Effect of AgNPs on the human reconstructed epidermis

Jana FRANKOVÁ1,2, Jana JURÁŇOVÁ1,2, Vojtěch KAMARÁD3, Bohumil ZÁLEŠÁK3, Jitka ULRICHOVÁ1,2

1Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Hněvotínská 3, Olomouc, Czech Republic
2Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacký University, Hněvotínská 5, Olomouc, Czech Republic
3Department of Histology and Embryology, Faculty of Medicine and Dentistry, Hněvotínská 3, Olomouc, Czech Republic
4Department of Plastic and Aesthetic Surgery, University Hospital Olomouc, I.P.Pavlova 6, Olomouc, Czech Republic

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.
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$_4$ (Franková 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).

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...
AgNPs 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 multilayer 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.

Acknowledgements

This paper was supported by grants: LF_2017_011, LF_2018_012, NPU I (LO1304), IGA 15-2776A and RVO 61989592. The authors are thankful to I. Travnickova for helping with histological analysis.

REFERENCES

Ambozova N, Ulrichova J, Galandakova A. (2017). Models for the study skin wound healing. The role of Nrf2 and NF-κB. Biomed Pap Med Fac Palacky Olomouc Czech Repub 16: 1–13.
Akhavan-Tavakoli M, Fard M, Khanjani S, Zare S, Edalatkhah H, Mehrabani D, Zarnani AH, Shirazi H, Kazemnejad S. (2017). In vitro differentiation of menstrual blood stem cells into keratinocytes: A potential approach for management of wound healing. Biologics 48: 66–73.
Bhowmlic S, Koul V (2016). Assessment of PVA/silver nanocomposite hydrogel patch as antimicrobial dressing scaffold: Synthesis, characterization and biological evaluation. Mater Sci Eng C Mater Biol Appl 59: 109–119.
Bianco C, Visser MJ, Platt OA, Vetićic V, Pletikapic G, Jakasa I. (2016). Characterization of silver particles in stratum corneum of healthy subjects and atopic dermatitis patients dermally exposed to a silver-containing agent. Nanotoxicology 10: 1480–1491.
Boonkaew B, Kempf M, Kimble R, Supaphol L, Cuttle L. (2014). Antimicrobial efficacy of a novel silver hydrogel dressing compared to two common silver burn wound dressing: Acticoat ™ and PolyMem Silver™. Burns 40: 89–96.
Coquette A, Berna N, Vandenbosch A, Rosdy M, De Wever B, Poumay Y. (2013). Analysis of interleukin-1α (IL-1α) and interleukin-8 (IL-8) expression and release in in vitro reconstructed human epidermis for the prediction of in vivo skin irritation and/or sensitization. Toxicol In Vitro 17: 311–321.
Filon FL. (2015). Nanoparticles skin absorption: New aspects for a safety profile evaluation. Regul Toxicol Pharmacol 72: 310–322.
Frankart A, Malaisse J, De Vuyst E, Minner F, de Rouvroit CL, Povmou Y. (2012). Epidermal morphogenesis during progressive in vitro 3D reconstruction at the air-liquid interface. Exp Dermatol 21: 871–875.
Frankova J, Pivodová J, Várgnerová H, Juráňová J, Ulrichová J. (2016). Effects of silver nanoparticles on primary cell cultures of fibroblasts and keratinocytes in a wound healing model. J Appl Biomater Funct Mater 14(2): e137–e142.
Galandáková A, Franková J, Ambrožová N, Habartová K, Pivodová V, Zálešák B, Sáfárová K, Smekalová M, Ulrichová J. (2016). Effects of silver nanoparticles on human dermal fibroblasts and epidermal keratinocytes. Hum Exp Toxicol 35(9): 946–957.
Gibs S. (2009). In vitro Irritation Models and Immune Reactions. Skin Pharma Col Physiol 22: 103–113.
Hanel KH, Cornelissen C, Lüscher B, Baron JM. (2013). Cytokines and the skin barrier. Int J Mol Sci 14(4): 6720–45.
Jung MK, Lee SH, Jung HS, Heo Y, Park YH, Bae S, Lim KM, Seok SH. (2014). KeraSkin ™ – VM: A novel reconstructed human epidermis model for skin irritation tests. Toxicol In Vitro 28: 742–750.
Juráňová J, Frankova J, Ulrichova J. (2017). The role of keratinocytes in inflammation. J Appl Biomed 15: 169–179.
Kandarova H, Hayden P, Klauser M, Kubilus J, Kearney P, Sheasgreen J. (2009). In vitro skin irritation testing: Improving the sensitivity of the EpiDerm skin irritation test protocol. ATLA 37 (6): 671–689.
Kim BE, Howell MD, Guttman E, Gilleaudeau PM, Cardinale IR, Boguniewicz M, Kreuger JG, Leung DYM. (2011). TNF-α downregulates fillagrin and loricrin through c–Jun N–terminal kinase: Role of TNF-α antagonists to improve skin barrier. J Invest Dermatol 131: 1272–1279.
Li N, Liu Y, Qiu J, Zhong L, Alpérod N, Cotovio J, Cai Z. (2017). In vitro skin irritation assessment becomes a reality in China using a reconstructed human epidermis test method. Toxicol In Vitro 41: 159–167.
Mathes SH. (2014). The use of skin models in drug development. Adv Drug Deliv Rev 66: 79–81.
Saito K, Nukada Y, Takenouchi O, Miyazawa M, Sakaguchi H, Nishiyama N. (2013). Development of a new in vitro skin sensitization assay (Epidermal Sensitization Assay: Epi SenSA) using reconstructed human epidermis. Toxicol In Vitro 27: 2213–2224.
Sondi I, Salopek-Sondi B. (2004). Silver nanoparticles as antimicrobial agent: a case study on E. coli as a model for Gram-negative bacteria. *J Colloid Interface Sci* **275**: 177–182.

Turner D, Nedjai B, Hurst T, Pennington DJ. (2014). Cytokines and chemokines: At the crossroad of cell signalling and inflammatory disease. *Biochim Biophys Acta* **1843**: 2563–2582.

Wang XP, Schunck M, Kallen KJ, Trautwein C, Rose – John S, Prokch S. (2004). The interleukin-6 cytokine system regulates epidermal permeability barrier homeostasis. *J Invest Dermatol* **123**: 124–131.

Watkinson AC, Bunge AL, Hadgraft J, Lane ME. (2013). Nanoparticles do not penetrate human skin – a theoretical perspective. *Pharm Res* **30**(8): 1943–1946.

Zhang S, Liu X, Wang H, Peng J, Wong KK. (2014). Silver nanoparticle-coated suture effectively reduces inflammation and improves mechanical strength at intestinal anastomosis in mice. *J Pediatr Surg* **49**: 606–613.