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ORIGINAL ARTICLE

Effect of AgNPs on the human reconstructed epidermis

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ABSTRACT

Nanoparticles are utilized in a wide range of industries. The most studied silver nanoparticles (AgNPs) are used in medicine and also in several wound dressings due to their antimicrobial properties. The inflammatory response or potential morphological changes of skin cells after their application are not well known yet. In our study we used the model of human reconstructed epidermis (RHE), prepared in our laboratory, to evaluate whether the AgNPs penetrate through RHE, induce some morphological changes of keratinocytes or influence the production of pro-inflammatory cytokines (IL-6 and IL-8). After the application of three different concentrations (25 ppm, 2.5 ppm, 0.25 ppm) of AgNPs to of RHE for 24 hours we verified that AgNPs did not affect the production of pro-inflammatory cytokines (IL-6 and IL-8) and neither did they influence the expression of keratin K14 and loricrin. The morphology of the cells was likewise unchanged. Based on these results we conclude that AgNPs do not have any negative effect on the morphological changes and do not increase the production of pro-inflammatory cytokines.

KEY WORDS: human reconstructed epidermis; AgNPs; IL-6; IL-8; loricrin; keratin K14

Introduction

Nanoparticles display unique physical and chemical properties and can be used in numerous applications (Sondi & Salopek-Sondi, 2004). Due to their low toxicity to humans, and their antibacterial properties, silver and silver nanoparticles (AgNPs) are of interest in medicine and dermatology.

The establishment and application of alternative *in vitro* models for safety assessment is of growing interest of toxicology research today (Li *et al.,* 2017, Kandarova *et al.,* 2009). Some of the existing 3D models consist of one type of cells (e.g. reconstructed epidermis prepared of keratinocytes) (Mathes *et al.,* 2014). When keratinocytes grow on a solid surface, or on polycarbonate porous filter and were subjected to cyclic pressure treatment, they started to differentiate into a multilayer system with a protein expression pattern (keratins, fillagrin, loricrin, *etc.*), which is typical of differentiated epidermis. Immunohistochemistry studies often monitor the abundance and distribution of these proteins within the given 3D model.

Cytokines are the key modulator of inflammation, participating in acute and chronic inflammation via a complex and sometimes seemingly contradictory network of interactions (Turner *et al.,* 2014, Ambrozova *et al.,* 2017). In response to physical and chemical stress, keratinocytes produce inflammatory cytokines such as interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrotic factor α (TNF-α), *etc.* (Coquette *et al.,* 2013). The most frequently detected cytokines are IL-6 and IL-8. IL-6 is able to increase keratinocyte proliferation (Hänel *et al.,* 2013, Juráňová *et al.,* 2017) and may also enhance the barrier function of the skin (Wang *et al.,* 2004). However, the critical pro–inflammatory chemokine IL-8 participates in the initiation phase of cutaneous inflammation but does not correlate with cytotoxicity either as an irritant or sensitizer.

Based on the literature, we prepared an *in vitro* model of RHE that mimics normal human epidermis and is useful for toxicological testing. The aim of the study was to demonstrate the safety of AgNPs on the RHE model that simulated intact (healthy) epidermis.

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Materials and methods

Preparation and characterization of AgNPs

AgNPs were prepared by Nano Trade Company (Czech Republic). In brief, $AgNO_3$ was dissolved in distilled water and $NaBH_4$ added under constant magnetic stirring. Formation of AgNPs occurred rapidly upon addition of $NaBH_4$ (Frankova *et al.,* 2016). The AgNPs were characterized by ultraviolet-visible (UV-VIS) 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 and the iTEM program (SIS, Olympus). Zeta Plus analyzer (Brookhaven) was used to measure the zeta potential. The silver nanoparticles used in our study had an average size of approximately 10 nm (more than 50%).

Preparation of RHE model

The RHE was prepared using keratinocytes isolated from tissue sections of healthy volunteers with approval from the Ethical Committee of the University Hospital Olomouc and the patients' consent. After the third passage, the 500 µL of suspension of keratinocytes was seeded on special inserts (pore size 0.4 µm and surface of the insert 1.2 cm^2) and allowed to grow under differentiation conditions for 14 days (Frankart *et al.,* 2012). After 14 days the 50 µL of AgNPs were applied on the top of RHE for 24 hours at either 25 ppm, 2.5 ppm or 0.25 ppm. These concentrations were used as they had been found to be non-toxic in previous experiments (Frankova *et al.,* 2016, Galandakova *et al.,* 2016). The RHE, only with the serum free medium (without AgNPs), was used as a negative control. Following the incubation period, we studied the histological changes of RHE and production of pro-inflammatory cytokines in collected medium (store at –80 °C).

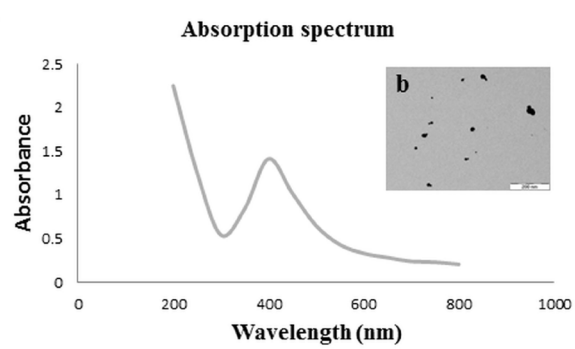

Figure 1. Characterization of AgNPs. a) UV-VIS spectrum of AgNPs. b) TEM image of AgNPs.

Histology and immunofluorescent staining

Following 24 hours of exposure to AgNPs, RHE was checked for morphological changes. RHE was then cut from the insert and fixed with Baker´s solution for 1 hour followed by incubation in methanol and toluene. The fixed samples were embedded in paraffin. Sections were then cut and stained in hematoxylin and eosin, or with immunofluorescent antibodies, after deparaffinization. Differentiation markers were carried out with keratin 14 (1:500, Abcam) and loricrin (1:500, Abcam). The secondary antibody used was Alexa fluor 594 and 488 IgG (1:2000, Molecular Probes). Sections were mounted, covered and visualized by microscopy.

Detection of IL-6 and IL-8

After the treatment of RHE with three different concentrations of AgNPs for 24 h, the levels of interleukins IL-6 and IL-8 were measured in the cell supernatant (Human Quantikine ELISA Kit, R&D Systems, Bio-Techne) according to the manufacturer's instructions.

Results

Characterization of AgNPs

AgNPs (1 ml) were diluted in 50 ml of distilled water for UV-VIS characterization (Figure 1a). Visualization of AgNPs by TEM is on figure 1 (Figure 1b). 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 NPs. The mean diameter of the AgNPs was 10±5 nm (>50% of the NPs) as confirmed by TEM. The pH of the AgNPs was found to be 7.1, with a zeta potential of -22 mV.

Histological and immunofluorescent staining

We visualized RHE morphology by hematoxylin and eosin staining and by immunofluorescent staining for the detection of keratin 14 and loricrin. No morphological changes were observed between RHE treated with AgNPs at three different concentrations (25 ppm, 2.5 ppm, 0.25 ppm) and control RHE. However for the highest concentrations, AgNPs were visible on the top of RHE (Figure 2a, b, c and d). All RHE showed characteristic epidermal stratification consisting of fully differentiated epidermis. Distributions of two markers which are important for the function of the skin as a barrier, keratin 14 for the basal layer of epidermis and loricrin for terminal differentiation were unchanged after topical application of AgNPs (Figures 2e, f, g and h).

Production of IL-6 and IL-8

We proposed that production of IL-6 and IL-8 could be affected by exposure to AgNPs. For this reason, the production of these cytokines was evaluated after 24 h incubation of RHE with AgNPs for all concentrations tested. We detected these two frequently studied cytokines by ELISA and found the same level of cytokines in medium

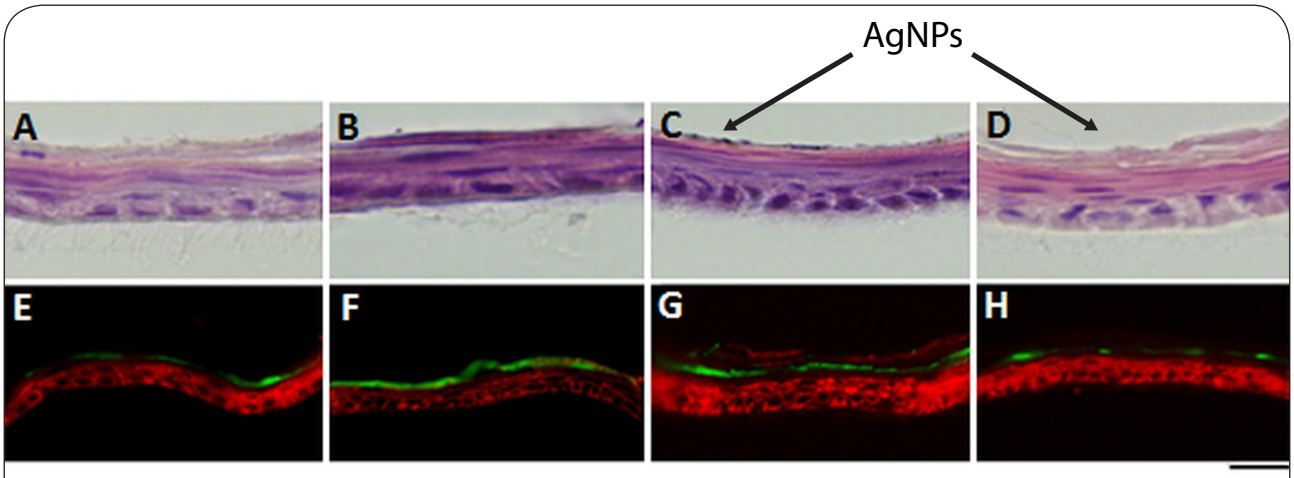

Figure 2. Morphology of the RHE model. Hematoxylin and Eosin staining of a) control RHE and RHE after application of AgNPs b) 25 ppm, c) 2.5 ppm and d) 0.25 ppm. Immunofluorescent staining of e) control RHE or RHE after application of AgNPs f) 25 ppm, g) 2.5 ppm and h) 0.25 ppm. K14 is indicated by red fluorescence and loricrin is indicated by green fluorescence. Scale bar: 50 µm. Representative picture of n = 3 experiments.

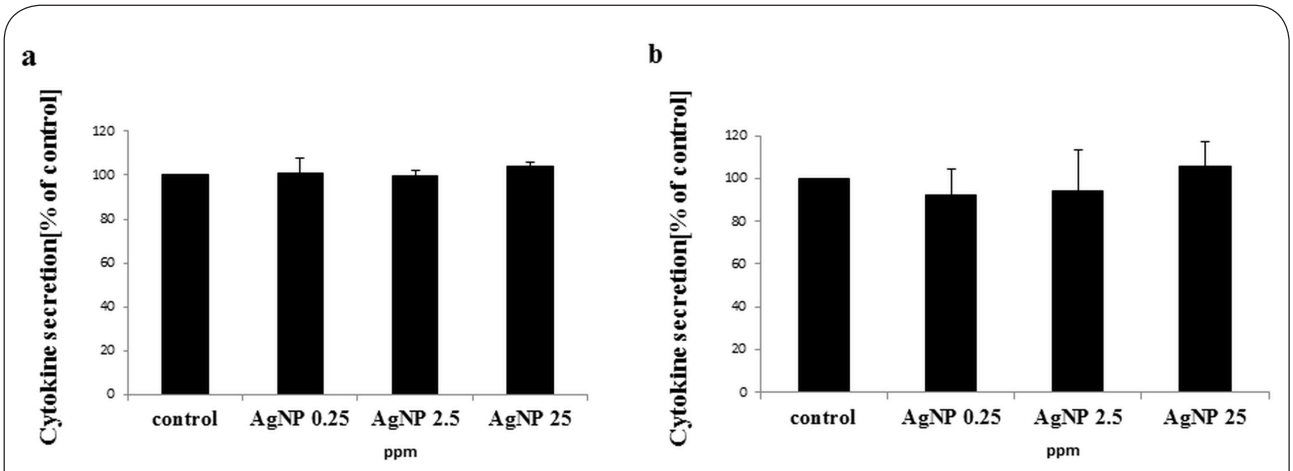

Figure 3. Production of pro-inflammatory cytokines a) IL-6 and b) IL-8 after application of three different concentrations of AgNPs on RHE.

samples from treated RHE as in control RHE ($p<0.05$) (Figure 3).

Discussion

RHE models have been validated for hazard prediction and are used for identifying irritant and cytotoxic properties of chemicals or natural substances. This highly differentiated mulitlayer model of human epidermis (Saito *et al.,* 2013) retains pro-inflammatory and immune regulatory functions (Mathes, 2014, Jung *et al.,* 2014).

Our RHE, derived from human skin tissue, exhibited the same cell layers as native human skin. It is divided into: stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and the external layer of epidermis – stratum corneum. The basal layer is composed of keratinocytes with highly expressed cytokeratin 14 as a marker of dividing basal keratinocytes assisting in maintenance of the shape of keratinocytes and provides resistance to physical stress (Akhavan-Tavikoli *et al.,* 2017). The expression of cytokeratin 14 demonstrated that RHE was composed of normal skin keratinocytes. On the other hand, the expression of loricrin, a cross–linked envelope of keratinocytes (Jung *et al.,* 2014), which has a key role in keratinization of the skin (Akhavan-Tavikoli *et al.,* 2017), indicates that the stratum corneum serves as a functional physical barrier. Loricrin is initially expressed in the stratum granulosum and comprises 70% of the total protein mass of the cornified layer (Kim *et al.,* 2011).

AgNPs are incorporated into several wound dressings (e.g. Acticoat™ (Bhowmic & Koul, 2016) or PolyMem Silver®) with the size of the AgNPs released from the silver

hydrogel ranging from 2.1 nm to 15.6 nm, as determined by TEM (Boonkaew *et al.,* 2014). The results of studies characterizing which sizes of nanoparticles can penetrate the skin are far from consistent. Due to their small size, some particles could penetrate through the upper layers of the epidermis or into the deeper dermal layer and might influence the production of pro-inflammatory cytokines or affect the morphology of skin cells. Filon *et al.* (Filon *et al.,* 2015) postulated that the silver nanoparticles of 25 nm could penetrate through the intact skin, and Bianco *et al.* confirmed that smaller AgNPs (19 nm) were also able to penetrate through the skin (Bianco *et al.,* 2016). Our results, with RHE prepared from the skin tissue from three different volunteers and AgNPs with an average size of 10 nm, support the claim of Watkinson *et al.* that only nanoparticles below 1 nm are able to penetrate through intact skin (Watkinson *et al.,* 2013). Interaction between skin and nanoparticles (or certain irritants) may damage the stratum corneum and trigger the production of pro-inflammatory cytokines, which is followed by morphological changes. Our histological evaluation found that AgNPs have no negative influence on RHE and did not cause any morphological changes, but at the highest concentration AgNPs are visible at the top of the stratum corneum.

The interaction of AgNPs with skin cells is still under investigation. Following their application, AgNPs have first to penetrate the stratum corneum and reach the living cells below before a biological effect might be observed. If AgNPs could cause inflammation of the stratum corneum, release of pro-inflammatory cytokines would probably be detectable. For example kinases and cytokines, such as IL-1α, IL-6, IL-8, PGE2, SKALP and HSP70, have been described to act as biomarkers of changes in metabolic activity and cytosolic leakage (Gibbs, 2009). IL-8 promotes the migration of dendritic cells and is a requirement of monocytes and neutrophils as key steps in the initiation phase of cutaneous inflammation (Coquette *et al.,* 2013) and could be linked to the product applied, either irritant or sensitizer. IL-6 is involved in the growth and differentiation of numerous cells. In addition, a deficiency in IL-6 causes a more pronounced reduction in barrier repair (Wang *et al.,* 2004). Not only does the inhibition of infiltration by inflammatory cells correspond to the reduced level of pro-inflammatory cytokines, but it is also known that various anti-inflammatory agents are effective in enhancing tissue repair and wound healing (Zhang *et al.,* 2014). We propose that if the production of pro-inflammatory cytokines is decreased (or unchanged), AgNPs should not be toxic. In our model of healthy epidermis, treated with AgNPs, we did not observe any deleterious effects on the production of pro-inflammatory cytokines IL-6 and IL-8.

In conclusion, AgNPs did not affect the production of IL-6 and IL-8, did not cause any morphological changes of RHE and may therefore be safe for further application. However for the full characterization of the mechanism of action of the AgNPs, or the metabolic pathways that are activated during their application, additional experiments are required.

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