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ORIGINAL ARTICLE

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Effects of silver nanoparticles on primary cell cultures of fibroblasts and keratinocytes in a wound-healing model

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ABSTRACT

Background: Nanoparticles are widely used in different technological fields, one of which is medicine. Because of their antibacterial properties, silver nanoparticles (AgNPs) are used in several types of wound dressings for the treatment of burns and nonhealing wounds, but their influence on each component of the wound-healing process remains unclear. In the present study, we evaluated the effects of AgNPs on normal human dermal fibroblasts (NHDFs) and normal human epidermal keratinocytes (NHEKs). Both cell types are important for wound healing, including with regard to inflammation, proliferation and tissue remodeling. Each phase of wound healing can be characterized by the secretion of cytokines, chemokines and growth factors.
Methods: The production of inflammatory parameters (tumor necrosis factor α [TNF-α], interleukin-6 [IL-6], IL-8 and IL-12 and cyclooxygenase-2 [COX-2]), angiogenesis parameters (vascular endothelial growth factor [VEGF], granulocyte macrophage colony-stimulating factor) and matrix metalloproteinases (MMP-1, MMP-2, MMP-3 and MMP-9) by NHDFs and NHEKs were examined by ELISA or Western blot after 24 and 48 hours of incubation with AgNPs.
Results: We found that AgNPs decreased some inflammatory cytokines (TNF-α and IL-12) and growth factors (VEGF) that were produced by NHDFs and NHEKs after 24 and 48 hours and decreased the expression of COX-2 after 24 hours but only at the highest concentration of AgNPs (25 parts per million).
Conclusions: The results indicate that NHEKs are more susceptible to the application of AgNPs than NHDFs, and AgNPs may be useful for medical applications for the treatment of wounds.

Keywords: Fibroblasts, Inflammation, Keratinocytes, Silver nanoparticles, Wound healing

Introduction

Skin wound healing is a dynamic process that involves inflammation, a proliferation phase (i.e., the formation of granulation tissue and angiogenesis) and tissue remodeling (1). These 3 phases involve well-organized interactions between various tissues and cells. Wound healing begins immediately after injury and is controlled by several factors, including cytokines (antiinflammatory and proinflammatory), chemokines, growth factors and enzymes (2).

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Disruption of the skin barrier leads to the production of proinflammatory cytokines, including interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α) (3). TNF-α production can be blocked by cyclooxygenase-2 (COX-2) inhibitors. Clot formation initiates homeostasis, and macrophages begin to form granulation tissue and release cytokines (IL-1 and IL-6) and growth factors (fibroblast growth factor [FGF], transforming growth factor [TGF] and epidermal growth factor [EGF]). The production of these growth and chemotactic factors (e.g., IL-8) activate the migration and proliferation of keratinocytes. Macrophages promote angiogenesis and secrete vascular endothelial growth factor (VEGF) and FGF (4, 5). Tissue remodeling involves collagen formation by fibroblasts, and the final product is a scar. The last part of wound repair depends on a combination of tissue inhibitors and matrix metalloproteinases (MMPs). MMPs degrade the newly formed extracellular matrix and control the activity of cytokines and growth factors (6). Proinflammatory cytokines (e.g., TNF-α) have been identified as potent inductors of MMPs, and the overproduction of TNF-α can result in nonhealing wounds.

Silver compounds have been well known for centuries as antimicrobial agents and are widely used for the treatment of

bacterial infections (7-9), burns and open wounds (10). Non-healing wounds that are abundantly bacterially colonized can also be treated with silver compound (11). With the elimination of bacteria, the later stages of wound healing progress more effectively (12). Because of the importance of the inflammatory phase in wound healing, silver nanoparticles (AgNPs) have mostly been tested with regard to their inflammatory properties (13, 14). Wound dressings that contain AgNPs have been suggested to enhance wound healing by decreasing inflammation through the modulation of cytokines (15). Several wound dressings with AgNPs are used because of their antibacterial properties (12, 15, 16), but the influence of AgNPs on the complete mechanism of wound healing has not been extensively investigated.

In the present study, we evaluated the effects of AgNPs on the main cells that are involved in the wound-healing process: normal human dermal fibroblasts (NHDFs) and normal human epidermal keratinocytes (NHEKs). We examined the production of wound-healing parameters (interleukins, growth factors and MMPs) using an in vitro wound-healing model.

Materials and methods

Preparation and characterization of AgNPs

AgNPs were obtained from NanoTrade Company as commercial samples, and were prepared by dissolving $AgNO_3$ in distilled water. After adding $NaBH_4$ under magnetic stirring, the AgNPs were formed very quickly. They were characterized by ultraviolet-visible (UV-VIS) spectrum spectroscopy (from 200 nm to 800 nm) and transmission electron microscopy (TEM). The analysis was performed using a JEOL JEM 2011 transmission electron microscope at an accelerating voltage of 100 kV. Photographs were taken with a Morada or Keen View II digital camera, using the iTEM program (SIS, Olympus). Zeta Plus analyzer (Brookhaven) was used for the detection of the zeta potential.

Cell cultivation and maintenance

NHDFs and NHEKs were isolated from plastic surgery skin sections with approval from the ethics committee of the University Hospital Olomouc and the patients' consent. The study was performed in accordance with the Code of Ethics of the World Medical Association. The morphology and origin of the cells were authenticated in the Histology Department of the University Hospital Olomouc.

NHDFs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin under standard culture conditions (5% CO_2, 37°C). Cells were used between the second and third passages. For the experiments, the cells were seeded in 6-well plates at a final concentration of 0.5×10^6 cells/well.

NHEKs were cultured in keratinocyte basal medium-2 (KBM-2) supplemented with KGM-2 SingleQuots and 2% FBS for 3 days (17). After the incubation period, KBM-2 was substituted with EpiLife growth medium supplemented with the Human Keratinocyte Growth Supplement Kit, 100 mg/mL penicillin, 100 mg/L streptomycin and 250 µg/mL ampicillin, and incubated at 37°C in a 5% CO_2 atmosphere. The medium was changed every other day until the keratinocytes reached 50%-60% confluence. Cells were used at the third passage. For the experiments, the cells were seeded in 6-well plates at a final concentration of 3×10^4 cells/well.

Scratch wound assay

Cells were seeded in 6-well plates at 0.5×10^6 cells/well for NHDF and 3×10^4 cells/well for NHEK, and grown to 100% confluence. After 1 day of culture, the scratch wound assay was performed using a 10-cm portion of a plastic pipette, making 1 scratch across each well. The cells were washed with phosphate-buffered saline, and AgNPs at different concentrations were added (0.25, 2.5 and 25 parts per million [ppm]), diluted in serum-free cultivation medium. The concentrations were chosen based on our previous experiments (data not shown). As a positive control, we used scratched cells without AgNPs.

The medium was collected for the enzyme-linked immunosorbent assay (ELISA) and Bio-Plex analyses, and the cells were lysed with RIPA buffer supplemented with protease inhibitors at specified times (24 and 48 hours) for the Western blot analysis.

Bio-Plex analysis

The healing mechanism is a complex system that is regulated by cytokines and growth factors. The levels of TNF-α, IL-6, IL-8, IL-10, IL-12, basic FGF, VEGF and granulocyte macrophage colony-stimulating factor (GM-CSF) were measured using a Bio-Plex cytokine assay (Human Group I 8-Plex Panel; Bio-Rad) according to the manufacturer's instructions. The detection range was 0.18 to 37,000 pg/mL. All of the Bio-Plex results are expressed as the ratio between the concentrations of markers (pg/mL) in treated cells vs. the concentrations of markers (pg/mL) in the positive control.

ELISA

MMP-1, MMP-2, MMP-3 and MMP-9 in the cell supernatant were examined using the Human Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) at 24 and 48 hours according to the manufacturer's instructions.

Western blot

Western blot was used for the detection of COX-2. Cell lysates were briefly ultrasonicated in a cleaning sonicator bath before being centrifuged for 10 minutes at 10,000 *g* at 4°C. The protein concentration was quantified by the Bradford method, and 50 µg of protein was loaded in each well with 10% sodium dodecyl sulfate–polyacrylamide gel for the analysis of COX-2 and β-actin. Gels were wet-transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% low-fat milk in Tris-buffered saline (TBS) with 0.1% Tween 20 for 2 hours at room temperature and washed with 0.1% Tween 20 TBS before incubation with primary antibodies overnight at 4°C. The dilutions of the antibodies were

1:500. The membranes were washed in TBS, and secondary antibody (goat antirabbit or rabbit antigoat) was added for 2 hours and imaged (18). A chemiluminescence system was applied to each membrane for 2 minutes. Excess substrate was removed, and the film was exposed to the membrane for 1-10 minutes. The films were then developed with a film processor.

Statistical analysis

All of the experiments were repeated at least 3 times using different donors in duplicate. The data are expressed as the means and standard deviation. Student's t-test was used for the statistical analysis. Values of $p<0.05$ were considered statistically significant.

Results

Characterization of silver nanoparticles

Silver nanoparticles (1 mL) were diluted in 50 mL of water for the UV-VIS characterization (Fig. 1A). The silver colloid was characterized by strong absorption in the visible region (called the surface plasmon resonance band) at 400 nm. The position of the maximum and width of an absorption band provide information about the form, average size and size distribution of nanoparticles (NPs). The average size of AgNPs was 10 ± 5 nm (>50% of the NPs), confirmed by TEM (Fig. 1B). The pH of the AgNPs was 7.1, with a zeta potential of-22 mV.

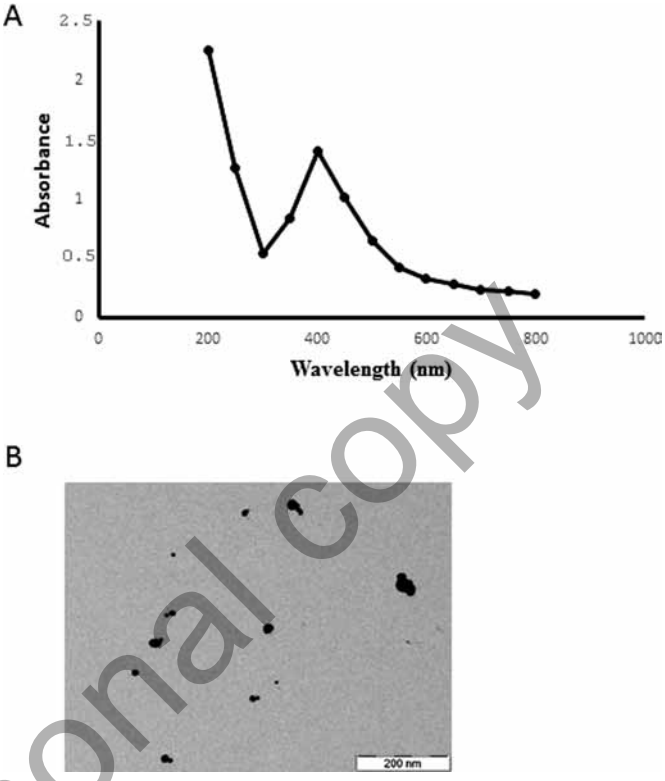


Fig. 1 - Characterization of silver nanoparticles (AgNPs). (**A**) Ultraviolet-visible (UV-VIS) spectrum of AgNPs. (**B**) Transmission electron microscopy image of AgNPs.

Bio-Plex

Cytokines play an important role in the wound-healing process. We tested whether AgNPs are able to change the production of proinflammatory cytokines, antiinflammatory cytokines and growth factors after 24 and 48 hours. The secretion of these cytokines was detected by the Bio-Plex multiple-bead system. Only the levels of cytokines that were altered by AgNPs compared with the positive control cells are discussed. The production of TNF-α and VEGF by NHDFs and NHEKs decreased after 24 and 48 hours at all of the tested concentrations (Fig. 2A-D). AgNPs decreased the secretion of IL-12 by NHDFs at all of the tested concentrations after 24 and 48 hours compared with untreated cells. AgNPs also decreased the secretion of IL-12 by NHEKs after 48 hours at all of the tested concentrations (Fig. 3A, B). AgNPs (2.5 and 0.25 ppm) slightly increased the production of IL-12 by NHEK after 24 hours (Fig. 3A).

ELISA

Extracellular matrix remodeling is mainly influenced by MMPs through the removal of damaged tissue and decrease in the overproduction of extracellular matrix proteins. We evaluated the effects of AgNPs on the remodeling of wound healing in cell supernatants and detected the volume of MMP-1, MMP-2, MMP-3 and MMP-9. The production of MMP-2 by NHDFs was unchanged, and MMP-9 production was below the limit of detection (data not shown). MMP-3 levels in NHEKs decreased compared with positive control after 48 hours (Fig. 4B), whereas AgNPs increased the production of MMP-1 after 24 and 48 hours (Fig. 4A).

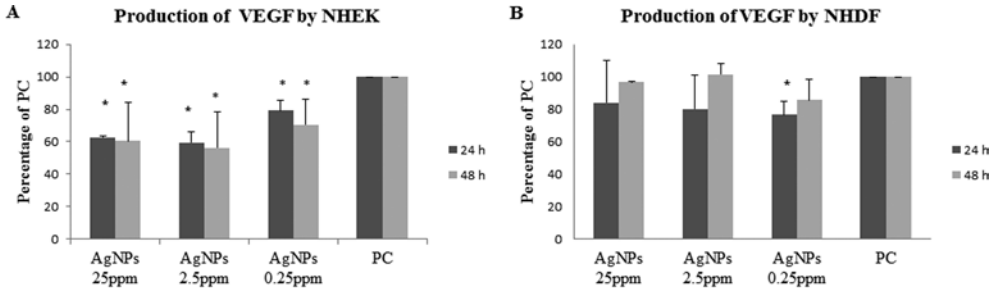
Western blot

COX-2 is one of the most important proinflammatory mediators and is involved in acute inflammation. In the present study, we determined whether AgNPs influence the expression of COX-2 in NHEKs and NHDFs after AgNP application. The expression of COX-2 in NHEKs was monitored by Western blot. It decreased after the application of AgNPs after 24 hours at concentrations of 2.5 and 25 ppm (Fig. 5A). AgNPs at a concentration of 25 ppm decreased the expression of COX-2 in NHDFs after 24 hours (Fig. 5C).

Discussion

The aim of the present study was to determine the response of primary human keratinocytes and fibroblasts after incubation with AgNPs. Both types of cells (NHDFs and NHEKs) were isolated from skin sections and used for the evaluation of wound healing parameters in vitro after AgNP application.

For the characterization of AgNPs, we used TEM and UV-VIS spectroscopy. The optical spectrum of metal AgNPs at sizes of 2 to 100 nm is characterized by strong absorption in the visible region, called the surface plasmon resonance band (19, 20). The position of the plasmon resonance band describes the size and shape of NPs and is caused by movement



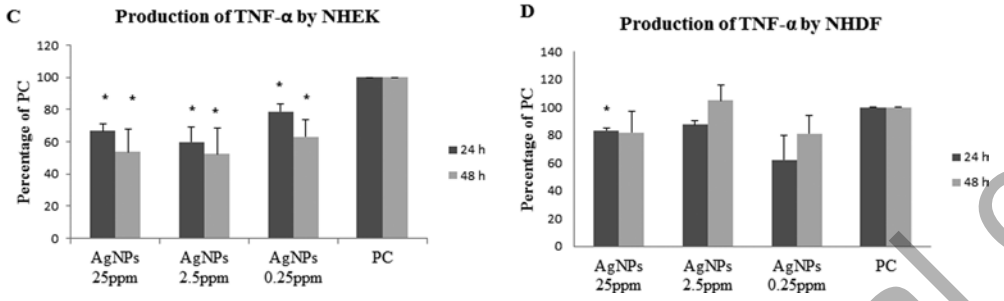


Fig. 2 - Production of VEGF by normal human epidermal keratinocytes (NHEKs) (**A**), VEGF by normal human dermal fibroblasts (NHDFs) (**B**), TNF-α by NHEKs (**C**) and TNF-α by NHDFs (**D**) in cell supernatants after 24 and 48 hours of incubation with silver nanoparticles (AgNPs). *p<0.05, vs. positive control (PC). ppm = parts per million.

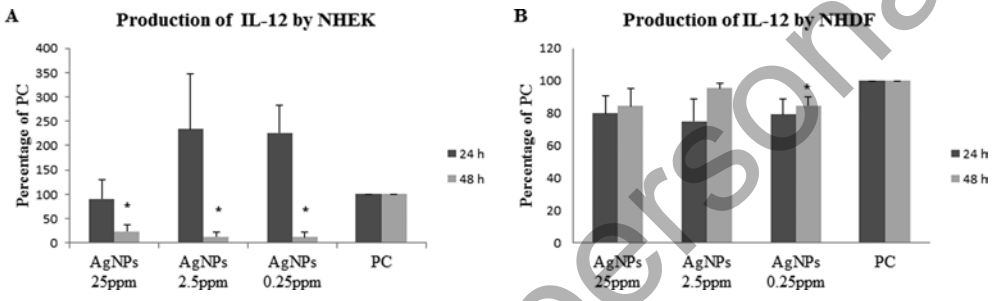


Fig. 3 - Production of IL-12 by normal human epidermal keratinocytes (NHEKs) (**A**) and normal human dermal fibroblasts (NHDFs) (**B**) in cell supernatants after 24 and 48 hours of incubation with silver nanoparticles (AgNPs). *p<0.05, vs. positive control (PC). ppm = parts per million.

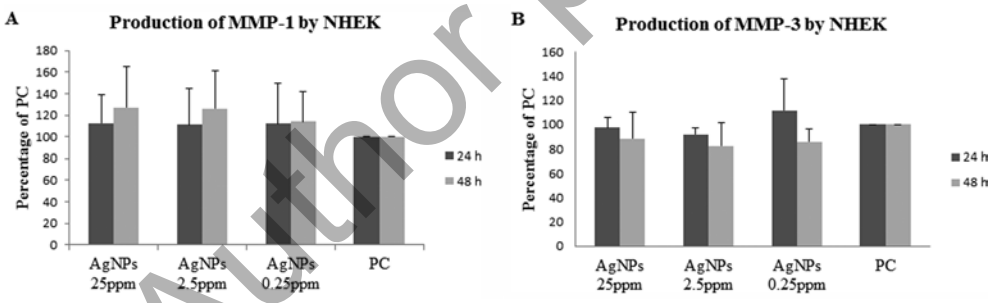


Fig. 4 - Production of MMP-1 (**A**) and MMP-3 (**B**) by normal human epidermal keratinocytes (NHEKs) in cell supernatants after 24 and 48 hours of incubation with silver nanoparticles (AgNPs). *p<0.05, vs. positive control (PC). ppm = parts per million.

of conduction electrons in the particles (21, 22). As the particle size increases, the surface plasmon resonance band of the optical absorption spectra of metal NPs shifts toward longer wavelengths (23). We detected the plasmon resonance band at 400 nm, corresponding to an average size of the AgNPs of 10 nm and indicating spherical NPs (confirmed by TEM).

AgNPs inhibit not only bacterial growth but also the production of proinflammatory cytokines. In normal tissue repair, especially without bacterial contamination, the inhibition of inflammatory cells and down-regulation of proinflammatory cytokines lead to earlier wound healing (14). Conversely, prolongation of the inflammatory phase of wound healing results in the formation of nonhealing wounds. Keratinocytes and fibroblasts produce growth factors (e.g., VEGF) (24) and proinflammatory cytokines (e.g., IL-8, IL-6, IL-12 and TNF-α) that subserve inflammatory and immunological reactions to irritants (25, 26). In the present study, we compared the influence of AgNPs on NHDFs and NHEKs (i.e., the most exposed cells during the wound-healing process). Our results suggest that NHEKs are more sensitive than NHDFs to the actions of AgNPs and decrease the production of proinflammatory cytokines (i.e., TNF-α and IL-12) and VEGF more significantly. No previous studies of which we are aware have evaluated the effects of AgNPs on NHDFs and NHEKs. However, Kalishwaralal et al (27) found that AgNPs may influence the production of VEGF in endothelial cells, similar to our observations with

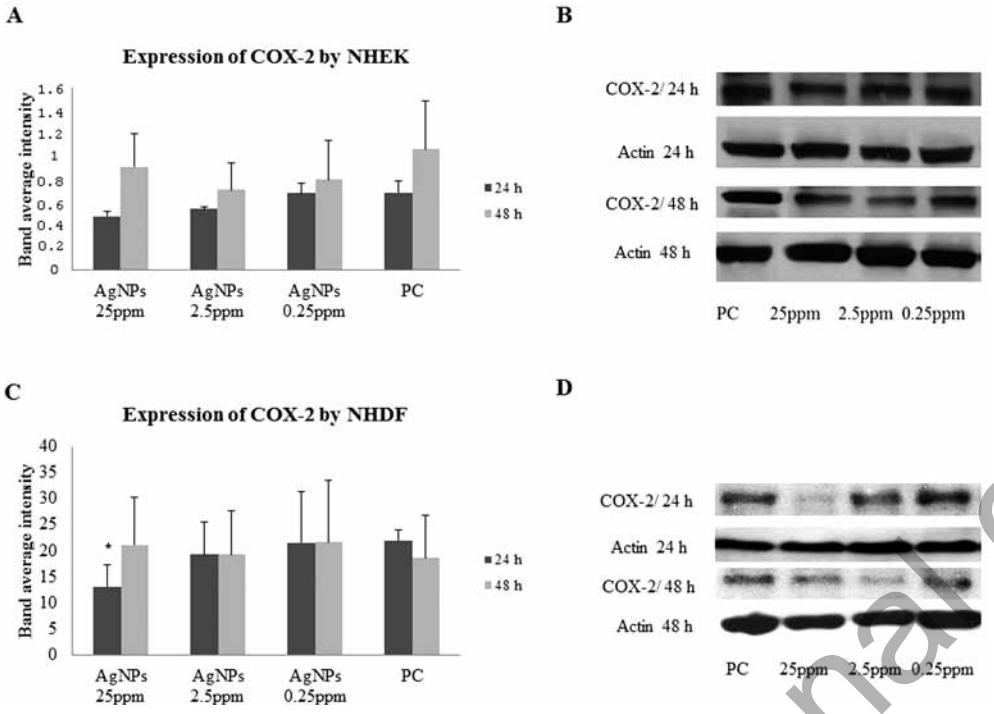


Fig. 5 - (**A**) Expression of cyclooxygenase-2 (COX-2) in normal human epidermal keratinocytes (NHEKs) after 24 and 48 hours. The data are expressed as means and standard deviation from 3 independent experiments. (**B**) Representative Western blots of NHEKs. (**C**) Expression of COX-2 in normal human dermal fibroblasts (NHDFs) after 24 and 48 hours. The data are expressed as means and standard deviation from 3 independent experiments. (**D**) Representative Western blots of NHDFs. *p<0.05, vs. positive control (PC). ppm = parts per million.

NHDFs and NHEKs. This finding is significant because these markers play central roles in some pathological conditions, such as wound healing and nonhealing wounds.

COX-2 is induced in cutaneous wound healing and expressed more intensively in injured skin than in normal tissue (28, 29). It is induced by various stimuli, including proinflammatory cytokines (e.g., TNF-α and IL-1), growth factors, and lipopolysaccharides (30, 31). Because of the antibacterial and antiinflammatory properties of AgNPs, we hypothesized that they may also decrease the production of COX-2 as a marker of inflammation. Indeed, we observed this effect on NHDFs and NHEKs after applying the 2 highest concentrations of AgNPs (2.5 and 25 ppm). Our experiments did not confirm the previous findings of Atieh et al (32). They found that fibroblasts appear to be more sensitive than keratinocytes to silver because it stimulates the proliferation of fibroblasts but inhibits its keratinocyte proliferation in vitro. We presumed a connection between the production of proinflammatory cytokines (TNF-α) and expression of COX-2, but in the present study, the level of TNF-α produced by NHDFs and NHEKs decreased after the application of AgNPs, but the expression of COX-2 decreased only at the highest concentration of AgNPs (25 ppm).

MMPs are zinc-dependent endopeptidases and key molecules in the healing process and tissue remodeling. According to their structure and substrate specificity, MMPs can be divided into several groups (33). Usually, excessive amounts of MMPs can prevent wound closure (34). Keratinocytes predominantly secrete MMP-1 and MMP-3, and fibroblasts secrete MMP-2 and MMP-9 during the wound healing process (35). The association between MMPs, wound healing and inflammatory mediators is supported by other studies (36). Increases in the levels of TNF-α enhance the production of some MMPs. We observed the down-regulation of MMP-3 production by NHEKs after 24 hours, with concomitant down-regulation of TNF-α. These findings are consistent with the observation that when granulation tissue has formed, the levels of proinflammatory cytokines and MMPs decrease in normal wound healing (37). In the present study, MMP-2 and MMP-9 production was unaltered, but other authors (38) reported that NPs increased the production of MMP-2 and MMP-9 in monocytes.

In summary, AgNPs decreased the production of proinflammatory cytokines (TNF-α and IL-12) and VEGF by NHDFs and NHEKs and also decreased MMP production (MMP-3) by NHEKs after 24 hours at all of the tested concentrations. Based on these results, we suggest that AgNPs can support the wound-healing process.

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Disclosures

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Conflict of interest: None of the authors has any financial interest related to this study to disclose.

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