot.

Results: Silver nanoparticles were successfully synthesized with a size range of 18-26 nm. Proliferation of HaCaT cells decreased with increasing concentration of silver nanoparticles (24-hour exposure). After seven days in culture medium without silver nanoparticles, the number of HaCaT cells was nearly the same as the control group, except cells that had been treated with 10 µg/ml silver nanoparticles. Proteins involved in cell proliferation signaling pathways were determined by immunoblot. Immunoblot of HaCaT cells that were exposed to silver nanoparticles for 24 hours showed that phosphorylation of the ERK1/2 was increased, but no significant change was found in the phosphorylation of p38.

Conclusion: Silver nanoparticles of about 20 nm could decrease HaCaT cell proliferation via ERK1/2 rather than p38 signaling pathway.

J Physiol Biomed Sci. 2014; 27(2): 48-53

Keywords: *Silver nanoparticles, HaCaT cell, ERK1/2, p38 MAPK*

Silver nanoparticles (AgNPs) are clusters of silver atoms with particle size of 1 to 100 nm. The AgNPs were introduced to the healthcare products due to their broad spectrum bactericidal activity. Antibacterial activity of silver has long been used in the treatment of burn wounds in the form of silver sulphadiazine. The mechanism of the antibacterial property of silver is disrupting the peptidoglycan bacterial cell wall and inhibition of ATP synthesis.[1,2] AgNPs, used in burn wound treatment e.g., silver-Acticoat™ dressing, has been shown to be superior in promoting burn wound healing and bacterial clearance than silver nitrate and silver sulphadiazine.[3] The mechanism of AgNPs in wound healing might be due to anti-inflammatory activity of AgNPs.[3,4] Treatment of thermal injury wounds in the BALB/C mice with AgNPs increased anti-inflammatory cytokines IL-10 expression in the keratinocytes. IL-10 can inhibit synthesis of several pro-inflammatory cytokines, including monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) which could promote faster wound healing.[4] AgNPs are beneficial in treating thermal injury wounds; however, AgNPs also exert toxicity to mammalian cells.[5-7] AgNPs toxicity depends on concentration, exposure time, particle size and particle coating.[8-10] Chronic use of topical silver products cause argyria (silver toxicity) which is characterized by gray to gray-black staining of the skin and mucous membranes produced by silver deposition.[11,12] There are reports that AgNPs from wound dressing containing silver were cytotoxic to cultured keratinocyte and fibroblast. Moreover, cells derived from other organs were also susceptible to the toxicity of AgNPs, including lung, liver, kidney,

This work was presented at Siriraj International Conference in Medicine and Public Health 2014 (SICMPH 2014), July 21-25, 2014, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

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brain and reproductive organs. The mechanism of AgNPs cytotoxicity, as studied in fibroblast cell culture, is postulated to be induction of oxidative stress and apoptosis.[13,14] Moreover, AgNPs induce reactive oxygen species and release of cytochrome C into the cytosol of fibroblast, as well as induce a p53-mediated apoptotic pathway.[14] Because fibroblasts are more susceptible to silver cytotoxicity than keratinocytes, the effects of AgNPs on keratinocytes have been shown to reduce proliferation rather than promote apoptosis.[15,16] At least two signaling pathways have been reported on proliferative pathway of keratinocytes, MEK/ERK1/2 and p38 MAPK signaling cascade.

In this study, HaCaT cells exposed to AgNPs at various concentrations for 24 hours showed reduced cells proliferation via ERK1/2 signaling pathway but not p38 MAPK. HaCaT cell could increase cell number similar to control after removal of AgNPs for seven days, except at concentrations higher than 50 µg/ml.

Materials and Methods

Cell culture

Immortalized human skin keratinocyte (HaCaT) cell line[17] was a kind gift from Assoc. Prof. Dr. Uraiwan Panich (Department of Pharmacology, Faculty of Medicine Siriraj Hospital, Mahidol University). HaCaT cells were seeded and grown in Dulbecco's modified Eagle's medium (DMEM/F-12) supplemented with 2.5 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS), at 37 °C, in 95% O_2 and 5% CO_2.

Silver nanoparticles

Silver nanoparticles were synthesized by chemical reduction reaction according to method reported by Liu *et al.*[18] Briefly, 100 mg sodium borohydride (Fisher Scientific, UK) was dissolved in deionized water containing 0.7 mM tri-sodium citrate (Fisher Scientific, UK) and mixed on magnetic stirrer plate. Then 0.1 M silver nitrate (Fisher Scientific, UK) was dropped into solution. After centrifugation, the pellet of particles were dissolved in deionized water. The final concentration of a stock solution was adjusted to 1 mM (100 µg/ml). The mean diameter of silver nanoparticles was measured by transmission electron microscopy (TEM, JEM-1230 Electron Microscope, JEOL, USA). The dynamic light scattering (DLS) (NanoZS : ZEN3600, Malvern Instruments, UK) was used to analyze hydrodynamic size and particle stability in solution.

Silver nanoparticle treatment

Various concentrations of AgNPs (1, 3, 5 and 10 µg/ml) were added into the culture medium for 24 hours and then removed by washing twice with phosphate buffered saline (PBS). HaCaT cells were grown in DMEM culture media without AgNPs.

Cell viability and proliferation

AgNPs cytotoxicity on HaCaT cell viability and proliferation were assayed by analysis under microscope and CellTiter-Blue® cell viability assay (Promega, USA) according to the manufacturer's protocols. HaCaT cells were seeded in 96-well microplates, at a density of 6 x 10^3 cells/well, in 100 µl medium containing 10% fetal bovine serum (FBS). Control group was done in triplicate, using only culture medium. For treatment groups, after 24 hours exposure to AgNPs of various concentrations, the medium was washed twice with PBS. After incubating cells with 100 µl of CellTiter®-Blue reagent at 37 °C for 4 h, the reactions were measured at 570 nm wavelength and background was measured at 600 nm in Multi-Detection Microplate Reader (Synergy HT, Bio-Tek Instrument, USA). The average values from triplicate readings were calculated and the blank was subtracted from the average value.

Immunoblot analysis

Immunoblot analysis was performed to evaluate the changes in protein expression in ERK1/2 and p38 MAPK pathways. The protein expression in these two pathways, including their phosphorylated forms, was analyzed by immunoblot. Treated HaCaT cells were lysed with 2x Laemmli buffer. Protein concentration was determined with Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific, USA). Total protein of 120 µg was loaded on a 12% SDS-PAGE and transferred to nitrocellulose membrane (Bio-RAD, Hercules, CA, USA). The membrane was then blocked with 5% skim milk for 1 h at room temperature, followed by primary antibody at 4 °C overnight. The primary antibodies (Cell Signaling Technology, USA) were rabbit monoclonal anti-p42/44 MAPK (ERK1/2) (1:1000), rabbit monoclonal anti-phospho-p42/44 MAPK (ERK1/2) (1:1000), rabbit monoclonal anti-p38 MAPK (1:5000), rabbit monoclonal anti-phospho-p38 MAPK (1:1000) and rabbit monoclonal anti-β-Actin (1:1000). Secondary antibody was peroxidase-conjugated Affini Pure goat anti-rabbit IgG (1:1000) (Jackson ImmunoResearch Laboratories, USA). Chemiluminescence was performed using TMB One Component HRP Membrane Substrate (BioFx Laboratories, USA).

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). Statistically significant difference between treated groups and the respective control group was determined by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. $P < 0.05$ was considered statistically significant.

Results

Characterization of AgNPs

Primary size and morphology of AgNPs in solution

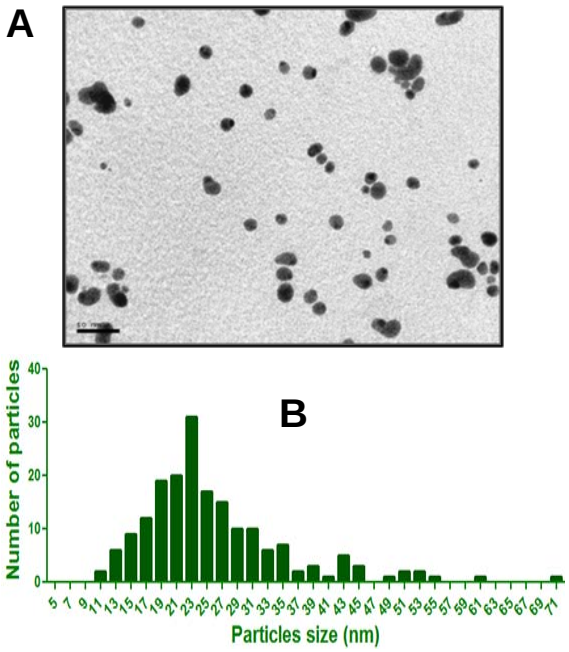

Figure 1 ***(A)*** Transmission electron microscopic image of AgNPs solution dried on copper grid; scale bar represents 50 nm. ***(B)*** Size distribution histogram of AgNPs in colloidal solution.

Table 1 Characterization of AgNP size in solution

Sample	Agglomerate size (nm)		Zeta potential (mV)	
	Deionized water	DMEM/F-12 with FBS	Deionized water	DMEM/F-12 with FBS
AgNPs	66.38 ± 10.9	120.05 ± 47.2	-38.10 ± 7.1	-9.65 ± 1.1

Data are mean ± SD.

were determined by TEM. The number and diameter of particles were analyzed by the program CellProfiler™. TEM image of AgNPs in deionized water showed spherical and monodispersed particles (Figure 1a). The mean diameter of the particles was 26 ± 9.6 nm, averaging from 186 nanoparticles (Figure 1b).

Dynamic light scattering (DLS)

DLS was performed to investigate particle size in solution. Zeta potential (ZP) value demonstrated a surface charge of the particles in solution. DLS and ZP data represent the particles' agglomerate size and stability, respectively, when they were dispersed in solution. Agglomerate size of particles in deionized water is smaller than in medium with fetal bovine serum (Table 1). The particles in deionized water showed more negative surface charge than in medium with serum. These results indicated that particles in deionized water have less aggregate state and higher stability than in medium with serum.

Effects of various AgNPs concentrations on cell viability and proliferation

Treating HaCaT cells with AgNPs for 24 hours caused a concentration-dependent decrease in cell numbers. The data are presented as percentage of

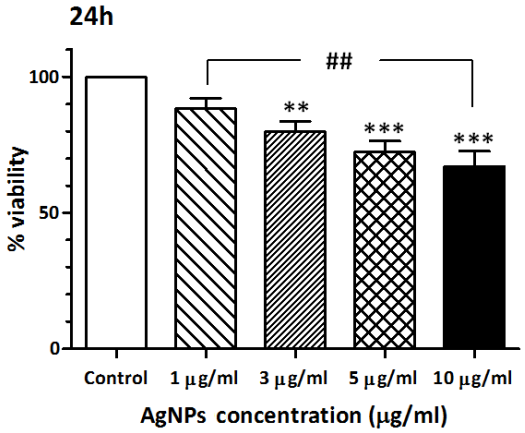

Figure 2 The percentage of cell viability after 24 hour exposure to AgNPs at various concentrations. All results are expressed as mean ± SEM; n = 6 per group. $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the control group; $^{\#\#}P < 0.01$, compared among AgNPs-treated groups.

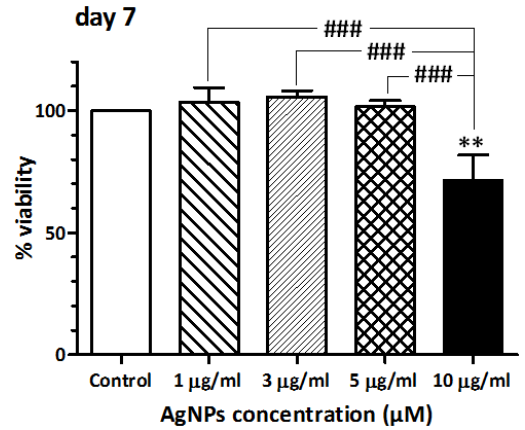

Figure 3 The percentage of cell viability after 7 days of AgNPs removal. All results are expressed as mean ± SEM; n = 6 per group. $^{**}P < 0.01$, compared with the control group; $^{\#\#\#}P < 0.001$, compared among AgNPs-treated groups.

viable cells obtained from the CellTiter®-Blue assays. AgNPs concentration of 3, 5 and 10 µg/ml reduced the number of viable cells to 79.82% ($P < 0.01$), 72.49% ($P < 0.001$) and 67.01% ($P < 0.001$), respectively, when compared to control (Figure 2). After 24 hours, cells were grown in culture media\um without AgNPs for 7 days. Cell numbers of AgNP-treated HaCaT were similar to the control group (1, 3 and 5 µg/ml), except for HaCaT cells treated with 10 µg/ml AgNPs which showed reduced number of viable cells (Figure 3). These results suggested that AgNPs concentration at 10 µg/ml had an anti-proliferative effect on HaCaT cells.

Effects of AgNPs on ERK1/2 and p38 MAPK protein expression.

After treating HaCaT cell with AgNPs for 24 hours, the ERK1/2 and p38 protein levels were assessed by immunoblot analysis. The expression of ERK1/2 and p38 proteins was normalized to β-actin protein. Expression levels of phospho-ERK1/2 protein were increased in all AgNPs concentrations (1, 3, 5 and 10

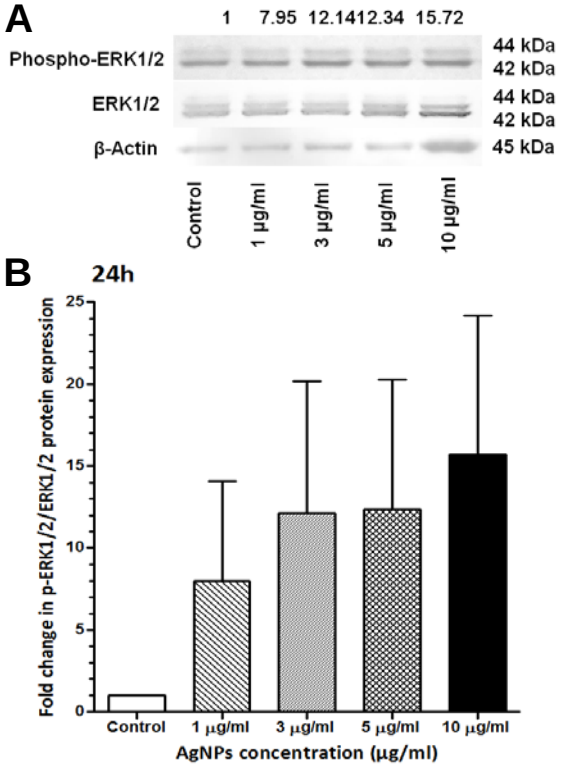

Figure 4 Immunoblot analysis of ERK1/2 and phospho-ERK1/2 protein expression in HaCaT cultured in various of AgNPs concentrations for 24 hours. ***(A)*** Representative ERK1/2and phospho-ERK1/2 bands by immunoblot analysis. ***(B)*** The ratio of phospho-ERK1/2 / ERK1/2 band density. The relative expression was normalized as percentage of β-actin protein expression. All data are presented as mean ± SEM; n = 3 per group. p-ERK1/2 = phospho-ERK1/2.

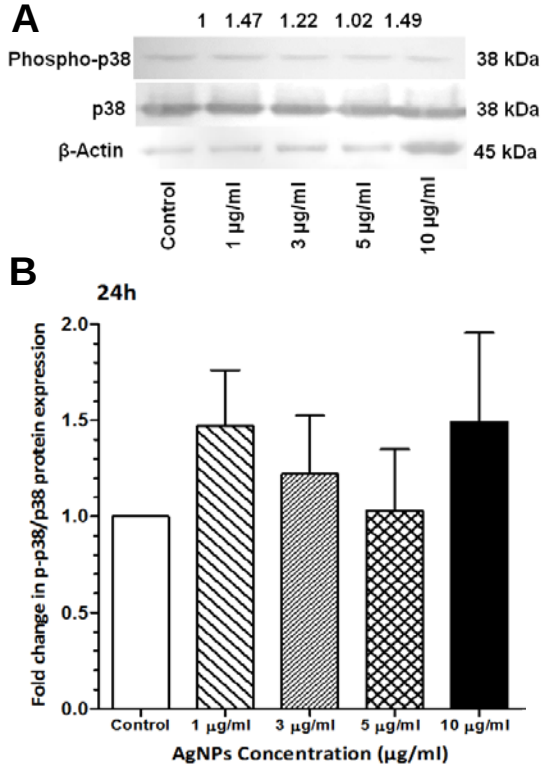

Figure 5 Immunoblot analysis of p38 and phospho-p38 protein expression in HaCaT cultured in various of AgNPs concentrations for 24 hours. ***(A)*** Representative p38 and phospho-p38 bands by immunoblot analysis. ***(B)*** The ratio of phospho-p38 / p38 band density. The relative expression was normalized as percentage of β-actin protein expression. All data are presented as mean ± SEM; n = 3 per group. p38 = phospho-p38.

μg/ml) to 7.95 (95% CI = -38.94 to 25.03), 12.14 (95% CI = -43.12 to 20.84), 12.34 (95% CI = -43.32 to 20.64) and 15.72 (95% CI = -46.70 to 17.26) fold, respectively, compared to the control (Figure 4). While the expression of phosphorylated p38 protein in 1, 3 and 10 μg/ml AgNPs were increased to 1.47 (95% CI = -1.940 to 0.997), 1.22 (95% CI = -1.689 to 1.248) and 1.49 (95% CI = -1.497 to 1.440) fold, respectively, increased expression was not observed in 5 μg/ml concentration (Figure 5). These results demonstrated that AgNPs could activate more phosphorylation of ERK1/2 protein than p38 protein. This suggests that AgNPs could modulate HaCaT cell proliferation through ERK1/2 signaling pathway.

Discussion

In this study, 10 μg/ml AgNPs significantly decreased HaCaT cell viability and proliferation after 24 hours exposure compared to control. When AgNPs were removed from the medium for 7 days, the proliferation of HaCaT cells treated with 10 μg/ml AgNPs was significantly inhibited. This goes along with a previous study reporting that 24 hour exposure to 10 μg/ml AgNPs (diameter ranging from 10-50 nm) caused reduced HaCaT cell number even after culturing in AgNP-free medium for 7 days.[19] These data suggested that AgNPs affected HaCaT cell proliferation after 24 hour exposure. Similarly, other *in vitro* studies showed that AgNPs could inhibit proliferation of normal human lung fibroblasts and human glioblastoma cells,[20] human keratinocyte (HaCaT) cell line[19,21] and epithelial adenocarcinoma cell line (HeLa).[21] These data indicated that 24 hour exposure to 10 μg/ml AgNPs can cause strong inhibition of HaCaT cell proliferation.

The mechanism of AgNP inhibition of keratinocytes cell proliferation is not clearly understood. To investigate the effect of AgNPs on cell proliferation signaling pathways, we found that AgNPs dose-dependently increased ERK1/2 phosphorylation. On the other hand, AgNPs slightly activated p38 phosphorylation but not in a dose-dependent manner. In contrast, a previous study demonstrated that 24 hour exposure to 5 μg/ml AgNPs (10 nm in diameter) attenuated ERK1/2 phosphorylation in human epithelial A-431 cell line.[22] In another study, AgNPs, 15 nm in diameter, at 12.5 and 25 μg/ml increased p38 phosphorylation more than ERK1/2 phosphorylation in normal human primary keratinocytes (NHEK) cell.[23] This may suggest that modification of AgNP responses depend on cell types, AgNP concentration, particle size, particle coating and exposure time. This study suggested that exposure of keratinocyte to low

concentration of AgNPs can inhibit cell proliferation via activation of ERK1/2 rather than p38 signaling pathway. Removal of AgNPs from the culture medium could restore cell proliferation but not at high AgNPs concentration. According to a previous report, it is possible that AgNPs could penetrate through cell membrane via endocytosis and localized at nuclear target, resulting in cell cycle alterations, such as reduced sub G1, G2/M phase and S phase, leading to cell proliferation arrest.[24] These suggested that AgNPs could stimulate activation of ERK1/2 and induce downstream target proteins. It has been shown that the activation of ERK-downstream target proteins probably cause modulation of proliferation or apoptosis.[25,26] Thus, further study is necessary to elucidate the exact changes in ERK cascades and downstream signal transduction to define the alteration in survival and death pathways or other signaling pathways involved.

Acknowledgements

This work was supported by the Siriraj Graduate Thesis Scholarship and Siriraj Research Development Fund. We acknowledge facilities supported by Department of Physiology, Faculty of Medicine Siriraj Hospital, Mahidol University and the National Nanotechnology Center (NANOTEC), a member of National Science and Technology Development Agency (NSTDA).

Conflict of Interest

None.

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