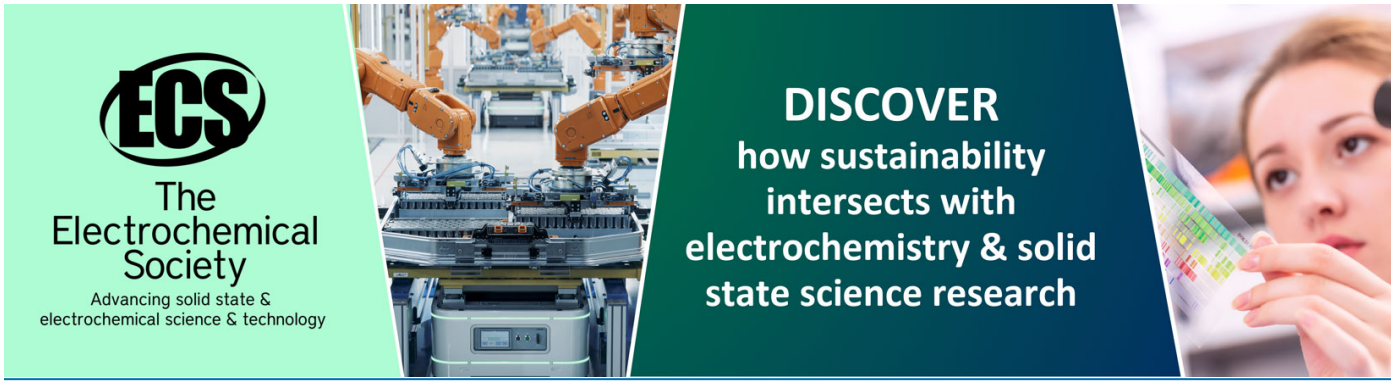
Toxicity of nano- and micro-sized silver particles in human hepatocyte cell line L02



Toxicity of nano- and micro-sized silver particles in human hepatocyte cell line L02

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Abstract. Silver nanoparticles (Ag NPs) previously classified as antimicrobial agents have been widely used in consumers and industrial products, especially food storage material. Ag NPs used as antimicrobial agents may be found in liver. Thus, examination of the ability of Ag NPs to penetrate the liver is warranted. The aim of the study was to determine the optimal viability assay for using with Ag NPs in order to assess their toxicity to liver cells. For toxicity evaluations, cellular morphology, mitochondrial function (3-(4, 5-dimethylazol-2-yl)-2, 5-diphenyl-tetrazolium bromide, MTT assay), membrane leakage of lactate dehydrogenase (lactate dehydrogenase, LDH release assay), Oxidative stress markers (malonaldehyde (MDA), glutathione (GSH) and superoxide dismutase (SOD)), DNA damage (single cell gel eletrophoresis, SCGE assay), and protein damage were assessed under control and exposed conditions (24 h of exposure).The results showed that mitochondrial function decreased significantly in cells exposed to Ag NPs at 25 $\mu g \cdot mL^{-1}$. LDH leakage significantly increased in cells exposed to Ag NPs (≥ 25 μg mL^{-1}) while micro-sized silver particles tested displayed LDH leakage only at higher doses (100 $\mu g \cdot mL^{-1}$). The microscopic studies demonstrated that nanoparticle-exposed cells at higher doses became abnormal in size, displaying cellular shrinkage, and an acquisition of an irregular shape. Due to toxicity of silver, further study conducted with reference to its oxidative stress. The results exhibited significant depletion of GSH level, increase in SOD levels and lead to lipid peroxidation, which suggested that cytotoxicity of Ag NPs in liver cells might be mediated through oxidative stress.

The results demonstrates that Ag NPs lead to cellular morphological modifications, LDH leakage, mitochondrial dysfunction, and cause increased generation of ROS, depletion of GSH, lipid peroxidation, oxidative DNA damage and protein damage. Though the exact mechanism behind Ag NPs toxicity is suggested oxidative stress and lipid peroxidation playing an important role in Ag NPs elicited cell membrane disruption, DNA damage, protein damage and subsequent cell death. Our preliminary data suggest that oxidative stress might contribute to Ag NPs cytotoxicity. To reveal whether apoptosis involved in Ag NPs toxicity, further studies are underway.

1. Introduction

Ag nanoparticles (Ag NPs) were previously classified as antimicrobial agents, and have been widely used in consumers and industrial products, especially food storage material [1,2]. However, the cytotoxicity of Ag NPs has caused wide concerns by scientists and engineers in the last decades.

In vitro cell line studies have shown decreased mitochondrial function after exposure to Ag NPs in murine neuroblastoma cells [3], rat liver cells [4], germline stem cells [5], human skin carcinoma cells [6], human hepatoma cells [7], human alveolar epithelial and macrophage cell lines [8], human epidermal keratinocytes (HEKs) and fibroblasts [9], human lung fibroblast cells (IMR-90) and human glioblastoma cells (U251) [10]. Ingestion of Ag can cause agyria, the benign condition characterized by the bluish-graying of the skin that occurs through the preferential deposition of Ag in the basal lamina of soft tissues such as the liver, and spleen [11]and blood vessels, gastrointestinal tract, liver, and kidney [12]. Thus, examination of the ability of Ag NPs to penetrate the liver is warranted.

Our objectives in this study were to determine the optimal viability assay for using with Ag NPs in order to assess their toxicity to liver cells. In this paper, we evaluated the toxicity of Ag NPs and analyzed cellular viability, oxidative stress, and DNA damage in AgNPs-treated cells.

2. Materials and methods

2.1. Materials

The nano- and micro-sized silver particles used in this study were purchased from Qinhuangdao Taiji Ring Nano-Products Co., Ltd, Republic of China. The nano- and micro-sized silver particles were dissolved in Milli-Q water at a final concentration of 10 $mg \cdot mL^{-1}$.

2.2. Synthesis of silver particles

The nano- and micro-sized silver particles were characterized by Scanning Electron Microscope (SEM) imaging (JEOL JSM-5610LV).

2.3. Cell culture and treatment

L02 cells were cultured in RPMI 1640 medium with fetal calf serum (10 %), L-glutamine (2.9 $mg \cdot mL^{-1}$), streptomycin (1 $mg \cdot mL^{-1}$), and penicillin (100 $units \cdot mL^{-1}$). Cells were cultured at 37°C in water-saturated air supplemented with 5% CO_2. Culture media were changed every 3 days. Cells were passaged once a week.

After the monolayer of cells became conXuent in 6, 24 or 96-well plate, L02 cells were treated with a range of concentrations of nano- and micro-sized silver particles suspended in RPMI 1640 medium without serum for 24 h. After the 24 h treatment, the various toxicity end points were evaluated in control and silver particles exposed cells.

2.4. Cell morphology

L02 cells were exposed as mentioned above at various concentrations of nano- and micro-sized silver particles for 24 h. After completion of the exposure period, cells (control, nano-Ag and micro-Ag exposed) were washed with PBS and observed by phase contrast inverted microscopy at 200 × magnification.

2.5. Cell activity

LDH leakage due to membrane damage was assessed by measuring the activity of LDH in the cells and medias described elsewhere with some modifications [13]. Mitochondrial function was evaluated spectrophotometrically by 3-(4, 5-dimethylazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay. The MTT assay helps in cell-viability assessment by measuring the enzymatic reduction of yellow tetrazolium MTT to a purple formazan, as measured at 570 nm using Enzyme-labeled instrument (Tecan Co.).

2.6. DNA damage

DNA damage by Ag NPs was further studied using comet assay. After treatment with nano- and micro-sized silver particles for 24 h, the cells were rinsed with ice-cold 1×PBS and trypsinized. Then the cells were washed once in ice-cold 1×PBS and resuspended at 1×10^5 cells mL^{-1} in ice-cold 1×PBS. An aliquot of 10 µL cell suspension was mixed with 100 µL molten agarose (37°C), and 75µL of this mixture was immediately applied to a glass slide. The slide was held horizontal at 4 °C for 30 min to improve adherence. Then the slide was immersed in cold lysis solution to lyse the cells. After 50 min at 4 °C in the dark, the slide was immersed in an alkaline solution (300 mM NaOH, 1 mM EDTA, pH>13) at room temperature in the dark to denature the DNA. After 30 min, the slide was placed on a horizontal electrophoresis unit and the unit was filled with fresh buffer (300 mM NaOH, 1 mM EDTA, pH>13) to cover the slide. Electrophoresis was conducted at 27 V (300 mA) for 40 min at 4°C in the dark. The slide was then washed gently with distilled water and immersed in 70% ethanol for 5 min. After the slide was air dried, 50µL of Ethidium bromide (EB) working solution was applied to each circle of dried agarose. All steps described above were conducted under yellow light to prevent additional DNA damage.

Slides were viewed using an epifluorescence Leica DMI 4000B microscope equipped with a fluorescein filter. Observations were made at a final magnification of 400×. Fifty randomly selected cells per experimental point were imaged and analyzed using CASP software (download from http://www.casp.of.pl/). Results were reported as tail moment, a parameter describing the number of migrated fragments, represented by the fluorescence intensity in the tail, expressed as the mean of the 50 cells.

2.7. Oxidative stress

Cells were cultured in 75-cm^2 culture flask and exposed to Ag NPs (5-100µg·mL^{-1}) for 24 h. After exposure, the cells were harvested in chilled PBS by scraping and washed twice with 1× PBS at 4°C for 6 min at 1500 rpm. The cell pellet was then sonicated at 15 W for 10 s (3 cycles) to obtain the cell lysate.

Oxidative stress markers (MDA, GSH and SOD) were estimated by Nanjingjiancheng Kit (China) according to manufacturer's protocol. Protein content was measured by the method of Lowry [14] using BSA as the standard.

2.8. Statistical analysis

The data were expressed as mean ± standard deviation of three independent experiments. Wherever appropriate, the data were subjected to statistical analysis by one-way analysis of variance (ANOVA) followed by Dunnett's method for multiple comparisons. A value of $p<0.05$ was considered significant. SPSS 16.0 software was used for the statistical analysis.

3. Results

3.1. Characterization studies

The average size of nano-Ag and micro-Ag reported by SEM was 37.8±6.7 nm and 985.4±283.5 nm. Figure **1** shows a representative SEM image recorded nano-Ag and micro-Ag.

3.2. Cell morphology

Figure **2** shows the comparative morphologies of unexposed, and nano- and micro-sized silver particles exposed cells. At the low dose of nano-Ag (10 µg·mL^{-1}), the cells appear similar to control cells with brownish particles most likely associated with the cell membranes. With increasing doses of nano-Ag, the cells started to shrink and became irregular in shape (Figure **2**.*e*). The microscopic studies demonstrated that nano-Ag exposed cells at higher doses became abnormal in size, displaying cellular shrinkage, and an acquisition of an irregular shape.

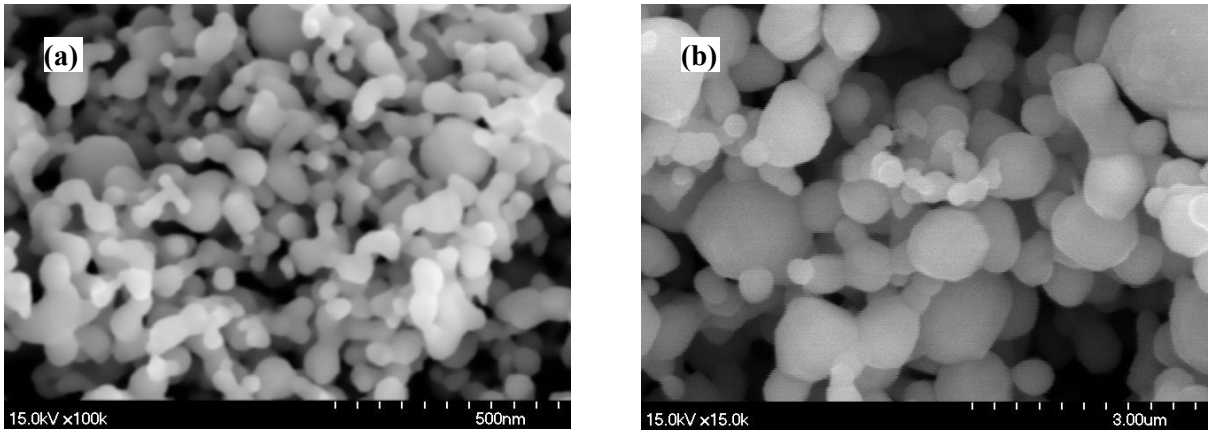

Figure 1. SEM images of nano-Ag and micro-Ag
a: nano-Ag, 37.8±6.7 nm; b: micro-Ag, 985.4±283.5 nm.

3.3. Cell activity

L02 cells were exposed to nano- and micro-sized silver particles (5-100 $\mu g \cdot mL^{-1}$) for 24h and cytotoxicity was determined with MTT assay and LDH release assay.

The MTT assay results demonstrated a concentration dependent cytotoxicity after exposure to Ag NPs (Figure **3**). The percentage (%) MTT reduction (relative to control) observed after nano-Ag exposure at concentrations 25, 50 and 100 $\mu g \cdot mL^{-1}$ was 49.35%, 26.61% and 14.44% respectively with reduction to 83.61%, 68.64% and 58.82% after micro-Ag exposure. It was noted that there is a statistically significant difference between different sizes of silver particle, where the nano-Ag showed higher toxicity at 25-100 $\mu g \cdot mL^{-1}$ than the micro-Ag($p<0.05$).

The LDH release assay results demonstrated that exposure to Ag nanoparticles for 24h resulted in concentration-dependent increase in LDH leakage and exhibited a significant ($p<0.05$) cytotoxicity at 25-100 $\mu g \cdot mL^{-1}$ (Figure 4). This is only ture for nano-Ag.

3.4. DNA damage

DNA damage by Ag NPs was further studied using comet assay. Chromosome abnormalities are a direct consequence of DNA damage such as double-strand breaks and misrepair of strand breaks in DNA, resulting in chromosome rearrangement.

Extensive and dose-dependent damage to DNA was observed after treatment of the cells with Ag NPs. Comet assay of Ag-NPs treated cells showed a concentration-dependent increase in tail moments (Figure **5**.*c*) as compared to control cells (Figure **5**.*a*), which gave the extent of DNA damage (Figure **5**.*c*). A comet-like tail implies presence of a damaged DNA strand that short DNA fragments are migrating faster than the intact nucleus. The length of the tail increases with the extent of DNA damage. Tail moments of control DNA was compared with nano-Ag treated cells, and extent of damage was assessed. An increase in DNA damage with increase in nano-Ag concentration was observed, whereas the cell showed no further increase in DNA damage beyond a micro-Ag concentration of 100 $\mu g \cdot mL^{-1}$ (Figure **5**.*d*).

3.5. Oxidative stress markers

3.5.1. *Effect of Ag NPs on MDA*. Lipid peroxidation was examined by measuring Malondialdehyde (MDA) concentration. A significant increase ($p<0.05$) in MDA formation was observed at all concentrations above 10 $\mu g \cdot mL^{-1}$ of Ag NPs as evident from Table 1.

3.5.2. *Effect of Ag NPs on GSH level.* Cells exposed to Ag NPs showed depletion of GSH level in a dose dependent manner, exposure concentrations exhibiting statistically significant (p<0.05) depletion of 57.51% and 26.53% at 50 and 100 µg·mL^{-1} respectively after 24 h (Table 1).

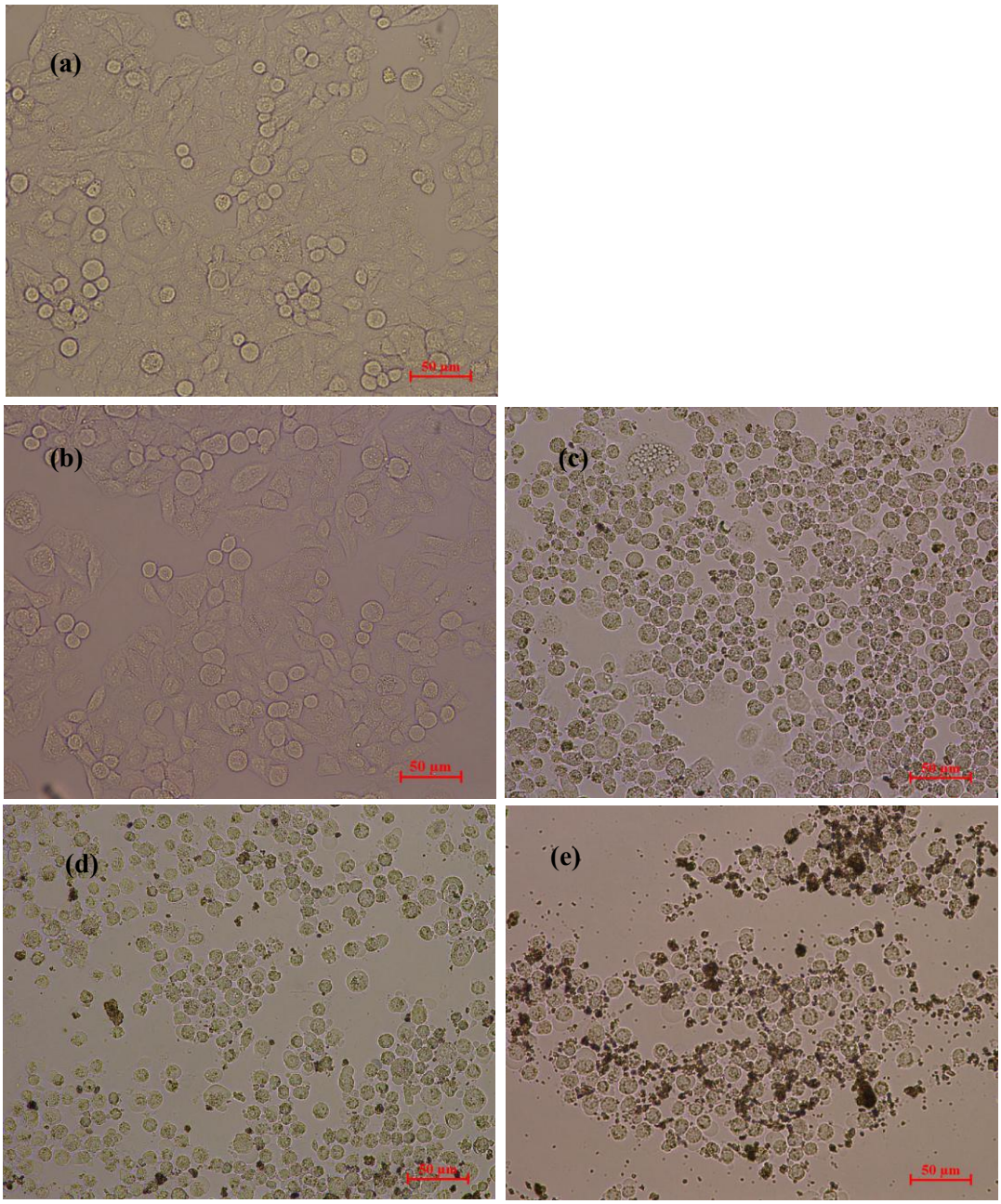

Figure 2. Morphology of human liver cells (L02)
(a) normal, and micro-Ag treated (b) 10 µg·mL^{-1} for 24 h, (c) 100 µg·mL^{-1} for 24 h, and nano-Ag treated (d) 10 µg·mL^{-1} for 24 h, (e) 100 µg·mL^{-1} for 24 h.

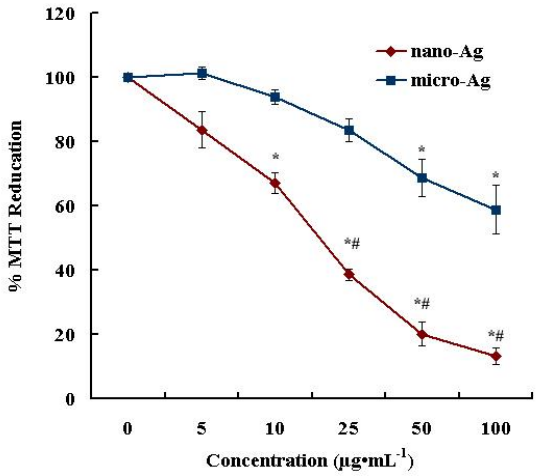

Figure 3. Cytotoxicity of Ag NPs on human liver cells (L02). MTT assay

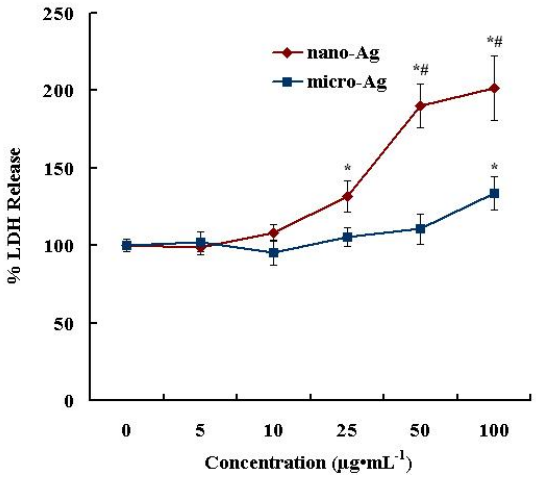

Figure 4. Cytotoxicity of Ag NPs on human liver cells (L02).LDH release assay

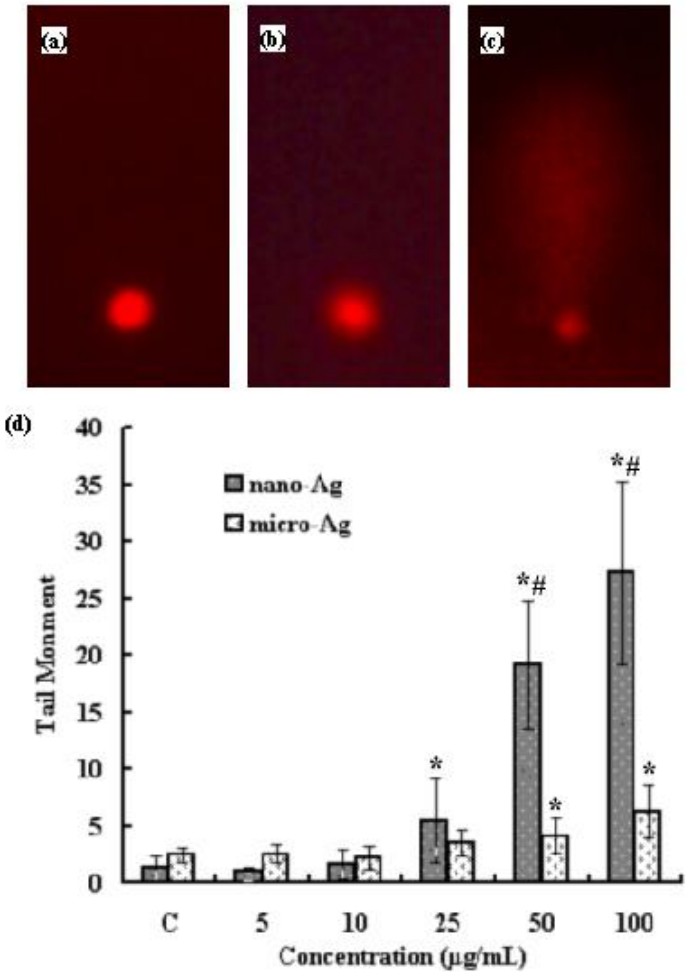

Figure 5. Comet analysis:
(a) control
(b) micro-Ag treated
(c) nano-Ag treated
(d) the Tail Moments of DNA

* $p<0.05$, compared with the control group.
# $p<0.05$, compared with micro-Ag group.

3.5.3. *Effect of Ag NPs on SOD activity.* SOD activity in cells treated with 25-100μg·mL^{-1} Ag NPs was significantly (p<0.05) reduced after 24 h of exposure when compared to unexposed cells as evident from Table 1.

Table 1. Effect of Ag NPs on Oxidative stress.

Conc.(μg·mL^{-1})	MDA(nmol·mgprot^{-1})	GSH(mg·gprot^{-1})	SOD(U·mgprot^{-1})
Control	0.188±0.017	4.26±0.62	31.56±2.56
5	0.195±0.037	4.31±0.26	30.62±1.26
10	0.248±0.022*	3.49±0.36	28.53±2.45
25	0.267±0.029**	3.00±0.23*	25.12±1.02*
50	0.312±0.036**	2.45±0.34**	22.83±0.95**
100	0.404±0.043**	1.13±0.25**	18.23±1.34**

* p<0.05, compared with control group.
** p<0.01, compared with control group.

4. Discussion

The field of nanotechnology has rapidly been growing worldwide for the past few years showing tremendous applicability in a variety of different sectors for the betterment of human life. However, a major and simultaneous outcome of these increasing nano-based applications is that humans now are at a greater risk of exposure to nanomaterials which can enter the biological systems through different routes [15,16].

Ag nanoparticles (Ag NPs) were previously classified as antimicrobial agents, and have been widely used in consumer and industrial products, especially food storage material. However, the cytotoxicity of Ag NPs has caused wide concerns by scientists and engineers in the last decades. Therefore in this study, cellular responses on exposure of Ag NPs to human liver cells (L02) were investigated. Our data demonstrate that exposure of Ag NPs to human liver cells causes cytotoxicity and oxidative stress. We have also observed DNA damaging effects of Ag NPs on L02 cells for which lipid peroxidation and oxidative stress may be attributed as one of the probable cause.

The cytotoxicity of Ag NPs was evident by morphological changes that appeared in L02 cells. Loss of normal morphology started appearing even at 10 μg·mL^{-1} after 24 h of exposure. With a consequent increase in exposure time, cells retracted into spherical shape and formed clusters in media after detachment from surface. A high tendency of Ag NPs adhering to cell membrane was observed at higher magnification. There is an earlier report that Ag NPs exposed human hepatoma cells reflect abnormal morphology, cellular shrinkage, detachment from the surface of the flask as well as decreased mitochondrial function and significantly increased LDH release at concentrations 0.7-2μg·mL^{-1} after 24 h exposure [7].

The production of free radicals has been found in a diverse range of nanomaterials which is one of the primary mechanisms of NPs toxicity [15]. It may result in oxidative stress, inflammation and consequent damage to proteins, membranes and DNA [17]. Thus in our study we investigated the levels of GSH and other antioxidant marker enzymes in the cells exposed to Ag NPs. A depletion in the GSH and SOD level were found at 25 μg·mL^{-1} on 24 h exposure. This indicates a condition of oxidative stress in cells which may arise due to imbalance in the ROS formation and antioxidant defence system of cells. As formation of ROS by Ag NPs is still unclear, the mechanism of ROS formation by Ag NPs needs further investigations. We also studied lipid peroxidation which represents another marker of oxidative stress. An increase in the formation of hydroperoxide compared to control was observed indicating lipid peroxidation on exposure to Ag NPs. This increase in lipid peroxidation may lead to membrane damage indicated by enhanced LDH released on Ag NPs exposure.

Our results also demonstrate that Ag NPs cause statistically significant DNA damage at concentrations of 25 $\mu g \cdot mL^{-1}$ after an exposure period of 24 h. This is supported by a previous study conducted by Ahamed *et al* [6] who have shown that Ag NPs caused different levels of DNA damage in mouse embryonic stem (mES) and mouse embryo fibroblast (MEF) cells, induced p53 protein expression, DNA double strand breakage and apoptosis.

As there is a well documented link between NPs and oxidative stress, one of the possible modes that can be suggested for Ag NPs induced DNA damage may be lipid peroxidation and oxidative stress [18]. ROS are known to react with DNA molecule causing damage to both purine and pyrimidine bases as well as DNA backbone [19]. Another important outcome of ROS production is lipid peroxidation which generates a variety of products reactive towards cellular macro molecules including DNA. One of the major products of lipid peroxidation malondialdehyde, is a proven mutagen and carcinogenic compound which reacts with DNA to form adducts to deoxyguanosine, deoxyadenosine and deoxycytidine [20].

This Ag NPs induced genotoxicity, if in actuality mediated by oxidative stress can be further linked to different pathways [21,22]. Principle modes that can be considered for ROS generation by Ag NPs are—intrinsic ROS generation from particles or their adsorbed species and ROS generation in target cells on exposure to Ag NPs. DNA damage resulting from any of these probable mechanisms may trigger signal transduction pathways leading to apoptosis or cause interferences with normal cellular processes thereby causing cell death [23]. A schematic diagram has been depicted in Figure 6 for the plausible mechanisms of Ag NPs induced cellular toxicity. However, further studies are needed to unravel the exact mechanism behind Ag NPs induced genotoxicity.

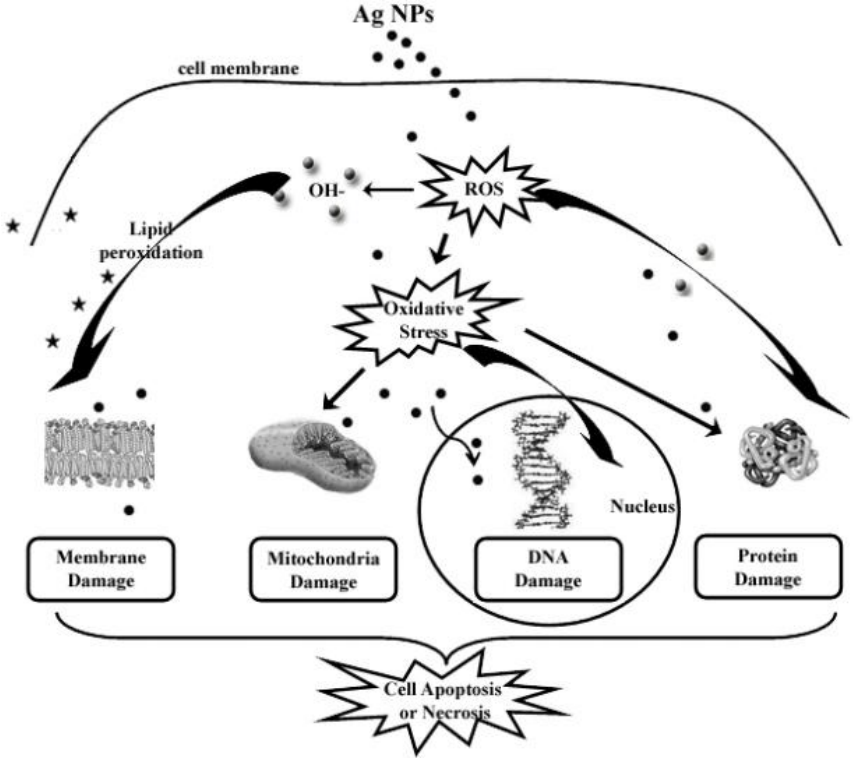

Figure 6. A schematic representation of results from this study representing the Ag NPs induced cytotoxicity.

Acknowledgement

The project supported by National High Technology Research and Development Program of China (2007AA100403), Food safety Supervision Program of General Administration of Quality Supervision,

Inspection and Quarantine of the People's Republic of China (AQSIQ2009SP26), Science and Technology Development Program of Hangzhou (20091832B50) and Science and Technology Development Program of Hangzhou (20101032B18).

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