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# Cytotoxicity, oxidative stress, apoptosis and the autophagic effects of silver nanoparticles in mouse embryonic fibroblasts

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ABSTRACT

With the advancement of nanotechnology, nanomaterials have been comprehensively applied in our modern society. However, the hazardous impacts of nanoscale particles on organisms have not yet been thoroughly clarified. Currently, there exist numerous approaches to perform toxicity tests, but common and reasonable bio-indicators for toxicity evaluations are lacking. In this study, we investigated the effects of silver nanoparticles (AgNPs) on NIH 3T3 cells to explore the potential application of these nanoparticles in consumer products. Our results demonstrated that AgNPs were taken up by NIH 3T3 cells and localized within the intracellular endosomal compartments. Exposure to AgNPs is a potential source of oxidative stress, which leads to the induction of reactive oxygen species (ROS), the up-regulation of Heme oxygenase 1 (HO-1) expression, apoptosis and autophagy. Interestingly, AgNPs induced morphological and biochemical markers of autophagy in NIH 3T3 cells and induced autophagosome formation, as evidenced by transmission electron microscopic analysis, the formation of microtubule-associated protein-1 light chain-3 (LC3) puncta and the expression of LC3-II protein. Thus, autophagy activation may be a key player in the cellular response against nano-toxicity.



## 1. Introduction

Nanoparticles (NPs) are an emerging class of functional materials. Application fields for NP range from medical imaging and new drug delivery technologies to various industrial products. Recent advances in particle-forming chemistries used for developing nanotechnology has not only widened applications for nanoscale materials but has also induced significant concern regarding their adverse biological effects [1–3]. With the rapid development of nanotechnology and the widespread use of nano-products, the risk of human exposure rapidly increases, and reliable toxicity test systems are urgently needed [3,4]. Evidence is accumulating that NPs differ significantly from traditional materials and may cause different toxicities [5]. Therefore, understanding the detailed mechanism of cell-specific cytotoxicity of NPs will be helpful for assessing their risk [6,7]. Nevertheless, some of the important and basic characteristics of NPs, including the process of cellular uptake, the mechanism of cytotoxicity, the intracellular location and the translocation of NPs, remain unclear [8]. More studies are needed to focus on the processes of nanoparticle–cell interactions, their intracellular fate and their relationships. Silver nanoparticles (AgNPs) are used in many consumer products. Because of superior their anti-microbial activity, AgNPs are involved in the production of several medical products, including catheters, implants and other materials to prevent infection [9,10]. In addition to their medical uses, AgNPs are used in clothing, the food industry, paints, household products and other fields [11–13]. Although the use of AgNPs is increasingly widespread in medicine and in daily life, comprehensive biologic and toxicological information is lacking [10].

Several different mechanisms contributed to AgNPs toxicity, notably the production of excess reactive oxygen species (ROS). ROS are both physiologically necessary and potentially destructive. ROS may contribute to tissue damage in many pathophysiological

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conditions and participate in several cellular events, including signal transduction, proliferative response, gene expression and protein redox regulation [14,15]. High ROS levels are indicative of oxidative stress and can influence cellular signal transduction pathways, such as proinflammatory signaling pathways, and can modulate the expression of numerous genes [16]. In mammalian systems, the heme oxygenase-1 (*Ho-1*) gene represents one of the most widely studied examples of a redox-regulated gene [17]. ROS can induce HO-1, the rate-limiting enzyme in heme degradation, as well as steal electrons from lipids in the cell membrane, resulting in cellular injuries and cell death. Excessive production of ROS in the cell is known to induce apoptosis [18,19]. ROS generation has been shown to play an important role in apoptosis induced by AgNPs [20–22]. It is now known that different types of cell death (apoptosis, necrosis and autophagy) contribute to the pathophysiology of different human disorders [23]. One of the cell death types that has received much attention in the recent years is autophagy. Autophagy is a process of bulk degradation of toxic protein aggregates and damaged organelles in which portions of the cytoplasm are sequestered into double-membrane vesicles known as autophagosomes, which then fused with lysosomes to form single-membrane autolysosomes; ultimately, the contents of the autolysosomes are degraded by lysosomal hydrolases and recycled for energy utilization [24]. Unlike apoptosis, autophagy acts as either a survival or death safeguard mechanism on different environmental stresses and cell types. Several markers of the autophagic process have been discovered and various strategies have been reported for studying this molecular process in different biological systems in both physiological and stress condition. In this study, we selected AgNPs, which have been commonly used in the industry, as candidate toxicants and looked for the nanoparticle toxicity bioindicators in a mammalian cell line.

## 2. Materials and methods

### 2.1. Preparation and characterization of AgNPs

AgNPs were prepared from the $NaBH_4$ reduction of $AgNO_3$. Briefly, aqueous sodium citrate solution (1.2 ml, 80 mM) and $AgNO_3$ solution (1.5 ml, 20 mM) were mixed with $H_2O$ (26.7 ml). Ice-cold aqueous $NaBH_4$ solution (0.6 ml, 100 mM) was added dropwise under vigorous stirring. The rapid formation of AgNPs was indicated by the color change of the mixture from colorless to yellow. The Ag hydrosol was then aged for 24 h to completely decompose the residual $NaBH_4$. Characterization of the AgNPs was performed using transmission electron microscopy (TEM) (JEOL Co., MA, USA). AgNPs were examined after suspension in M.Q. water or DMEM medium and subsequently deposition onto copper-coated carbon grids. TEM software was calibrated to measure the sizes of the AgNPs. The composition of the AgNPs was determined by electron dispersive X-ray (EDX) analysis. The hydrodynamic sizes, zeta potential and polydispersity index (PDI) of AgNPs examined by *dynamic light scattering (DLS)* (Delsa™ Nano C, Beckman, U.S.A.). The zeta potential of the AgNPs was analyzed in aqueous dispersion using a phase analysis light scattering (PALS) (Delsa™ Nano C, Beckman, U.S.A.).

### 2.2. Cell culture and co-incubation with nanoparticles

The NIH 3T3 mouse embryonic fibroblast *cell* line was obtained from the American Type Culture Collection (ATCC). NIH 3T3 cell lines were cultured in DMEM (Gibco BRL, Grand Island, NY) supplemented with antibiotics containing 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco BRL, Grand Island, NY) and 1% fetal bovine serum (HyClone, South Logan, UT, USA). Cells were incubated in a humidified atmosphere containing 5% $CO_2$ at 37 °C. Exponentially growing cells were detached with 0.05% trypsin-EDTA (Gibco BRL, Grand Island, NY) in DMEM. All of the AgNPs solutions were fresh prepared from stock solutions and sonicated for 5 min before addition to cell cultures.

### 2.3. Electron microscopy

After NIH 3T3 cells were incubated for 24 h with silver nanoparticles (2 or 5 μg/ml), the cells were washed with PBS and then centrifuged at 2000 r/min for 5 min. The supernatants were removed. The cell pellets were fixed with a solution containing 2.5% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3, for 1 h. After fixation, the samples were postfixed in 1% $OsO_4$ in the same buffer for 30 min. Ultrathin sections were then observed under a transmission electron microscope (JEOL JEM-1200EX, Japan) at 100 kV.

### 2.4. Cell morphology by phase-contrast microscopy

Cells were seeded on a 6-well plate at a density of $1.5 \times 10^5$ cells per well in 2 ml of growth medium. After overnight growth, the culture supernatants were aspirated, and fresh growth medium containing AgNPs at the indicated concentration (0–30 μg/ml) was added. After incubation for 24 h, the cells were washed with 0.1 M PBS, pH 7.4, and morphological changes were observed under an inverted phase contrast microscope at 200× magnification.

### 2.5. Cell viability assay

Cellular viability was determined by the MTS assay, which observes the reduction of (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) (MTS) to formazan in viable cells. Briefly, cells were plated onto 96 multiwell plates (Costar, Corning, NY). After incubation with the indicated dose of AgNPs for various lengths of time at 37 °C, formazan absorbance was measured at 490 nm. The mean absorbance of the non-exposed cells was the reference value for calculating 100% cellular viability.

Cytotoxicity was quantified using a Live/Dead viability/cytotoxicity kit (Invitrogen, CA, USA), according to the manufacturer's protocol. Briefly, cells were treated with AgNPs for 24 h and the combined Live/Dead assay reagents were added. The cells were incubated with the assay reagents for 30–45 min at room temperature. The labeled cells were analyzed by a fluorescence microscope (Olympus, Japan) and flow cytometry (BD Biosciences, Germany).

### 2.6. Intracellular reactive oxygen species (ROS) and glutathione (GSH) measurement

ROS production was monitored by flow cytometry using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). This dye is a stable, nonpolar compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield the DCHF, which trapped within the cells. Hydrogen peroxide ($H_2O_2$) or low molecular weight peroxides produced by the cells oxidize DCHF to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Thus, the fluorescence intensity is proportional to the amount of hydrogen peroxide produced by the cells. The thioreactive fluorescent dye 5-chloromethylfluorescein diacetate (CMFDA) was used for GSH determination. CFMDA forms a GSH adduct in a reaction catalyzed by glutathione-*S*-transferase. After conjugation with GSH, CMFDA is hydrolyzed to the fluorescent 5-chloromethylfluorescein by cellular esterase [25]. After treatment with AgNPs for 3 and 6 h, cells were incubated with DCFHDA (20 μM) or CFMDA (30 μM) for a further 30 min. The cells were harvested, washed once and resuspended in PBS. Fluorescence was monitored by flow cytometry. Histograms were analyzed using Winmdi software and were compared with the histograms of untreated control cells.

### 2.7. Detection of apoptosis using Annexin V/PI and DAPI staining

Apoptosis was assessed by observing the translocation of phosphatidyl serine to the cell surface, as detected with an Annexin V apoptosis detection kit (Calbiochem, San Diego, CA) as described previously [26]. Cells were treated with AgNPs in a concentration-and time-dependent manner. After the exposure time, cells were trypsinised, washed with 1 X PBS and centrifuged at 3000 rpm for 5 min. Cells were resuspended in 100 μl of 1 X Annexin V-binding buffer (10 mM HEPES (pH 7.4), 0.14 M NaCl and 2.5 mM $CaCl_2$) that contained 5 μl of Annexin V-FITC (Becton Dickinson, San Jose, CA, USA) alone or in combination with 10 μl of PI (50 μg/ml) and were incubated at room temperature for 15 min. The 1× binding buffer (400 μl) was added to stop the reaction, and the stained cells were collected for flow cytometry analyses. For the observation of nuclear morphology, cells treated under the indicated conditions were fixed in methanol, incubated with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co.), and analyzed using a fluorescence microscope.

### 2.8. Detection and quantification of acidic vesicular organelles with acridine orange staining

Cell staining with acridine orange (Sigma Chemical Co.) was performed according to the published procedures [27,28], adding a final concentration of 1 μg/ml for a period of 20 min. Photographs were obtained with a fluorescence microscope (Axioscop) equipped with a mercury 100-W lamp, 490-nm band-pass blue excitation filters, a 500-nm dichroic mirror, and a 515-nm long-pass barrier filter. Flow cytometric analysis was used to detect acidic vesicular organelles (AVOs), which are a characteristic of autophagy [27].

### 2.9. Immunofluorescence microscopy for LC3

After NIH 3T3 cells were incubated for 18 h with silver nanoparticles (10 μg/ml), the cells were fixed in 4% paraformaldehyde and blocked with 1% BSA for 30 min. Fixed cells were incubated with primary antibodies specific for LC3 (MBL, Japan) for 1 h. After washing, cells were labeled with a DyLight™ 488-conjugated affinipure goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, PA, USA) for 1 h. After washing again, the cells were counterstained with DAPI and visualized with a confocal microscope (Carl Zeiss LSM780, Instrument Development Center, NCKU).