HIGHLIGHTED ARTICLE

Eleyon NANOSCALE REPORTS

DOI: 10.26524/nr1921

Silver Nanoparticle-Mediated Cellular Responses in Human Keratinocyte Cell Line HaCaT *in Vitro*

Khaled Habas [a], Lijun Shang [b, *]

[a] School of Chemistry and Biosciences, Faculty of Life Sciences, University of Bradford, Bradford, Richmond Road, West Yorkshire, BD7 1DP, UK.

***Corresponding Author**
L.Shang1@bradford.ac.uk
(**Lijun Shang**)
Tel.: + 44 (01274) 234676

Received : 02-02-2019
Accepted : 21-04-2019

ABSTRACT: The interactions between cells and nanoparticles has been the focus of recent research in the area. The effects of AgNPs on skin cell lines for further potential biological applications are highlighted. This study aimed to investigate the mechanism of cytotoxic and genotoxic effects of AgNPs nanoparticles on human skin keratinocytes (HaCaT). Genocytotoxic effects of AgNPs was assessed using changes in various cellular parameters of HaCaT cells involving viability, superoxide anion radical production, lactate dehydrogenase release and the levels of the antioxidant enzymes, namely, Catalase, Glutathione peroxidase (GPX) and Superoxide Dismutase (SOD). Superoxide anion was detected using nitroblue tetrazolium NBT reduction assay. LDH levels was evaluated using the standard kit, and activity of antioxidant enzymes such as catalase (CAT), glutathione peroxidase 1 (GPX-1) and superoxide dismutase 1 (SOD-1) were quantified using qPCR. Our results indicated that AgNPs caused severe HaCaT oxidative damage, accompanied by increased the production of superoxide anion levels as well as significant decrease in endogenous antioxidant enzyme of SOD, CAT, GPX expression involved in HaCat cells *in vitro*. Our study suggests that AgNPs exposure increased oxidative stress levels. Moreover; the low cytotoxic effect observed on human HaCaT keratinocytes suggested that these nano-compounds have a potential toxic effect at the skin level only after long-term exposure.

Keywords: Nanoparticles; Silver nanoparticle; HaCat cell lines; Cytotoxicity; gene-expression

1. Introduction

Nanoparticles (NPs) of physicochemical properties and different size have been presented to various areas of medical sciences since last century [1]. Among these NPs, AgNPs are commonly used for medical applications in many disciplines of medicine [2, 3]. A number of studies have shown their interests to examination of the toxicity NPs to human [2-5]. The new developing area of nanotechnology have developed further risk to human life, particularly if exposed to several NPs during childhood [1]. The increased risk could be related to their unique properties, which make them easily to enter into the human body, cross the several biological barriers and can reach the most sensitive tissues [6]. AgNPs express unique optical, electrical, and thermal properties and thus are commonly used in conductive inks, pastes and fillers and major known uses for AgNPs are as antimicrobial agents [7]. The action by which AgNPs operate in an antimicrobial manner is still yet to be fully elucidated. However, it is claimed that the bactericidal effects are mediated by oxidative stress [8]. Study reported that smaller AgNPs are more likely to produce ROS and cause apoptosis [9]; therefore, in this study we opted for 20 nm silver nanospheres. This is in line with other studies where the use of 20 um AgNPs has proven effective against other cell types [10].

The mechanisms of cytotoxicity of NPs is more complexed. It might due to generated reactive oxidative species (ROS) through disruption of intracellular metabolism [11]. It also could be damaging the endogenous antioxidant defence system [12]. AgNPs can induce DNA damage and downregulated DNA repair-related genes as result of generation of ROS [13]. AgNPs can also enter the cell during diffusion, leads mitochondrial dysfunction and ROS generation and leads

to destruction of protein and nucleic acid, resulting in inhibition of cell proliferation [14]. Based on its antimicrobial activity, AgNPs have been widely used in serval commercially available products designed to be directed interaction with the skin [15].

In this study, we investigated AgNPs nanoparticle-induced cytotoxicity and oxidative stress in human keratinocyte HaCaT cell line. Accordingly, cytotoxicity was measured by several cell viability assays: MTT assay and lactate dehydrogenase (LDH) assay. The superoxide anions production of the cultured Sertoli cells was assessed and mRNA level of SOD, CAT and GPX genes expression were quantified. Thus, we mapped the key stages of pathways predicted to be manipulated by nanoparticle exposure and suggested a possible mechanism for this cytotoxic behaviour.

2. Materials and methods

2.1 Cell culture and treatment with gold and silver nanoparticles

Human keratinocyte cells (HaCat) were cultured in 75 cm^2 culture flasks at $1x10^5$ cells ml in Roswell Park Memorial Institute (RPMI) medium (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL. The cells were incubated 37 °C with 5% CO_2 saturation, RPMI was changed every 48 h until a desired confluency of 85% was reached.

Prior to treatment the nanoparticle stock solutions were pulse sonicated in 1 second intervals for 5-10 minutes to disturb any nanoparticle aggregates. The HaCat cells were aspirated, washed twice with PBS and trypsinised. Fresh RPMI was added, containing 15 µM Silver nanoparticles in 6 well plates at volume of 1ml per well for 6, 12 and 24 h. Cell viability and cytotoxicity was measured using MTT and LDH assays, superoxide anion levels were also measured using NBT assay. The cells were detached from the plate using a cell scraper and plated out in 96 well plates at 100µl per well for all assays. Total RNA was extracted from the remaining cell suspension for use in qPCR.

2.2 MTT cell proliferation assay

Cells (1 × 10^5 cells/well) were plated in 96-well plates for 24 h and then exposed to AgNPs nanoparticles (15 µM). After 6, 12 and 24 h of exposure, a15µl of MTT dye solution per 100 µl cell suspensions was added to each well and the plates were incubated for 2 hours at 37°Cwith 5% CO_2 saturation. A control well with MTT reagents only, was also plated for incubation. 100µl of solubilisation solution was added to each well and the plates were incubated for 1 h at room temperature. Plates were read in a microplate reader MRX II (Dynex Technologies, Chantilly, USA) at 570 nm. Data are reported as a percentage of control and are the mean ± SE of three independent experiments performed in triplicate.

2.3 Lactate Dehydrogenase Cytotoxicity Assay

The Cell membrane integrity of HaCat cell line was assessed by performing the activity of lactate dehydrogenase (LDH) in the medium leaked from the cell according to the manufacturer's instructions (CytoTox 96® Non-Radioactive Cytotoxicity Assay (LDH) (Promega. Madison, WI; USA). This assay is relied on the release of the cytosolic enzyme (LDH) from cells with damaged cellular membranes. Briefly, cells were exposed to 15 µM of AgNPs nanoparticles for 6, 12 and 24 h, then 10 µl lysis solution was added to a triplicate of 0µg/ml wells and incubated for 45 minutes at 37°c with 5% CO_2 saturation, this forms the LDH positive control. A control well with LDH reagents only, was also plated for incubation. 50µl of cell suspension from each well was transferred to a flat-bottomed 96-well plate; the cells were released from the plate prior to transfer by cell scraping. 50µl of the CytoTox 96® reagent was added to each well and incubated at room temperature for 30 minutes. 50µl of stop solution was added to each well and the plate was read immediately at a wavelength of 490 nm using a microplate reader MRX II (Dynex Technologies, Chantilly, USA).

2.4 Nitro Blue Tetrazolium (NBT) Assay

Intracellular superoxide anion levels were measured measured using a kit (Sigma, Poole, UK). Cells were seeded onto 24-well plates at a density of 1 × 10^5cells per well and cultured for 24 h. After washing twice with PBS, fresh phenol red-free media (M199) (Sigma-Aldrich, UK) containing 15 µM of AgNPs nanoparticles was added, and the cells were incubated for 6, 12 and 24 h.The cells then were incubated with 1 mg/ml NBT at 37 ˚C and 5% CO_2 for 90 min. The cells were directly lysed with lysis

solution (90% DMSO, 0.1% SDS and 0.01 M of NaOH). The resulting blue coloured solution was assessed spectrophotometrically at a wavelength of 750 nm using a microplate reader MRX II (Dynex Technologies, Chantilly, USA).

2.5 Total RNA isolation and cDNA synthesis

Total cellular RNA was isolated from HaCaT cell line (2 ×10^5) using the GenElute Mammalian Total RNA Miniprep Kit according to the manufacturer's instructions (Sigma-Aldrich, UK). The yield and purity of RNA samples were assessed by the absorbance at 260 and 280 nm ($A_{260/280}$) ratios, using a NanoDrop™ Spectrophotometer. Random hexamer primed reverse transcription reactions were performed with 100 ng total RNA from HaCaT cells in a 20 µl setup using ImProm-II™ Reverse Transcription System reaction following the manufacturer's instructions (Promega). The samples were eluted in 20 µl elution solution and stored at −20 °C for further use.

2.6 Quantitative real time RT-PCR (RT-qPCR)

The StepOnePlus™ real-time PCR instrument (Applied Biosystems) was used to determine the expression level of selected target genes.The qPCR was used to measure the mRNA expression level of endogenous antioxidants SOD, CAT and GPx in HaCaT cell line. For SYBR® Green PCR Master Mix (Applied Biosystems), 10 µL of 2× SYBR® Green, 1 µL of each 10 µM (0.4 µM) forward and reverse primer and 8 µL of nuclease-free water was made to a total volume of 20 µL. The cycling conditions included a denaturing step at 95°C for 10 min and 50 cycles of 95°C for 15 sec and 60°C for 1 min. Reactions are typically run in triplicates.

The data were analysed by StepOne™ Software v 2.2.2. Thus, the transcript levels of each specific gene were calculated according to the 2−ΔΔCt method [16].

3. Results

3.1 Effects of AgNPs on cellular morphology

The morphological changes of HaCat cells exposed to AgNPs at 15 µM for 24 h using inverted microscope. Significant morphological changes of cell death, including restricted spreading patterns and increased floating cells were observed in HaCaT cells exposed to AgNPs (Figure 1).

3.2 MTT assay

The results of MTT assays showed a time-dependent decrease in viability percentage of HaCat cell line after 2, 6 and 24 h exposure to AgNPs (Figure 2). Viability percentage measured by MTT assay on HaCat cell line exposed to 15 µM of AgNPs for 2, 6 and 24 h represented a time-response pattern as shown in Figure 2. Data were reported as mean ± SEM of three independent experiments performed in quadruplicate (*p < 0.05, **p < 0.01and ***p < 0.001 and *p < 0.001).

3.3 Cellular Effects of AgNPs in HaCat cell line

The LDH assay is a cytotoxicity assay that measures membrane damage by quantifying the amount of LDH released from the cytoplasm. HaCat cell lines were exposed to AgNPs (15 µM) for 2, 6 and 24 h. No significant toxicity was observed after 2 h for any of the AgNPs (Figure 3). However, significant toxicity was observed after 6 h (*$p \leq 0.05$) and (***$p \leq 0.001$) for 24 h.

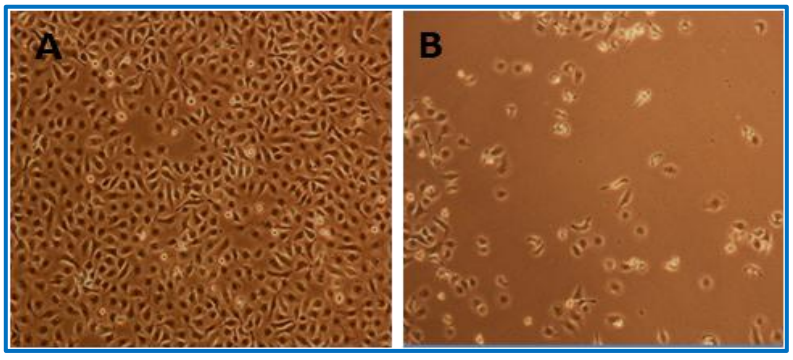


Figure 1. Morphological characterization of HaCat cell line: A. Untreated HaCaT cell line, B and B. treated HaCaT cell line at of 15 µM of AgNP for 24 hours

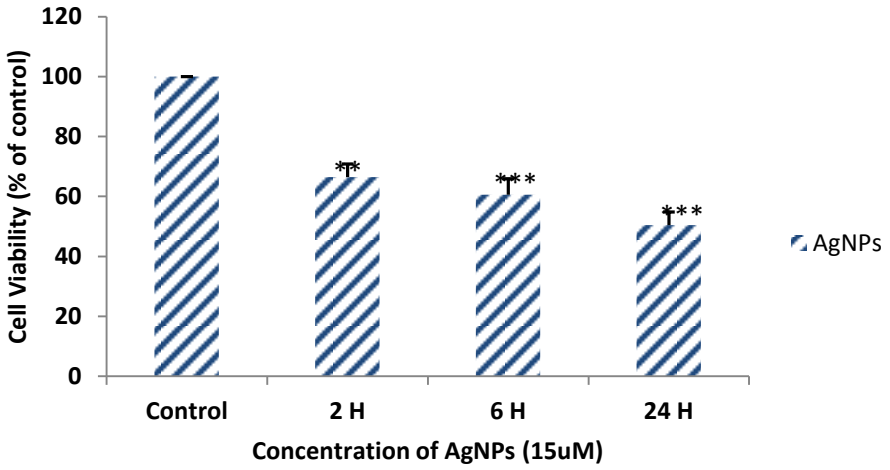


Figure 2. Time-dependent toxicity of AgNPs in HaCaT cells. Cells were treated with 15 µM of AgNPs for 2, 6 and 24 h. The cell viability was measured by MTT assay at different time points. $^{**}p < 0.01$ and $^{***}p < 0.001$. Values represent mean ± SE (n = 3).

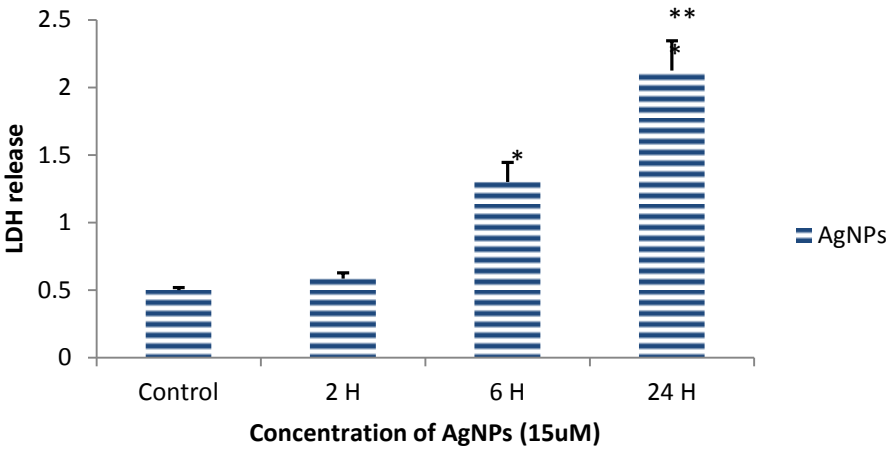


Figure 3. Effect of AgNPs on LDH release from HaCat cells. Cells were treated with 15 µM of AgNPs for 2, 6 and 24 h. LDH was evaluated by changes in optical density due to NAD+ reduction monitored at 490 nm. $^{*}p < 0.05$ and $^{***}p < 0.001$. Values represent mean ± SE (n = 3).

3.4 Effect of AgNPs on superoxide anion production

Inside the cell, superoxide anions decreased membrane-permeable water-soluble nitroblue-tetrazolium salt to form formazan a blue precipitate. This blue formazan was detected spectrophotometrically at 450 nm. Figure 4 showed that the 15 µM of both AgNPs particles had significant difference in O2– production in HaCat cells at 2, 6 and 24 h. ($^{**}p < 0.01$ and $^{***}p < 0.001$).

3.5 Effect of AgNPs on superoxide anion production

We further evaluated gene expression on HaCaT cells with 15 µM of AgNPs. The *B*-actin gene was used as a reference; changes in the expression levels of the SOD, CAT and GPX were evaluated and compared following exposure to AgNPs for 2, 6 and 24 h (Figure 5). qPCR revealed that mRNA expression of SOD, CAT, GPX were decreased gradually ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p<0.001$) respectively.

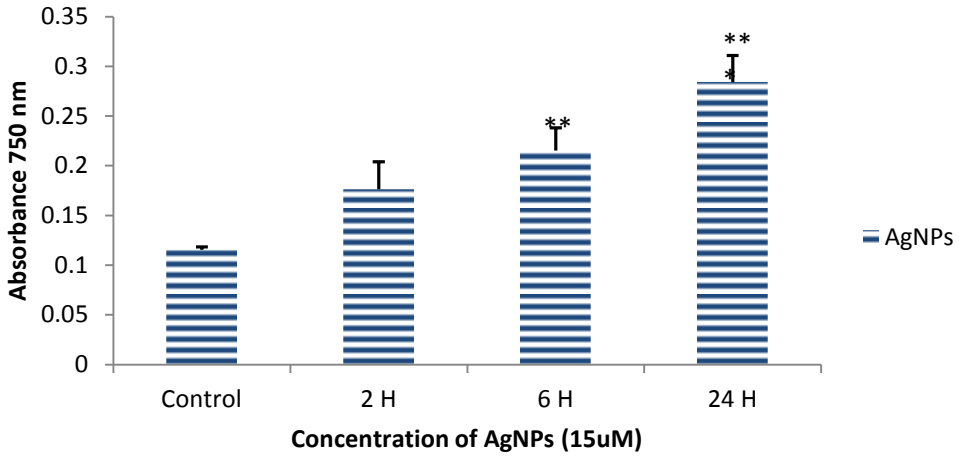
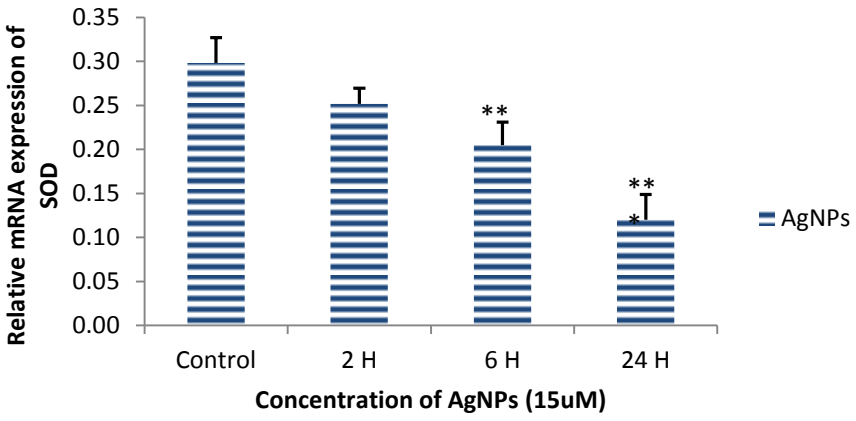
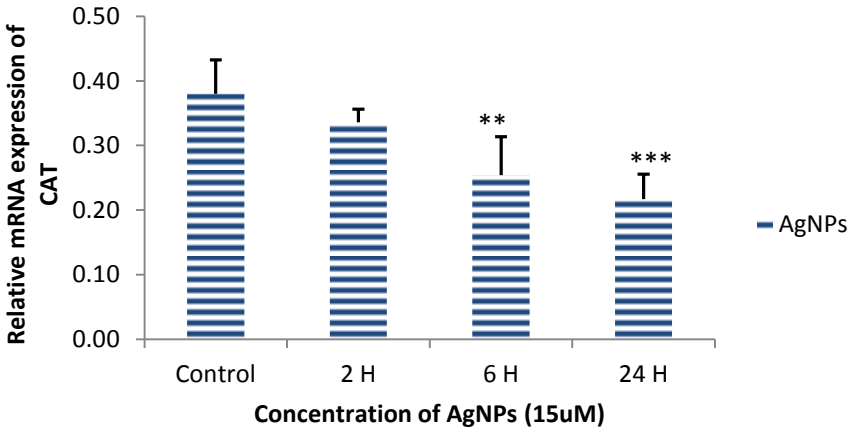


Figure 4. Effect of AgNPs on superoxide anion production in HaCat cells using the NBT assay. Cells were grown in complete medium and treated with and without 15 µM AgNPs 2, 6 and 24 h. Non-treated cells were considered as negative control. ***p* < 0.01 and ****p* < 0.001. Values represent mean ± SE (n = 3).





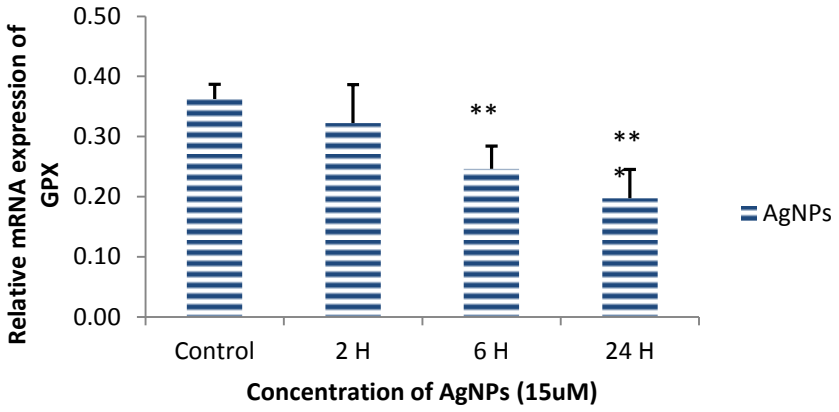


Figure 5. Alterations in the mRNA expression of antioxidative enzymes-related genes SOD, CAT and GPX HaCat cells treated with AgNPs for 2, 6 and 24 h. $^{**}p < 0.01$and $^{***}p < 0.001$. Values represent mean ± SE (n = 3).

4. Discussion

The rapid development of nanotechnologies and their applications in clinical research have increased concerns about the adverse effects of NPs on human health [17]. NPs can be directly taken up by organs exposed and enter the human body most often through the skin [18]. Cellular oxidative and ROS have been proposed as one of the major mechanisms for nanoparticles toxicity [19]. Studies have been reported that oxidative stress via nanoparticles related to increased ROS [20]. To assess the toxic potential of AgNPs at the skin cells, a preliminary study was performed on human HaCaT keratinocytes. AgNPs were induced a slight cytotoxic effect, reducing cell viability using MTT assay after 2, 6 and 24 h of exposure Figure 2. These results are concordant with earlier reports preformed on HaCaT cells reporting the ability of AgNPs to induce oxidative stress and mitochondrial damage [15, 21]. Our results are concordant with earlier reports; AgNPs have a toxic potential effect on HaCaT cells and decrease of cell viability [15]. These results showed that on HaCaT keratinocytes a relatively short time of contact with AgNPs causes a long-lasting inhibition of cell growth.

Lactate dehydrogenase is an enzyme extensively existing in cytosol which converts lactate to pyruvate. LDH released from cells into the medium is an indicator for cell death [22]. Disrupted integrity of the plasma membrane, leads to LDH leaks into the media and its extracellular levels increase displaying cytotoxicity by NPs [23]. Cells treated with AgNPs showed significantly increased the LDH with time-depended and more potent in producing cytotoxicity in HaCat cells compared to untreated cells (Figure 3). Different types of NPs such as AgNPs have been investigated with respect to skin absorption [24, 25]. ROS, including the superoxide anion (O2−) are highly toxic oxidants that a bile to induce cellular damage [26]. Quantification of superoxide anion production is indicator for adverse environmental conditions and pathogenesis of many chronic diseases like cancer and other inflammation [27, 28]. HaCaT cells treated with AuNPs and AgNPs clearly showed that increased production of superoxide anions levels which were significantly increased compared to non-treated cells (Figure 4). ROS generation are extremely destructive to organisms and has been found to be associated with cell death DAN damage [29]. It has been showed that oxidative stress is the most sources for NPs mediated toxicity [30, 31]. Generation of oxidative stress is a significance of the imbalance between ROS formation and the response of the endogenous antioxidant system [32]. In normal cells the cellular defence system is controlled the ROS levels under several of normal conditions including metabolic and protect against damaging oxidants [32].

It has also been shown that oxidative stress occurred when the balance among antioxidants enzymes and ROS are disrupted due to reduction of antioxidants enzymes [33]. This defence system involves enzymatic antioxidant mechanisms for removal of ROS produced during oxidative stress [34]. This main enzymatic defence include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) that essential for maintaining

physiological levels of O2•− to H2O2 [35]. SOD is considered to be the first line defence enzymes in combating ROS, that catalyze dismutation of O2•− into O2 and H2O2. CAT and GPx complement the process of ROS removal via transforming H2O2 into water and molecular oxygen [33]. In the present study, results showed that exposure to AgNPs significantly decreased in the levels of mRNA SOD, CAT and GPX, resulting in a higher level of ROS production in HaCaT cells. The decrease in SOD, CAT and GPX level in HaCaT cells treated with AgNPs, observed in our studies, was time-dependent (Figure 5). It has been shown that exposure to AgNPs decreased the antioxidant enzymes like SOD and GSH levels in human skin cells such as carcinoma and fibrosarcoma cell lines [36]. Our result indicates that AgNPs, substitute on different cellular targets, might differentially affect specific intracellular pathways depend on the NPs type used in this study. In the keratinocyte HaCaT cell line the mechanisms involved in the endogenous antioxidants effect that appear to be primarily independent on increased ROS production particular superoxide anions levels are presently under investigation.

5. Conclusions

In this study, we showed that AgNPs affects HaCat Cells and caused severe cellular oxidative damage, accompanied by excessive production of superoxide anion and a significant reduction in antioxidant enzyme. Our findings are consisted with the knowledge and presented that AgNPs could produce cytotoxic and genotoxic effects, oxidative damage in the human keratinocyte cell line HaCaT involved. This suggests that the commercial and medical application of AgNPs should be carefully evaluated, as they could have potentially hazardous effects on human health as the low cytotoxic effect observed on human HaCaT keratinocytes suggested that these nano-compounds have a potential toxic effect at the skin level only after long-term exposure.

References

[1]. G. Oberdorster, E. Oberdorster, J. Oberdorster, Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles, *Environmental Health Perspectives*, 113 (2005) 823-839.

[2]. H. Jans, Q. Huo, Gold nanoparticle-enabled biological and chemical detection and analysis, *Chemical Society Reviews*, 41 (2012) 2849-2866.

[3]. P.V. AshaRani, G.L.K. Mun, M.P. Hande, S. Valiyaveettil, Cytotoxicity and Genotoxicity of Silver Nanoparticles in Human Cells, *Acs Nano*, 3 (2009) 279-290.

[4]. A. Alkaladi, N.A.M.N. El-Deen, M. Afifi, O.A. Abu Zinadah, Hematological and biochemical investigations on the effect of vitamin E and C on Oreochromis niloticus exposed to zinc oxide nanoparticles, *Saudi Journal of Biological Sciences*, 22 (2015) 556-563.

[5]. A. Avalos, A.I. Haza, D. Mateo, P. Morales, Effects of silver and gold nanoparticles of different sizes in human pulmonary fibroblasts, *Toxicology Mechanisms and Methods*, 25 (2015) 287-295.

[6]. A. Pourmand, M. Abdollahi, Current Opinion on Nanotoxicology, *Daru-Journal Of Faculty Of Pharmacy*, 20 (2012).

[7]. T.C. Dakal, A. Kumar, R.S. Majumdar, V. Yadav, Mechanistic Basis of Antimicrobial Actions of Silver Nanoparticles, *Frontiers in microbiology*, 7 (2016) 1831.

[8]. P. Korshed, L. Li, Z. Liu, T. Wang, The Molecular Mechanisms of the Antibacterial Effect of Picosecond Laser Generated Silver Nanoparticles and Their Toxicity to Human Cells, *Plos One*, 11 (2016).

[9]. L.K. Braydich-Stolle, B. Lucas, A. Schrand, R.C. Murdock, T. Lee, J.J. Schlager, S.M. Hussain, M.C. Hofmann, Silver Nanoparticles Disrupt GDNF/Fyn kinase Signaling in Spermatogonial Stem Cells, *Toxicological Sciences*, 116 (2010) 577-589.

[10]. M. Poirier, J.C. Simard, F. Antoine, D. Girard, Interaction between silver nanoparticles of 20 nm (AgNP20) and human neutrophils: induction of apoptosis and inhibition of de novo protein synthesis by AgNP20 aggregates, *Journal of Applied Toxicology*, 34 (2014) 404-412.

[11]. T.C. Long, N. Saleh, R.D. Tilton, G.V. Lowry, B. Veronesi, Titanium dioxide (P25) produces reactive oxygen species in immortalized brain microglia (BV2): implications for nanoparticle neurotoxicity, *Environmental Science and Technology*, 40 (2006) 4346-4352.

[12]. D.M. Brown, K. Donaldson, P.J. Borm, R.P. Schins, M. Dehnhardt, P. Gilmour, L.A. Jimenez, V. Stone, Calcium and ROS-mediated activation of transcription factors and TNF-alpha cytokine gene expression in macrophages exposed to ultrafine particles, *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 286 (2004) L344-L353.

[13]. J.J. Li, L. Zou, D. Hartono, C.N. Ong, B.H. Bay, L.Y.L. Yung, Gold nanoparticles induce oxidative damage in lung fibroblasts in vitro+, *Advanced Materials*, 20 (2008) 138-142.

[14]. Y. Li, W. Zhang, J.F. Niu, Y.S. Chen, Surface-Coating-Dependent Dissolution, Aggregation, and Reactive Oxygen Species (ROS) Generation of Silver Nanoparticles under Different Irradiation Conditions, *Environmental Science and Technology*, 47 (2013) 10293-10301.

[15]. C. Zanette, M. Pelin, M. Crosera, G. Adami, M. Bovenzi, F.F. Larese, C. Florio, Silver nanoparticles exert a long-lasting antiproliferative effect on human keratinocyte HaCaT cell line, *Toxicology in Vitro*, 25 (2011) 1053-1060.

[16]. K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method, *Methods*, 25 (2001) 402-408.

[17]. E. Soderstjerna, F. Johansson, B. Klefbohm, U.E. Johansson, Gold- and Silver Nanoparticles Affect the Growth Characteristics of Human Embryonic Neural Precursor Cells, *Plos One*, 8 (2013).

[18]. X. Chen, H.J. Schluesener, Nanosilver: A nanoproduct in medical application, *Toxicology letters*, 176 (2008) 1-12.

[19]. B. De Berardis, G. Civitelli, M. Condello, P. Lista, R. Pozzi, G. Arancia, S. Meschini, Exposure to ZnO nanoparticles induces oxidative stress and cytotoxicity in human colon carcinoma cells, *Toxicology and applied pharmacology*, 246 (2010) 116-127.

[20]. F. Wang, F. Gao, M.B. Lan, H.H. Yuan, Y.P. Huang, J.W. Liu, Oxidative stress contributes to silica nanoparticle-induced cytotoxicity in human embryonic kidney cells, *Toxicology in Vitro*, 23 (2009) 808-815.

[21]. M. Auffan, J. Rose, M.R. Wiesner, J.Y. Bottero, Chemical stability of metallic nanoparticles: A parameter controlling their potential cellular toxicity in vitro, *Environmental Pollution*, 157 (2009) 1127-1133.

[22]. J.W. Han, S. Gurunathan, J.K. Jeong, Y.J. Choi, D.N. Kwon, J.K. Park, J.H. Kim, Oxidative stress mediated cytotoxicity of biologically synthesized silver nanoparticles in human lung epithelial adenocarcinoma cell line, *Nanoscale Research Letters*, 9 (2014) 459.

[23]. S. Gurunathan, J.W. Han, V. Eppakayala, M. Jeyaraj, J.H. Kim, Cytotoxicity of Biologically Synthesized Silver Nanoparticles in MDA-MB-231 Human Breast Cancer Cells, *BioMed Research International*, (2013).

[24]. F.L. Filon, M. Crosera, G. Adami, M. Bovenzi, F. Rossi, G. Maina, Human skin penetration of gold nanoparticles through intact and damaged skin, *Nanotoxicology*, 5 (2011) 493-501.

[25]. M.E. Samberg, S.J. Oldenburg, N.A. Monteiro-Riviere, Evaluation of Silver Nanoparticle Toxicity in Skin in Vivo and Keratinocytes in Vitro, *Environmental Health Perspectives*, 118 (2010) 407-413.

[26]. L.L. Bronsart, C. Stokes, C.H. Contag, Chemiluminescence Imaging of Superoxide Anion Detects Beta-Cell Function and Mass, *Plos One*, 11 (2016).

[27]. T. Finkel, N. J. Holbrook, Oxidants, oxidative stress and the biology of ageing, *Nature*, 408 (2000) 239-247.

[28]. G. Waris, H. Ahsan, Reactive oxygen species: role in the development of cancer and various chronic conditions, *Journal of Carcinogenesis*, 5 (2006) 14.

[29]. Y.H. Lee, F.Y. Cheng, H.W. Chiu, J.C. Tsai, C.Y. Fang, C.W. Chen, Y.J. Wang, Cytotoxicity, oxidative stress, apoptosis and the autophagic effects of silver nanoparticles in mouse embryonic fibroblasts, *Biomaterials*, 35 (2014) 4706-4715.

[30]. N. Li, T. Xia, A.E. Nel, The role of oxidative stress in ambient particulate matter-induced lung diseases and its implications in the toxicity of engineered nanoparticles, *Free Radical Biology and Medicine*, 44 (2008) 1689-1699.

[31]. A. Manke, L.Y. Wang, Y. Rojanasakul, Mechanisms of Nanoparticle-Induced Oxidative Stress and Toxicity, *BioMed Research International*, (2013) 15.

[32]. S.N.P. Voicu, D. Dinu, C. Sima, A. Hermenean, A. Ardelean, E. Codrici, M.S. Stan, O. Zarnescu, A. Dinischiotu, Silica Nanoparticles Induce Oxidative Stress and Autophagy but Not Apoptosis in the MRC-5 Cell Line, *International Journal of Molecular Sciences*, 16 (2015) 29398-29416.

[33]. E. Birben, U.M. Sahiner, C. Sackesen, S. Erzurum, O. Kalayci, Oxidative stress and antioxidant defense, *World Allergy Organization journal*, 5 (2012) 9-19.

[34]. P.L. Gratao, A. Polle, P.J. Lea, R.A. Azevedo, Making the life of heavy metal-stressed plants a little easier, *Functional Plant Biology*, 32 (2005) 481-494.

[35]. A. Bhattacharyya, R. Chattopadhyay, S. Mitra, S.E. Crowe, Oxidative Stress: An Essential Factor in the Pathogenesis of Gastrointestinal Mucosal Diseases, *Physiological Reviews*, 94 (2014) 329-354.

[36]. S. Arora, J. Jain, J.M. Rajwade, K.M. Paknikar, Cellular responses induced by silver nanoparticles: In vitro studies, *Toxicology letters*, 179 (2008) 93-100.

Acknowledgements

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors

Competing Interests: The author declares to have no competing interests

About The License