

# Silver nanoparticles – allies or adversaries?

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## Abstract

Nanoparticles (NP) are structures with at least one dimension of less than 100 nanometers (nm) and unique properties. Silver nanoparticles (AgNP), due to their bactericidal action, have found practical applications in medicine, cosmetics, textiles, electronics, and other fields. Nevertheless, their less advantageous properties which make AgNP potentially harmful to public health or the environment should also be taken into consideration. These nanoparticles are cyto- and genotoxic and accumulate in the environment, where their antibacterial properties may be disadvantageous for agriculture and waste management. The presented study reviews data concerning the biological effects of AgNP in mammalian cells *in vitro*: cellular uptake and excretion, localization in cellular compartments, cytotoxicity and genotoxicity. The mechanism of nanoparticle action consists on induction of the oxidative stress resulting in a further ROS generation, DNA damage and activation of signaling leading to various, cell type-specific pathways to inflammation, apoptotic or necrotic death. In order to assure a safe application of AgNP, further detailed studies are needed on the mechanisms of the action of AgNP on mammalian cells at the molecular level.

## Key words

nanoparticles, silver, cytotoxicity, genotoxicity, *in vitro* toxicity testing

## INTRODUCTION

Over the last 30 years, nanotechnology has developed many structures with at least one dimension of less than 100 nanometers. There are a variety of nanomaterials and their properties differ depending on shape and size. These have found many practical applications in medicine, especially in cancer treatment and drug delivery [1, 2], industry and everyday life, but they also are examined with increasing concern because of their toxicity [3, 4, 5, 6] and environmental risk [6, 7]. A number of comprehensive reviews on nanoparticle properties and applications have been published in the last few years, e.g. [8, 9, 10, 11, 12, 13]. Due to their bactericidal properties, silver nanoparticles (AgNP) are the most frequently applied nanomaterials. They are used in textiles, cosmetics, as products for domestic cleaning, air cleaners, food packaging, coating for refrigerators, water disinfection, in fact, in every application where bacteria may exert a harmful effect. In particular, an important use is that in hospitals and general medical practice, in medical devices, and in dressings for the treatment of wounds, burns and ulcers (review in [3]).

The antimicrobial properties of AgNP are the cause of their potential risk to the environment. AgNP released from industrial activities and consumer products accumulate in sewage sludge. Thus, they can interfere with beneficial bacteria in waste water and sewage treatment. Further, due to their presence in water, they accumulate in plants and animals entering their ecological food chains. The bioaccumulation and propagation of nanoparticles through food chains is one fundamental concern in nanotoxicology. Their impact on the environment is discussed in [6, 7, 13, 14].

Silver ions have antibacterial properties [15, 16]. They can be liberated from AgNP; however, most scientists consider silver ions as only partly responsible for the antibacterial effects of AgNP [17, 18, 19]. Rather, the AgNP properties, similarly to those of other nanoparticles, result from the unique physicochemical characteristics connected with their small size: such properties as surface energy, charge and solvation are relevant to their interactions with biomolecules and behaviour in the cell [20, 21, 22, 23]. Their shape and coating (if applied) can be modified to obtain the desired characteristics.

One important concept of nanoparticle activity *in vivo* is that of corona [22]. These authors indicate that:

the 'surfaces' of nanoparticles in a biological environment are modified by the adsorption of biomolecules such as proteins and lipids, leading to a biomolecular interface organization that may be loosely divided into two components named the 'hard' and 'soft' coronas with (respectively) 'long' and 'short' typical exchange times.

This concept explains the somewhat unpredictable behaviour of nanoparticles in the biological milieu, showing that the biological effect depends on the type of macromolecules met and bound upon interaction with the cell. Further, corona components exchange can take place while the particle travels inside the cell. The corona concept also explains the differences between apparently similar nanoparticles, because even small changes in their shape, size or coating may radically modify the composition of the corona [20, 23]. The impact of properties of the corona on the cellular response to nanoparticle treatment is still not sufficiently understood.

Because of the numerous practical applications of AgNP, they may enter living organisms, including the human body, in food, and also through skin or the respiratory system [4, 5], and even pass the blood-brain barrier [24]. The toxicity

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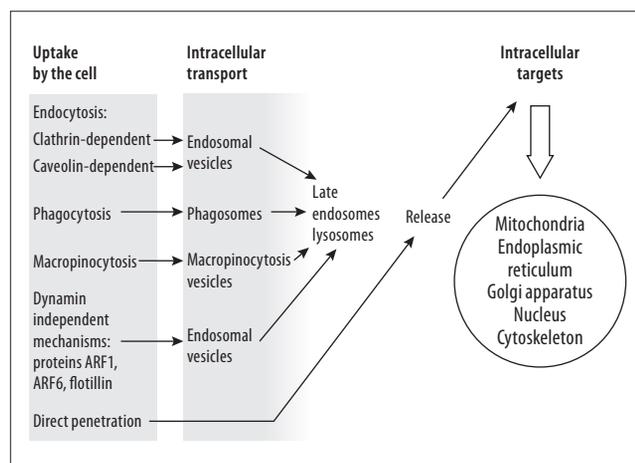
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of AgNP *in vivo* and *in vitro*, in eukaryotic cells was recently reviewed in [11]. Here, experimental data are reviewed concerning the impact of AgNP on biological functions, as studied in various mammalian cellular models, originating from human or animal normal and cancer cells.

## UPTAKE OF AgNP AND THEIR INTRACELLULAR LOCALIZATION

All nanoparticles are taken up by mammalian cells by such mechanisms as pinocytosis, endocytosis dependent on caveolae and lipid raft composition, clathrin-dependent endocytosis and phagocytosis [25]. AgNP are no exception in this respect: as shown in [25], normal human lung fibroblasts (IMR-90) and human glioblastoma (U251) take up AgNP by clathrin-dependent endocytosis and macropinocytosis. Uptake kinetics, intracellular localisation and exocytosis depend on nanoparticle size, surface characteristics (including coating type, if any), as well as on the ability to form aggregates. Also, the cell type affects the uptake kinetics. In the case of AgNP(6-20 nm), they were usually present inside the cells after a 2h treatment and the uptake was linear during the 2h-48h exposure [25] and resulted from a balance between exo- and endocytosis. It took 48 h to expel 66% of AgNP taken up during 2h incubation, showing that exocytosis was significantly slower than endocytosis in the examined cells and that probably some of the remaining AgNP were localized in an inaccessible cellular compartment. An example of the uptake dependence on cell type is given in [26]: AgNP were detected in human blood monocytes (CD14+) but not in T-cells (CD3+). A review paper [27] summarizes the data on relations between nanoparticle size and uptake mechanisms and points to their inconsistency. It seems that the particle size is less important than the physicochemical properties. The mechanisms of AgNP uptake, transport pathways inside the cell and cellular targets are diagrammatically presented in Fig. 1.

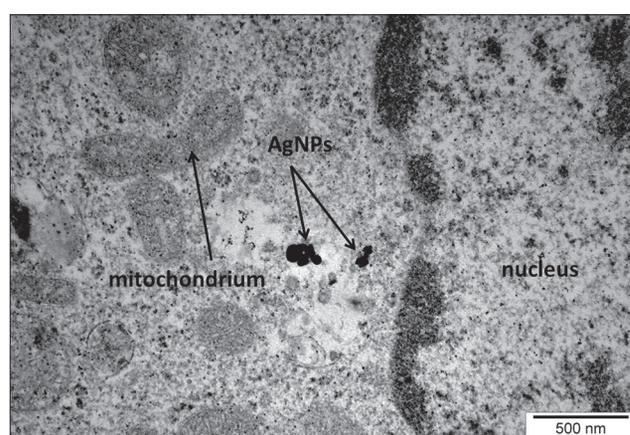


**Figure 1.** Diagram showing the AgNP uptake, transport pathways inside the cell and cellular targets

The intracellular sites of localization of AgNP vary depending on the cell type and the method applied. Specific staining of cellular structures (endo-lysosomes, nuclei, Golgi complex and endoplasmic reticulum) in human mesenchymal stem cells (hMSC) with the use of fluorescent

probes showed that AgNP ( $50 \pm 20$  nm) were present mainly within endo-lysosomal structures. They were absent in the cell nucleus, endoplasmic reticulum or Golgi complex. They also formed agglomerates in the perinuclear region [28].

In contrast with these observations with the use of a light microscope, the transmission electron microscopic (TEM) analysis indicated the presence of AgNP inside the mitochondria and nucleus, as stated in the paper by Asharani mentioned above [25]. According to those authors, this observation implicates the direct involvement of AgNP in the mitochondrial and DNA damage. Others found AgNP (20-40 nm) in rat alveolar epithelial cells (R3-1) as aggregates or single particles in the cytosol, in the nucleus and in the nuclear membrane 30 min. after AgNP exposure [29]. Similarly, AgNP (20 nm) were found in the cytoplasm, mitochondria and nuclei of human cell lines, A549 and HepG2 [11]. Fig. 2 shows an example of TEM microphotograph of AgNP (20 nm) in the cytoplasm of A549 cells.



**Figure 2.** Transmission electron microscope image of silver nanoparticles in cytoplasm of A549 (lung adenocarcinoma epithelial) cells treated with AgNP (20 nm) for 48 h at concentration 50 µg/ml

As can be concluded from this brief review, AgNP uptake and localisation in the cell depend on the cell type and the specific analysis method applied; the surface properties and size of AgNP also are important factors.

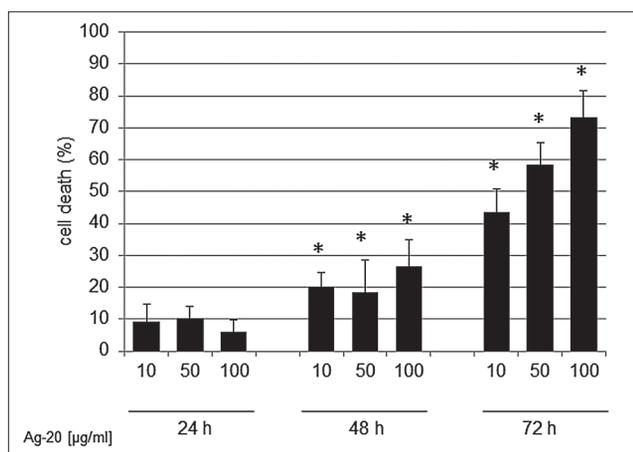
## INHIBITION OF PROLIFERATION AND CELL DEATH

Examination of AgNP action *in vivo* demonstrated the cytotoxic effects of AgNP in eukaryotic organisms (reviewed in [11]). Experiments *in vitro* with the use of various types of cellular models not only confirmed the cytotoxicity but also allowed analysis of the molecular mechanisms of AgNP action, such as proliferation inhibition, apoptotic or necrotic death, alterations in the expression of genes essential for survival, and the pathways leading to genetic damage.

Cytotoxicity *in vitro* is usually estimated with the use of colorimetric tests; their principle is the reduction of tetrazolium salts, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) or XTT (2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide) to formazan. The reduction is carried out by a mitochondrial reductase and is an indirect measure of cell population viability and proof of an undamaged mitochondrial respiratory chain. Examples of MTT test application for AgNP cytotoxicity

estimations can be found e.g. in [30]: AgNP (69 nm) inhibited proliferation of RAW264.7 cells in a concentration and time dependent way. Smaller size AgNP (20 nm) were shown to be more effective than the larger ones. Another paper [31] reported the dependence of the MTT-measured AgNP cytotoxicity on their size and agglomeration/ aggregation state. The 3 human cell lines tested were THP1 (leukemic monocyte-like cells), HepG2 (hepatocellular liver carcinoma) and A549 (lung adenocarcinoma epithelial cells). Among these cell lines, the HepG2 proved to be the most sensitive and THP1 the most resistant. Typically, the cytotoxic effect was in proportion to AgNP concentration and incubation time (the latter with the exception of HepG2 cells). The agglomeration state of AgNP affected the examined cell types to various extents apparently related to the cellular binding and uptake. Also, the resistance of THP1 cells which are 'professional phagocytes' seems to be related to their high endocytotic and exocytic activities.

The cytotoxic effect of AgNP treatment can also be evaluated by the propidium iodide (PI) assay. This dye is membrane impermeant and thus, viable cells are not stained, hence the common PI application for identifying dead cells. Fig. 3 shows the result of the PI assay for A549 cells [31]. The dependence of the cytotoxic effect on AgNP concentration and time of treatment can be seen.



**Figure 3.** Cytotoxic effect of treatment of A549 cells with increasing concentrations (10, 50 and 100 µg/ml) of AgNP (20 nm) evaluated by the propidium iodide assay. Standard deviation indicated. Asterisks denote statistically significant difference ( $P < 0.05$ ) from the respective control. Data for the plot taken from Lankoff et al. [31]

Similar cytotoxicity determinations were carried out by other authors who also determined the cell death type. Human fibrosarcoma HT-1080 and A431 human epithelial carcinoma cells were treated overnight with 7-20 nm spherical AgNP (0-50 µg/ml) and cytotoxicity determined by the XTT test [32]. The viability decreased in proportion to the AgNP concentration. The cell death type depended on the AgNP concentration. Apoptosis (with the characteristic pattern of DNA fragmentation) was seen at the lower concentration range; the onset of apoptosis monitored with caspase-3 assay was 0.78 µg/ml in HT-1080 cells and 1.56 µg/ml in A431 cells, whereas the concentration causing necrotic death was equal (12.5 µg/ml) in both cell lines.

One of the early studies on the difference in cellular sensitivity to the cytotoxic action of AgNP was that carried out on mouse fibroblasts NIH3T3 and human colorectal

carcinoma HCT116 cells [33]. The cells differed considerably in the response to AgNP (1 – 100 nm). Incubation of NIH3T3 cells with AgNP at the concentration of 50 µg/ml for 24h induced apoptosis (estimated from PARP cleavage), whereas after the same treatment apoptosis was not detected in HCT116 cells. Interestingly, in both cell lines p53 and c-Jun N-terminal kinase were activated. However, in HCT116 cells, an increased expression of the anti-apoptotic protein BCL-2 (B-cell lymphoma 2) was observed, thus explaining the resistance to AgNP. In contrast, ROS (reactive oxygen species) was generated by mitochondria in NIH3T3 cells, followed by JNK activation and translocation to mitochondria of the pro-apoptotic BAX (Bcl-2-associated X protein). The result was the release of the mitochondrial cytochrome c and apoptosis.

The cytotoxic effect of AgNP treatment often is often due to enhanced expression of apoptosis-related genes and the induction of apoptotic death. This is the case in 2 cell lines, human colon adenocarcinoma HT29 and baby hamster kidney, BHK-21, where AgNP (20 nm) treatment at 11 µg/ml induced apoptosis [34]. Using real-time polymerase chain reaction (RT-PCR) that enables quantitative estimation of the products of amplification of the chosen genes, expression profiles were obtained of pro-apoptotic and anti-apoptotic genes. Expression of the pro-apoptotic genes, such as *BAK*, *BAD*, *C-MYC* and caspase 3 gene, increased in AgNP treated cells, whereas that of the anti-apoptotic genes (*BCL-2*, *BCL-X<sub>L</sub>*) decreased. Importantly, there was a pronounced increase in the expression of *P53*, pointing to the p53-dependent apoptotic mechanism.

The size-dependent cellular toxicity of AgNP 10, 50, and 100 nm and cell death type were examined in MC3T3-E1 and PC12 cells [35]. The cytotoxic effect was size- and dose-dependent as well as cell type dependent: the treatment caused apoptotic death in the MC3T3-E1 cells, and necrosis in the PC12 cells. AgNP (10 nm) were more toxic than the larger (50 and 100 nm) particles.

Although usually inhibition of cell proliferation was observed upon incubation with AgNP, there is one example of its stimulation. In HepG2 cells incubated with AgNP at concentrations up to 0.5 mg/l no cytotoxic effect was observed, and the proliferation rate was higher than that in the untreated cell culture [18]. Exposure to AgNP >1.0 mg/l gave a considerable cytotoxicity and increased the frequency of micronucleus formation. In contrast, in the rat coronary endothelial cells (CEC), AgNP (45 nm) at low concentrations (1.0-10 µg/ml) inhibited proliferation, whereas at high concentrations (50-100 µg/ml) stimulated it. The stimulation was dependent on nitric oxide generation, in particular, on activation of nitric oxide synthase (eNOS) resulting from the phosphorylation of serine 1177. The stimulated proliferation was blocked by the eNOS inhibitor, NG-nitro-L-arginine methyl ester [36].

Growth stimulation by low concentrations of AgNP, such as described in [18], is caused by ROS generated in the treated cells. AgNP, similarly to other types of nanoparticles, cause oxidative stress (see the following section). This effect of ROS has been known for some time. Growth factor receptors that have tyrosine kinase activity are inactivated by tyrosine phosphatases, enzymes which contain cysteine residue in their catalytic site. Due to the specific structure of the site, the cysteine residue is extremely sensitive to oxidation. Hence, intracellular ROS generation is the cause of the phosphatase inactivation. There follows a shift in the

equilibrium between the active and inactive forms of the receptor kinases resulting in an apparent activation of the kinase activity. Thus, proliferation is stimulated, although there is no ligand-mediated activation of the receptor [37].

In summary, the results of the experiments with AgNP *in vitro* show that the cytotoxic effects depend on nanoparticle size and surface properties, concentration, time of treatment and cell type-specific response. The lack of standards in nanoparticle research makes the comparisons of cytotoxic effects difficult or impossible. Nevertheless, it is obvious that AgNP can exert toxic effects and are potentially harmful to public health and the environment.

## OXIDATIVE STRESS

Under physiological conditions, reactive oxygen species (ROS) are present in every cell, being produced by the mitochondrial and cytoplasmic oxidation processes. Under environmental stress, the cell reacts by increased ROS generation and this leads to imbalance between ROS generation and their neutralisation by antioxidative enzymes and low molecular weight antioxidants, among others by glutathione. This disturbance of the redox equilibrium is defined as oxidative stress. Under conditions of oxidative stress the cell accumulates ROS, and the antioxidative response that follows involves modifications in signalling pathways, among them – activation of mitogen activated protein kinases (MAPK) and release of pro-inflammatory cytokines. ROS are highly reactive and thus, able to modify cellular components, among them DNA. The oxidative damage thus inflicted leads to genotoxic effects, discussed further in the text.

ROS increase due to nanoparticle treatment has been shown to be the key factor in the biological effects *in vivo* and *in vitro* [17, 19, 29, 38, 39, 40, 41]. From the TEM microphotographs it can be judged that AgNP of various size and shape accumulate in the mitochondria. It is possible that this is the direct cause of mitochondrial damage and the disturbed function of the respiratory chain resulting in ROS generation and oxidative stress. An example of such response is that of BRL 3A rat liver cells to AgNP (15 and 100 nm) treatment [39]. The cellular level of ROS (determined using the dichlorodihydrofluorescein diacetate method) increased in a AgNP concentration-dependent manner and reached a maximum at 6h. Treatment at 25 and 50 µg/ml resulted in an approximately 10-fold increase in ROS generation as compared to the control. The effect of Ag NP on mitochondrial membrane potential (MMP) was evaluated after a 24h exposure to AgNP using the rhodamine 123 uptake assay. The results indicated that there was a significant decrease (80%) of MMP both at 25 and 50 µg/ml AgNP. Also, a significant depletion of GSH (by 70% relative to control) was observed at 25 µg/ml AgNP. In all these tests the results did not depend on AgNP size, in contrast with other reports. A decrease in the cellular glutathione content after AgNP treatment was also observed in mouse RAW264.7 cells [30].

The influence of AgNP size on the extent of oxidative stress was stated in rat macrophages: 24h treatment with AgNP 15 nm, AgNC -30 nm and AgNC- 55 nm gave the most pronounced ROS increase in the case of the 15 nm particles [42]. Consistently, antioxidant treatment exerted a protective

effect: N-acetyl-L-cysteine (NAC) prevented the significant ROS increase noted in human Chang liver cells treated with AgNP (28 -35 nm) [43]. NAC also decreased AgNP toxicity and DNA damage in human hepatoma cells, HepG2 [19]. Similarly, a strong protective effect of antioxidants from *Gentiana asclepiadea* flower and haulm extracts was observed in HEK 293 cells treated with AgNP (20 nm): oxidative DNA base lesions and strand breaks were considerably diminished [44, 45].

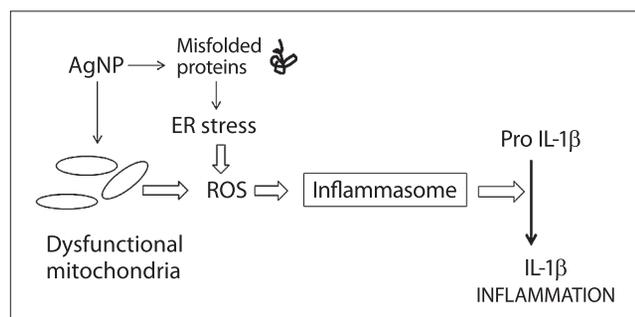
Enhanced expression of genes coding antioxidative defense proteins is a typical feature of the response to oxidative stress. In HepG2 cells treated for 24h with 0.2 µg/ml AgNP (5-10 nm), RT-PCR was applied to analyze the expression of SOD1 (superoxide dismutase 1), GPx1 (glutathione peroxidase 1) and catalase [19]. The mRNA levels of catalase and SOD1 (but not GPx1) were significantly higher than those in the untreated cells. Another set of genes was examined in HeLa cells after 4h incubation with AgNP (5-10 nm): heme oxygenase (HO-1), metallothionein-2A (MT-2A) and heat shock protein 70 (HSP70). Expression of MT-2A and HO-1 significantly increased, whereas that of HSP70 remained unchanged [46]. Similar observations were made in IMR-90 and U251 cells [25].

The expression of another group of genes is stimulated after treatment with inducers of oxidative stress, those coding the inflammation-related proteins, such as interleukins, cytokines (e.g. tumour necrosis factor, TNF-α) and VEGF (vascular endothelial growth factor). These proteins are released into the medium and can be estimated in cell cultures of AgNP treated cells. Thus, mesenchymal stem cells (hMSC) released significantly increased amounts of interleukin IL-8 and VEGF into the medium in result of treatment with AgNP (<50 nm) for 24h at a concentration as low as 1 µg/ml [47]. Rat macrophages treated with AgNP (15 nm, 30 nm and 55 nm) for 24h released TNF-α, MIP-2 (macrophage inhibitory protein-2) and IL-1β (interleukin-1β) at concentrations significantly different from the control only after treatment with AgNP-15 nm [42]. Higher cytokine expression was noted in IMR-90 and U251 [48]. In mouse macrophages RAW264.7 treated with AgNP (69 nm, 0.2-1.6 µg/ml) for 24-96h enhanced expression of TNF-α coding gene and increased TNF-α synthesis preceded apoptosis [30].

The proposed direct action AgNP on membrane receptors and subsequent ROS generation and activation of signaling pathways involving various protein kinases was recently reviewed in [27]. The 3 main groups of kinases are the extracellular signal regulated kinases, p38 and c- Jun N-terminal kinases. Redox-sensitive transcription factors, NRF2 (nuclear factor (erythroid-derived 2)-like 2) and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) also participate in the cellular response to AgNP. Depending on the nanoparticle size and concentration, as well as the cell type, the outcome is stimulation of proliferation, inflammation and/or apoptotic death.

The production of ROS is considered to specifically initiate the NLRP3 inflammasome [43, 50, 51, 53], the multiprotein complex specific for myeloid cells that activates caspase-1, which cleaves proIL1β, producing the active interleukin. The role of mitochondrial dysfunction was recently stressed [52] and – although not directly confirmed for AgNP – may be the case also in AgNP treated cells. Another possibility, not excluding the first one, is connected with the observation of causal link between endoplasmic reticulum (ER) stress

and inflammasome activation [51]. It is plausible to assume that the same type of mechanism is activated under the influence of AgNP (and also other types of nanoparticles) which interact directly with ER and disturb the folding of protein molecules, thus inducing the ER stress [53, 54] (Fig. 4).



**Figure 4.** Proposed pro-inflammatory pathways induced by AgNP

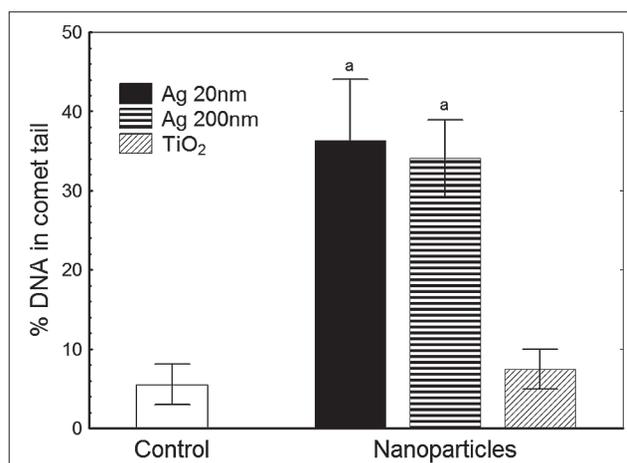
## GENOTOXICITY

AgNP-induced oxidative stress and resulting high level of ROS is the cause of DNA lesions, such as DNA breaks, oxidative adducts, oxidative single base damage causing point mutations. Apart from the direct damage, ROS is also the cause of lipid peroxidation and its products (e.g. nonenal) react with DNA, adding to the genotoxic effect. All these lesions – if left unrepaired by the cellular repair systems – are potentially carcinogenic.

DNA damage in cells of various origins is usually detected with the use of single cell electrophoresis or comet test which measures the sum of single and double strand breaks in individual cells (reviewed in [55]). In AgNP treated mammalian cells, the extent of DNA breakage was usually dependent on the particle size, concentration and time of treatment, as well as on cell type. Most experiments were carried out with human or mouse cells: L5178Y/Tk(+/-) mouse lymphoma cells [41], human peripheral blood cells [56], human mesenchymal stem cells (hMSCs) [47], NT2, human testicular embryonic carcinoma cell line, and primary testicular cells from C57BL6 mice of wild type and 8-oxoguanine DNA glycosylase knock-out (mOgg1(-/-) genotype [57], human normal bronchial epithelial (BEAS-2B) cells [16], human liver cells [22] and kidney cells [44].

The comet assay can also be used for determination of oxidative base damage by combining it with endonucleases that recognize specific lesions. An enzyme often applied is the formamidopyrimidine DNA glycosylase (FPG). It was used to estimate oxidative base damage in HepG2 cells treated with AgNP (20 nm), and compared with that inflicted by 200 nm Ag particles and TiO<sub>2</sub> nanoparticles [58] (Fig. 5).

One consequence of the oxidative stress caused by AgNP is lipid peroxidation and DNA lesions caused by the reaction of peroxidation products with base residues. To the resulting bulky DNA adducts belong among others, etheno adducts formed in reaction with the main lipid peroxidation product, *trans*-4-hydroxy-2-nonenal. They can be determined by <sup>32</sup>P-postlabelling assay, a method often used for the estimation of exposure effects to mutagens and carcinogenic chemicals present in the environment [50]. With this method, DNA adducts were shown to form after 24h incubation of A549



**Figure 5.** Oxidative DNA damage recognized by formamidopyrimidine DNA glycosylase in HepG2 cells for 2h. Untreated cells served as controls. Statistically significant difference from control was found for Ag particles. Mean values are shown  $\pm$  standard deviation,  $n = 4$ ,  $p < 0.05$ . Measure of damage was the % DNA in the comet tail. Reproduced with permission of the Nukleonika publisher from [58]

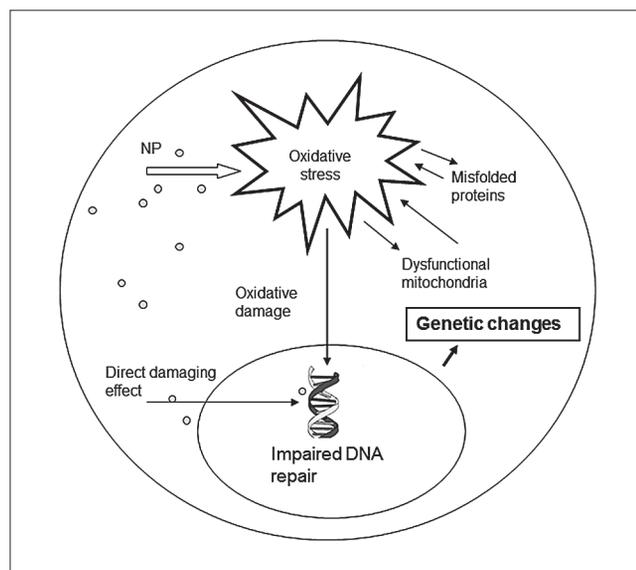
cells with AgNP (30-50 nm) in a concentration-dependent manner (0-15  $\mu$ g/ml). Treatment with NAC decreased adduct formation, thus indicating that they were formed as a result of oxidative stress [17].

A more recent and often applied method of DNA double strand break (DSB) estimation is the counting of gamma H2AX foci, which are formed at the sites of damage. Their number per cell corresponds with the number of DSB per cell [59]. It was shown that gamma H2AX foci (and hence, DSB) are formed in AgNP treated cells, for example, in HepG2 cells treated with AgNP (5-10 nm) at low concentrations (1-2  $\mu$ g/ml) for 24h [19]. Consistently, in the same cells, treatment with AgNP (7-10 nm) induced micronuclei formation [18]. The micronuclei test is a standard in genotoxicity estimation [60]. A recently established significant association between micronuclei frequency in healthy human subjects and cancer risk [61], as well as neurodegenerative diseases [62], points to the potential health risk linked with excessive use of AgNP.

Micronuclei indicate the presence of the chromosomal damage which can be characterized in more detail by a considerably more time consuming methods. In the already mentioned human cell lines, lung fibroblasts (IMR-90) and glioblastoma (U251) after AgNP (6-20 nm, 25  $\mu$ g/ml) treatment induced chromosomal aberrations. Their frequency was significantly higher in U251 cells than in the fibroblasts, corresponding with the DNA breakage level [38].

## CONCLUSIONS

Figure 6 shows the effects of AgNP at the cellular level and summarizes the present state of knowledge reviewed in the presented study. As in the case of other nanoparticles, the most important effect apparently is the oxidative stress. The enhanced generation of ROS affects the mitochondrial respiratory chain and increases the amount of unfolded and misfolded proteins in the endoplasmic reticulum (ER), inducing the ER stress and the unfolded protein response (UPR). Both these types of cellular damage lead to a further ROS generation, DNA damage and the activation of signaling resulting in various, cell type-specific pathways to inflammation, apoptotic or necrotic death.



**Figure 6.** Mechanisms of AgNP cytotoxicity in the mammalian cell

Generally, the reviewed data seem to indicate that mammalian cells respond to AgNP in a similar way as to other factors that induce oxidative stress, including silver ions. However, this is not the case: a recent paper [63] shows that a considerable difference exists between gene expression profiles in human lung epithelial A549 cells treated with AgNP (more than 2-fold upregulation of more than 1,000 genes) or with Ag<sup>+</sup> ions (133 genes), although the latter also induce oxidative stress. To achieve a full understanding of the molecular and cellular mechanisms of the response to AgNP, research must continue. So far, the experimental data point to mutagenic and carcinogenic properties of AgNP. In view of the potential health hazard, further basic research and elaboration of safety measure issues seem to be most important.

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### REFERENCES

- Lim ZZ, Li JE, Ng CT, Yung LY, Bay BH. Gold nanoparticles in cancer therapy. *Acta Pharmacol Sin.* 2011; 32: 983-990.
- Talekar M, Kendall J, Denny W, Garg S. Targeting of nanoparticles in cancer: drug delivery and diagnostics. *Anticancer Drugs.* 2011; 22(10): 949-962.
- Chopra I. The increasing use of silver-based products as antimicrobial agents: a useful development or a cause for concern? *J Antimicrob Chemother.* 2007; 59: 587-590.
- Schrand AM, Dai L, Schlager JJ, Hussain SM. Toxicity testing of nanomaterials. *Adv Exp Med Biol.* 2012; 745: 58-75.
- Schrand AM, Rahman MF, Hussain SM, Schlager JJ, Smith DA, Syed AF. Metal-based nanoparticles and their toxicity assessment. *Wiley Interdiscip Rev Nanomed Nanobiotechnol.* 2010; 2: 544-568.
- Wiechers JW, Musee N. Engineered inorganic nanoparticles and cosmetics: facts, issues, knowledge gaps and challenges. *J Biomed Nanotechnol.* 2010; 6: 408-431.
- Smita S, Gupta SK, Bartonova A, Dusinska M, Gutleb AC, Rahman Q. Nanoparticles in the environment: assessment using the causal diagram approach. *Environ Health.* 2012; 11 Suppl 1: S13.
- Ahamed M, Alsalhi MS, Siddiqui MK. Silver nanoparticle applications and human health. *Clin Chim Acta.* 2010; 411: 1841-1848.
- Dhawan A, Pandey A, Sharma V. Toxicity assessment of engineered nanomaterials: resolving the challenges. *J Biomed Nanotechnol.* 2011; 7: 6-7.
- Dhawan A, Sharma V. Toxicity assessment of nanomaterials: methods and challenges. *Anal Bioanal Chem.* 2010; 398: 589-605.
- Kruszewski M, Brzoska K, Brunborg G, Asare N, Dobrzyńska H, Dusinska M, et al. Toxicity of Silver Nanomaterials in Higher Eukaryotes. *Adv Mol Toxicol.* 2011; 5: 179-218.
- Singh N, Manshian B, Jenkins GJ, Griffiths SM, Williams PM, Maffei TG, Wright CJ, Doak SH. NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials. *Biomaterials* 2009; 30: 3891-3914.
- Snopczynski T, Goralczyk K, Czaja K, Strucinski P, Hernik A, Korcz W, Ludwicki JK. Nanotechnology – possibilities and hazards. *Rocz Panstw Zakl Hig.* 2009; 60: 101-111 (in Polish).
- Stensberg MC, Wei Q, McLamore ES, Porterfield DM, Wei A, Sepulveda MS. Toxicological studies on silver nanoparticles: challenges and opportunities in assessment, monitoring and imaging. *Nanomedicine (Lond)* 2011; 6: 879-898.
- Jung WK, Kim SH, Koo HC, Shin S, Kim JM, Park YK, Hwang SY, Yang H, Park YH. Antifungal activity of the silver ion against contaminated fabric. *Mycoses* 2007; 50: 265-269.
- Jung WK, Koo HC, Kim KW, Shin S, Kim SH, Park YH. Antibacterial activity and mechanism of action of the silver ion in *Staphylococcus aureus* and *Escherichia coli*. *Appl Environ Microbiol.* 2008; 74: 2171-2178.
- Foldbjerg R, Dang DA, Autrup H. Cytotoxicity and genotoxicity of silver nanoparticles in the human lung cancer cell line, A549. *Arch Toxicol.* 2011; 85: 743-750.
- Kawata K, Osawa M, Okabe S. In vitro toxicity of silver nanoparticles at noncytotoxic doses to HepG2 human hepatoma cells. *Environ Sci Technol.* 2009; 43: 6046-6051.
- Kim S, Choi JE, Choi J, Chung KH, Park K, Yi J, Ryu DY. Oxidative stress-dependent toxicity of silver nanoparticles in human hepatoma cells. *Toxicol In Vitro* 2009; 23: 1076-1084.
- Lundqvist M, Stigler J, Elia G, Lynch I, Cedervall T, Dawson KA. Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts. *Proc Natl Acad Sci USA* 2008; 105: 14265-14270.
- Monopoli MP, Bombelli FB, Dawson KA. Nanobiotechnology: nanoparticle coronas take shape. *Nat Nanotechnol.* 2011; 6: 11-12.
- Monopoli MP, Walczyk D, Campbell D, Elia G, Lynch I, Baldelli BF, Dawson KA. Physical-Chemical Aspects of Protein Corona: Relevance to in Vitro and in Vivo Biological Impacts of Nanoparticles. *J Am Chem Soc.* 2011; 133(8): 2525-2534.
- Walczyk D, Bombelli FB, Monopoli MP, Lynch I, Dawson KA. What the cell “sees” in bionanoscience. *J Am Chem Soc.* 2010; 132: 5761-5768.
- Kwon JT, Hwang SK, Jin H, Kim DS, Minai-Tehrani A, Yoon HJ, Choi M, Yoon TJ, Han DY, Kang YW, Yoon BI, Lee JK, Cho MH. Body distribution of inhaled fluorescent magnetic nanoparticles in the mice. *J Occup Health* 2008; 50: 1-6.
- Asharani PV, Hande MP, Valiyaveetil S. Anti-proliferative activity of silver nanoparticles. *BMC Cell Biol.* 2009; 10: 65.
- Greulich C, Diendorf J, Gessmann J, Simon T, Habijan T, Eggeler G, Schildhauer TA, Epple M, Koller M. Cell type-specific responses of peripheral blood mononuclear cells to silver nanoparticles. *Acta Biomater.* 2011; 7: 3505-3514.
- Marano F, Hussain S, Rodrigues-Lima F, Baeza-Squiban A, Boland S. Nanoparticles: molecular targets and cell signalling. *Arch Toxicol.* 2011; 85(7): 733-741.
- Greulich C, Diendorf J, Simon T, Eggeler G, Epple M, Koller M. Uptake and intracellular distribution of silver nanoparticles in human mesenchymal stem cells. *Acta Biomater.* 2011; 7: 347-354.
- Vanwinkle BA, de Mesy Bentley KL, Malecki JM, Gunter KK, Evans IM, Elder A, Finkelstein JN, Oberdorster G, Gunter TE. Nanoparticle (NP) uptake by type I alveolar epithelial cells and their oxidant stress response. *Nanotoxicology* 2009; 3: 307-318.
- Park EJ, Yi J, Kim Y, Choi K, Park K. Silver nanoparticles induce cytotoxicity by a Trojan-horse type mechanism. *Toxicol In Vitro.* 2010; 24: 872-878.
- Lankoff A, Sandberg WJ, Wegierek-Ciuk A, Lisowska H, Refsnes M, Sartowska B, Schwarze PE, Meczynska-Wielgosz S, Wojewodzka M, Kruszewski M. The effect of agglomeration state of silver and titanium dioxide nanoparticles on cellular response of HepG2, A549 and THP-1 cells. *Toxicol Lett.* 2012; 208: 197-213.

32. Arora S, Jain J, Rajwade JM, Paknikar KM. Cellular responses induced by silver nanoparticles: In vitro studies. *Toxicol Lett.* 2008; 179: 93-100.
33. Hsin YH, Chen CF, Huang S, Shih TS, Lai PS, Chueh PJ. The apoptotic effect of nanosilver is mediated by a ROS- and JNK-dependent mechanism involving the mitochondrial pathway in NIH3T3 cells. *Toxicol Lett.* 2008; 179: 130-139.
34. Gopinath P, Gogoi SK, Sanpui P, Paul A, Chattopadhyay A, Ghosh SS. Signaling gene cascade in silver nanoparticle induced apoptosis. *Colloids Surf B Biointerfaces* 2010; 77: 240-245.
35. Kim TH, Kim M, Park HS, Shin US, Gong MS, Kim HW. Size-dependent cellular toxicity of silver nanoparticles. *J Biomed Mater Res A.* 2012; 100(4): 1033-1043.
36. Rosas-Hernandez H, Jimenez-Badillo S, Martinez-Cuevas PP, Gracia-Espino E, Terrones H, Terrones M, Hussain SM, Ali SF, Gonzalez C. Effects of 45-nm silver nanoparticles on coronary endothelial cells and isolated rat aortic rings. *Toxicol Lett.* 2009; 191: 305-313.
37. van Montfort RL, Congreve M, Tisi D, Carr R, Jhoti H. Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B. *Nature.* 2003; 423(6941): 773-777.
38. Asharani PV, Low Kah MG, Hande MP, Valiyaveetil S. Cytotoxicity and genotoxicity of silver nanoparticles in human cells. *ACS Nano.* 2009; 3: 279-290.
39. Hussain SM, Hess KL, Gearhart JM, Geiss KT, Schlager JJ. In vitro toxicity of nanoparticles in BRL 3A rat liver cells. *Toxicol In Vitro.* 2005; 19(7): 975-983.
40. Kim HR, Kim MJ, Lee SY, Oh SM, Chung KH. Genotoxic effects of silver nanoparticles stimulated by oxidative stress in human normal bronchial epithelial (BEAS-2B) cells. *Mutat Res.* 2011; 726(2): 129-135.
41. Mei N, Zhang Y, Chen Y, Guo X, Ding W, Ali SF, Biris AS, Rice P, Moore MM, Chen T. Silver nanoparticle-induced mutations and oxidative stress in mouse lymphoma cells. *Environ Mol Mutagen.* 2012; 53(6): 409-419.
42. Carlson C, Hussain SM, Schrand AM, Hess KL, Jones RL, Schlager JJ. Unique cellular interaction of silver nanoparticles: size-dependent generation of reactive oxygen species. *J Phys Chem B.* 2008; 112: 13608-13619.
43. Piao MJ, Kang KA, Lee IK, Kim HS, Kim S, Choi JY, Choi J, Hyun JW. Silver nanoparticles induce oxidative cell damage in human liver cells through inhibition of reduced glutathione and induction of mitochondria-involved apoptosis. *Toxicol Lett.* 2011; 201: 92-100.
44. Hudecová A, Kusznierevicz B, Rundén-Pran E, Magdolenová Z, Hašplová K, Rinna A, et al. Silver nanoparticles induce premutagenic DNA oxidation that can be prevented by phytochemicals from *Gentiana asclepiadea*. *Mutagenesis.* 2012; 27: 759-769.
45. Hudecová A, Kusznierevicz B, Hašplová K, Huk A, Magdolenová Z, Miadoková E, Gálová E, Dušínská M. *Gentiana asclepiadea* exerts antioxidant activity and enhances DNA repair of hydrogen peroxide- and silver nanoparticles-induced DNA damage. *Food Chem Toxicol.* 2012; 50: 3352-359.
46. Miura N, Shinohara Y. Cytotoxic effect and apoptosis induction by silver nanoparticles in HeLa cells. *Biochem Biophys Res Commun.* 2009; 390(3): 733-737.
47. Hackenberg S, Scherzed A, Kessler M, Hummel S, Technau A, Froelich K, Ginzkey C, Koehler C, Hagen R, Kleinsasser N. Silver nanoparticles: Evaluation of DNA damage, toxicity and functional impairment in human mesenchymal stem cells. *Toxicol Lett.* 2011; 201: 27-33.
48. Asharani P, Sethu S, Lim HK, Balaji G, Valiyaveetil S, Hande MP. Differential regulation of intracellular factors mediating cell cycle, DNA repair and inflammation following exposure to silver nanoparticles in human cells. *Genome Integr.* 2012; 3: 2.
49. Cho KA, Suh JW, Lee KH, Kang JL, Woo SY. IL-17 and IL-22 enhance skin inflammation by stimulating the secretion of IL-1beta by keratinocytes via the ROS-NLRP3-caspase-1 pathway. *Int Immunol.* 2012; 24(3): 147-158.
50. Hu Y, Mao K, Zeng Y, Chen S, Tao Z, Yang C, Sun S, Wu X, Meng G, Sun B. Tripartite-motif protein 30 negatively regulates NLRP3 inflammasome activation by modulating reactive oxygen species production. *J Immunol.* 2010; 185(12): 7699-7705.
51. Menu P, Mayor A, Zhou R, Tardivel A, Ichijo H, Mori K, Tschopp J. ER stress activates the NLRP3 inflammasome via an UPR-independent pathway. *Cell Death Dis.* 2012; 3: e261.
52. Tschopp J. Mitochondria: Sovereign of inflammation? *Eur J Immunol.* 2011; 41(5): 1196-1202.
53. Christen V, Fent K. Silica nanoparticles and silver-doped silica nanoparticles induce endoplasmic reticulum stress response and alter cytochrome P4501A activity. *Chemosphere* 2012; 87(4): 423-434.
54. Zhang R, Piao MJ, Kim KC, Kim AD, Choi JY, Choi J, Hyun JW. Endoplasmic reticulum stress signaling is involved in silver nanoparticles-induced apoptosis. *Int J Biochem Cell Biol.* 2012; 44(1): 224-232.
55. Collins AR, Oscoz AA, Brunborg G, Gaivao I, Giovannelli L, Kruszewski M, Smith CC, Stetina R. The comet assay: topical issues. *Mutagenesis.* 2008; 23: 143-151.
56. Flower NA, Brabu B, Revathy M, Gopalakrishnan C, Raja SV, Murugan SS, Kumaravel TS. Characterization of synthesized silver nanoparticles and assessment of its genotoxicity potentials using the alkaline comet assay. *Mutat Res.* 2012; 742(1-2): 61-65.
57. Asare N, Instanes C, Sandberg WJ, Refsnes M, Schwarze P, Kruszewski M, Brunborg G. Cytotoxic and genotoxic effects of silver nanoparticles in testicular cells. *Toxicology.* 2012; 291(1-3): 65-72.
58. Wojewódzka M, Lankoff A, Dusinska M, Brunborg G, Czerwińska J, Iwaneńko T, Stępkowski T, Szumiel I, Kruszewski M. Treatment with silver nanoparticles delays repair of X-ray induced DNA damage in HepG2 cells. *Nukleonika.* 2011; 56: 29-33.
59. Rogakou EP, Boon C, Redon C, Bonner WM. Megabase chromatin domains involved in DNA double-strand breaks in vivo. *J Cell Biol.* 1999; 146(5): 905-916.
60. Fenech M. The micronucleus assay determination of chromosomal level DNA damage. *Methods Mol Biol.* 2008; 410: 185-216.
61. Bonassi S, El-Zein R, Bolognesi C, Fenech M. Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. *Mutagenesis.* 2011; 26(1): 93-100.
62. Migliore L, Coppede F, Fenech M, Thomas P. Association of micronucleus frequency with neurodegenerative diseases. *Mutagenesis.* 2011; 26(1): 85-92.
63. Foldbjerg R, Irving ES, Hayashi Y, Sutherland D, Thorsen K, Autrup H, Beer C. Global gene expression profiling of human lung epithelial cells after exposure to nanosilver. *Toxicol Sci.* 2012; 24: 145-57.

