Docosahexaenoic Acid and L-Carnitine Prevent ATP Loss in SH-SY5Y Neuroblastoma Cells After Exposure to Silver Nanoparticles

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ABSTRACT: Silver nanoparticles (AgNPs) are among the most commonly used nanomaterials, but thus far, little is known about ways to mitigate against potential toxic effects of exposure. In this study, we examined the potential effects of AgNPs on mitochondrial function and cellular ATP levels, and whether these could be prevented by treatment with docosahexaenoic acid (DHA) and L-carnitine (LC). Acute exposure of AgNPs for 1 h to SH-SY5Y cells resulted in decreased mitochondrial membrane potential, and decreased ATP and ADP levels, indicating mitochondrial damage and reduced production of ATP. Incubation of cells with DHA partially reduced, while treatment with LC and DHA completely abolished the AgNP induced decreases in ATP and ADP levels. This could be due to a LC-facilitated entry of DHA to mitochondria, for repair of damaged phospholipids. It is postulated that DHA and LC may be useful for treatment of accidental environmental exposure to AgNPs. © 2014 Wiley Periodicals, Inc. Environ Toxicol 31: 224–232, 2016.

Keywords: engineered nanomaterials; nanoparticles; health effects; nanotoxicology; mitochondria; silver nanoparticles; docosahexaenoic acid; DHA; carnitine

INTRODUCTION

The rapid development of nanotechnology recently likely results in increased exposure of workers to engineered nanomaterials. Silver nanoparticles (AgNPs) are among the most commonly used nanoparticles today (Woodrow Wilson International Center for Scholars, 2013), being incorporated as antibacterial agents in medical products, textiles, washing machines and clothing (Haase et al., 2012). The attractive characteristics of NPs could nonetheless be potentially harmful, when exposed to biological systems (Teodoro et al., 2011). AgNPs can cross the blood brain barrier (BBB), or enter the brain via the nose-to-brain route by travelling along the olfactory nerve (Haase et al., 2012).

NPs can induce toxicity to mitochondria resulting in interference to the electron transport chain and mitochondrial permeability transition pore (Costa et al., 2010). In vitro studies show that exposure to AgNPs results in decreased mitochondrial functions, decreased ATP production and increased reactive oxygen species (ROS) levels, resulting in DNA damage, apoptosis and necrosis (AshaRaniet al., 2009; Costa et al., 2010; Hwang et al., 2012; Kaur and Tikoo, 2013). Treatment of cells with AgNPs results in decreased mitochondrial membrane

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potential, ADP-induced depolarization and changes in respiration rate in response to ADP (Piao et al., 2011; Teodoro et al., 2011).

In contrast to AgNPs, there are mixed views on the potential toxicity of gold nanoparticles (AuNPs). The latter induce oxidative damage and cytotoxic effects in human lung fibroblasts, and inhibits cell proliferation via downregulation of cell cycle genes (Li et al., 2008), but are found to be less toxic than AgNPs in a zebrafish embryo model (Bar-Ilan et al., 2009), or even nontoxic, in some cell lines (Connor et al., 2005; Pan et al., 2007).

Docosahexaenoic acid (DHA) is the most common unsaturated fatty acid in nature and is incorporated in cell and mitochondrial membrane phospholipids (Ng et al., 2005). Being a polyunsaturated fatty acid, it is a target for lipid peroxidation (Stillwell et al., 1997) which can lead to damage to cellular membranes, loss of mitochondrial electrochemical gradient, release of mitochondrial Ca^{2+}, mitochondrial swelling, impaired ATP production, and cell death (Ng et al., 2005). Lipid peroxidation of DHA-containing mitochondrial membrane also produces toxic compounds such as α- and β-unsaturated aldehydes, which can interact with DNA resulting in mutagenic and genotoxic effects (Ng et al., 2005). DHA supplementation and incorporation into neural cellular membranes increases resistance against excitotoxic damage and has anti-apoptotic effect on HL-60 cells and Neuro-2A cells (Horrocks and Farooqui, 2004).

Fatty acids are transported into mitochondria through L-carnitine (LC), which is found in all mammals and occurs in the body primarily in its unesterified form (Walter and Schaffhauser, 2000). The main function of LC is to aid in transfer of fatty acids from the cytosol to the mitochondrial matrix, where they are metabolized for energy production. In addition, it modulates lipid peroxidation in aging rats (Alves et al., 2009) and inhibits mitochondrial-dependent apoptosis by regulating the concentrations of acyl-CoA and free CoA via enhanced β-oxidation (Sharma and Black, 2009). LC is reported to exert antioxidative and neuroprotective actions, under conditions of oxidative stress and mitochondrial dysfunction (Virmani and Binienda, 2004; Alves et al., 2009; He et al., 2011).

In this study, we examined the effects of AgNPs on mitochondrial function and ATP production, and determined whether any toxic effects of AgNPs could be prevented by supplementation with DHA and LC.

Abbreviations

$AgNO_3$	silver nitrate
AgNPs	silver nanoparticles
AK	adenylate kinase
AuNPs	gold nanoparticles
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
DMSO	dimethyl sulfoxide
DHA	docosahexaenoic acid
DMEM	Dulbecco's Modified Eagle Medium
EBSS	Earl's balanced salt solution
IMP	inosine monophosphate
JC-1 dye	5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide
LC	L-carnitine
NPs	Nanoparticles
PBS	phosphate buffered saline
TEM	transmission electron microscopy

MATERIALS AND METHODS

Cell Culture

SH-SY5Y, an immortalized human neuroblastoma cell line, was obtained from ATCC (CRL-2266). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco®, Life Technologies, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco®, Life Technologies), and 1% penicillin/streptomycin (Pen Strep) (Gibco®, Life Technologies). The fully supplemented medium is denoted as complete growth medium. SH-SY5Y cells were maintained in 100 mm dishes, and incubated under standard conditions of 37°C and 5% CO_2. Cells were regularly passaged using 25% Trypsin-EDTA (Gibco®, Life Technologies).

Synthesis of AgNPs

75 mL of reagent-grade water, and 5 mL of 2 mg/mL silver nitrate ($AgNO_3$) solution (Merck Millipore, Darmstadt, Germany) was heated in a 65°C silicone oil bath, and stirred at 1,000 rpm. A solution containing 4 mL of 10 mg/mL sodium citrate solution (Sigma-Aldrich, St. Louis, USA), 3.1 mL of 1 mg/mL tannic acid solution (Sigma-Aldrich) and 12.9 mL of reagent-grade water was heated for 20 min, and added to the silver nitrate solution. The temperature was adjusted to 100°C, and was further heated for another 20 min.

Purification of AgNPs

8 mL of synthesized AgNPs was centrifuged at 5,000 *g* for 30 min at 4°C. The supernatant (S1) was centrifuged with the same parameters to recover remaining AgNPs. 8 mL of reagent-grade water was used to resuspend the pellet (P1). After centrifugation, the supernatant (S2) was decanted, and the pellet (P2) was added to the remaining AgNPs suspension. The process was repeated, and final pellets (P3 and P4) were resuspended with 8 mL of complete growth medium.

Synthesis of AuNPs

AuNPs were synthesized and diluted to an equivalent number concentration as AgNPs in suspension for comparison. The protocol for synthesis of gold nanoparticles was

previously described in Balasubramanian et al. (2010). 95 mL of tetrachloroauric acid, containing 5 mg of Au (Sigma-Aldrich) was heated in a 100°C silicone oil bath, and stirred at 1,000 rpm for 20 min. 50 mg of trisodium citrate dihydrate (5 mL of 1% solution in water) (Sigma-Aldrich) was added and heated for another 20 min.

Purification of AuNPs

540 μL of synthesized AuNPs was centrifuged at 7,000 *g* for 20 min at 4°C. The supernatant (S1) was removed and centrifuged with the same parameters to recover remaining AuNPs. 540 μL of reagent-grade water was used to resuspend the pellet (P1). After centrifugation, the supernatant (S2) was decanted, and the pellet (P2) was added to the remaining AuNPs suspension. The process was repeated. Final pellets (P3 and P4) were resuspended with 540 μL of complete growth medium.

Characterization of AgNPs and AuNPs

Hydrodynamic size, concentration, and polydispersity of NPs were established by dynamic light scattering measurements using the Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Transmission electron microscopy (TEM) was used to analyze the size, general morphology and possible aggregation of NPs. Micrographs were taken using a Phillips CM120 BioTwin transmission electron microscope (FEI Philips, Oregon, USA).

Trypan Blue Exclusion Cell Viability Assay

SH-SY5Y cells were plated in a six-well plate at 0.5×10^6 cells/well, and allowed to attach overnight. At 70% confluence, cells were exposed to 78.7 μg/mL $AgNO_3$, 50 μg/mL AgNPs, and 91.3 μg/mL AuNPs for one hour. The concentration of AgNPs chosen, 50 μg/mL, is based on previous studies in AgNPs in cell culture, where effects on mitochondrial functions and cellular morphology were observed (Hussain, et al. 2005; Arora et al., 2008; Hsin et al., 2008).

The concentrations of $AgNO_3$ and AuNPs were selected to match the number of Ag and Au particles in the respective solutions to Ag particles in AgNPs suspension, at approximately 2.79×10^{17} particles/mL. Since AgNPs have been reported to release silver ions over time (AshaRani et al., 2009; Liu and Hurt, 2010), addition of $AgNO_3$ solution was used as a control for AgNPs, to try to distinguish between the effects of silver nanoparticulates, versus silver ions in solution. Based on number of silver nanoparticles in suspension, the total number of silver atoms was calculated, and converted to concentration of silver nitrate solution that needs to be added, in the event that all atoms went into solution as silver ions. We have also checked and found that very little AgNPs went into solution within 1 hour, in a parallel study (unpublished data).

Complete growth medium, supplemented with 1% water was used as vehicle controls. Cells were exposed to trypan blue for five minutes, and percentage of viable cells determined. Mean values were normalized to vehicle controls, and plotted on a bar chart. Standard errors were calculated, and comparison of values between groups carried out, using one-way ANOVA with Bonferroni's multiple comparison post-hoc test to determine the significance of possible differences in cell viability among various treatment groups. $p < 0.05$ was considered significant.

Mitochondrial Membrane Potential Assay

SH-SY5Y cells were plated in a 24-well plate at 1×10^5 cells/well, and allowed to attach overnight. At 70% confluence, cells were treated with complete growth medium containing 50 μg/mL AgNPs for one hour. Complete growth medium was used as controls. Following the treatment, cells were washed with phosphate buffered saline (PBS, pH 7.4), and incubated with 0.5 μM 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1 dye) (Molecular Probes, Invitrogen, CA, USA) at 37°C for 20 min in Earl's balanced salt solution (EBSS) (Sigma-Aldrich). Cells were washed with PBS, and EBSS containing 1% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) was added to the various wells, with the exception of positive controls, which were provided with 5 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Santa Cruz Biotechnology, CA, USA). The 24-well plate was placed in the Tecan Infinite® 200 microplate reader (Tecan Group, Männedorf, Switzerland) using an excitation wavelength of 488 nm, and emission wavelength of 535 nm to produce green fluorescence, and 590 nm to produce red fluorescence. Cells were read at 2-min intervals for 30 min. Mean values were normalized to negative controls, and normalized red/green fluorescence (590/535) ratios plotted on a line graph, as an expression of mitochondrial membrane potential. Comparison of values between groups was carried out using two-tail unpaired Student's *t* test to determine possible differences in 590/535 ratios between negative controls and various treatment groups. $p < 0.05$ was considered significant.

ADP/ATP Ratio Assay

SH-SY5Y cells were plated in a 96-well plate at 1.0×10^4 cells/well, and allowed to attach overnight. At 70% confluence, cells were treated with complete growth medium containing 50 μg/mL AgNPs, 91.3 μg/mL AuNPs, 10 μg/mL DHA (Cayman Chemical, Michigan, USA), and 100 μg/mL LC (Sigma-Aldrich) for one hour. Complete growth medium, supplemented with 1% ethanol was used as vehicle controls. The assay was carried out according to the manufacturer's instructions (Abcam, Cambridge, UK). Luminescence readings were taken at stipulated time points, and intracellular ADP/ATP ratios were determined by the following equation.

$$\frac{\text{Data C} - \text{Data B}}{\text{Data A}}$$

Mean values were normalized to vehicle controls, and plotted on a bar chart. Standard errors of different groups were calculated, and possible differences in ADP/ATP ratio, ATP, and ADP levels among different treatment groups were analyzed using one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. $p < 0.05$ was considered significant.

RESULTS

Characterization of AgNPs and AuNPs

AgNPs and AuNPs were observed to be roughly spherical in shape, and approximately 20–26 nm [Fig. 1(A)] in diameter. Concentrations of AgNPs and AuNPs suspensions were 50 μg/mL and 91.3 μg/mL, respectively (data not shown). Number of particles in both suspensions was approximately equal, at 2.79×10^{17} particles/mL, demonstrating that the experiments were controlled based on number of particles, which is a better design.

Trypan Blue Exclusion Cell Viability Assay

One-hour incubation of SH-SY5Y cells with 78.7 μg/mL $AgNO_3$ caused rapid cell death, resulting in a cell viability of 0.99% [Fig. 1(B)]. Significant differences were observed between vehicle controls, 78.7 μg/mL $AgNO_3$-, 50 μg/mL AgNPs-, and 91.3 μg/mL AuNPs-treatment groups ($p < 0.001$). Cell viability for vehicle controls, AgNPs-, and AuNPs-treated groups were 92.98%, 94.52%, and 92.77%, respectively.

Mitochondrial Membrane Potential Assay Analysis

Acute exposure of 50 μg/mL AgNPs to SH-SY5Y cells resulted in a significant decrease in 590/535 ratio ($p < 0.001$) compared to negative controls over time [Fig. 1(C)]. Likewise, incubation of SH-SY5Y cells with positive controls resulted in a significant reduction in the 590/535 ratio compared to negative controls ($p < 0.001$).

ADP/ATP Ratio Assay Analysis for AgNPs

ATP Levels

One-hour incubation of SH-SY5Y cells with 50 μg/mL AgNPs resulted in decrease in ATP levels to 0.63-folds ($p < 0.001$) compared to vehicle controls [Fig. 2(A)]. Addition of various rescue efforts—50 μg/mL AgNPs + 10 μg/mL DHA, 50 μg/mL AgNPs + 100 μg/mL LC, and 50 μg/mL AgNPs + 10 μg/mL DHA + 100 μg/mL LC—resulted in fold changes in ATP levels of 0.71 ($p < 0.001$), 0.79 ($p < 0.001$), and 1.05 ($p = 1.00$), respectively, compared to vehicle controls. A significant rise in ATP levels was observed following incubation of cells with various rescue efforts—50 μg/mL AgNPs + 10 μg/mL DHA ($p = 0.33$), 50 μg/mL AgNPs + 100 μg/mL LC ($p < 0.001$), and 50 μg/mL AgNPs + 10 μg/mL DHA + 100 μg/mL LC ($p < 0.001$), compared to cells incubated with only 50 μg/mL AgNPs. Cells incubated with 50 μg/mL AgNPs + 10 μg/mL DHA + 100 μg/mL LC showed a statistically significant increase in ATP levels, when compared to cells treated with either 50 μg/mL AgNPs + 10 μg/mL DHA ($p < 0.001$), or 50 μg/mL AgNPs + 100 μg/mL LC ($p < 0.001$).

ADP Levels

One-hour incubation of SH-SY5Y cells with 50 μg/mL AgNPs resulted in decrease in ADP levels to 0.79-folds ($p < 0.001$) compared to vehicle controls [Fig. 2(B)]. Addition of various rescue efforts—50 μg/mL AgNPs + 10 μg/mL DHA, 50 μg/mL AgNPs + 100 μg/mL LC, and 50 μg/mL AgNPs + 10 μg/mL DHA + 100 μg/mL LC—also showed a decrease in ADP levels to 0.80-folds ($p < 0.001$), 0.78-folds ($p < 0.001$), and 0.88-folds ($p = 0.003$), respectively. A significant rise in ADP levels was observed following incubation of cells with 50 μg/mL AgNPs + 10 μg/mL DHA + 100 μg/mL LC compared to cells incubated with only 50 μg/mL AgNPs ($p = 0.021$), and cells incubated with 50 μg/mL AgNPs + 100 μg/mL LC ($p = 0.011$).

ADP/ATP Ratio

One-hour incubation of SH-SY5Y cells with 50 μg/mL AgNPs resulted in increase in ADP/ATP ratio to 1.25-folds ($p < 0.001$) compared to vehicle controls [Fig. 2(C)]. The various rescue efforts—50 μg/mL AgNPs + 10 μg/mL DHA, 50 μg/mL AgNPs + 100 μg/mL LC, and 50 μg/mL AgNPs + 10 μg/mL DHA + 100 μg/mL LC—resulted in fold changes in ADP/ATP ratio of 1.13-folds ($p = 0.001$), 0.98-folds ($p = 1.00$), and 0.84-folds ($p < 0.001$), respectively, when compared to vehicle controls. Significantly lower ADP/ATP ratio levels were also observed in cells treated with 50 μg/mL AgNPs + 10 μg/mL DHA ($p = 0.002$), 50 μg/mL AgNPs + 100 μg/mL LC ($p < 0.001$), and 50 μg/mL AgNPs + 10 μg/mL DHA + 100 μg/mL LC ($p < 0.001$), when compared to cells exposed to only 50 μg/mL AgNPs. Similarly, cells that were incubated with 50 μg/mL AgNPs + 10 μg/mL DHA + 100 μg/mL LC showed a statistically significant drop in ADP/ATP ratio, when compared to cells treated with either 50 μg/mL AgNPs + 10 μg/mL DHA ($p < 0.001$), or 50 μg/mL AgNPs + 100 μg/mL LC ($p < 0.001$).

ADP/ATP Ratio Assay Analysis for AuNPs

ATP Levels

One-hour incubation of SH-SY5Y cells with 91.3 μg/mL AuNPs resulted in increase in ATP levels to 1.36-folds

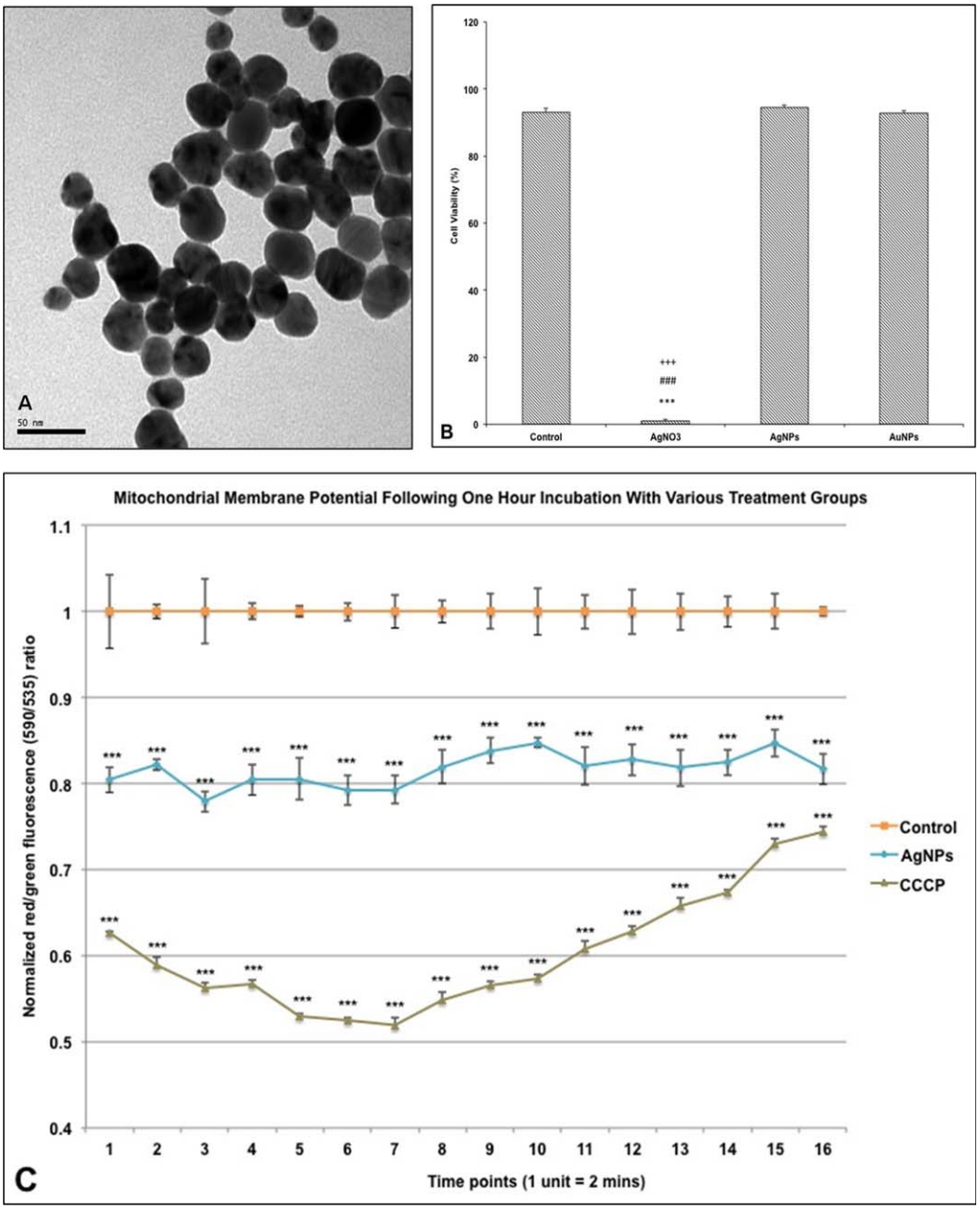
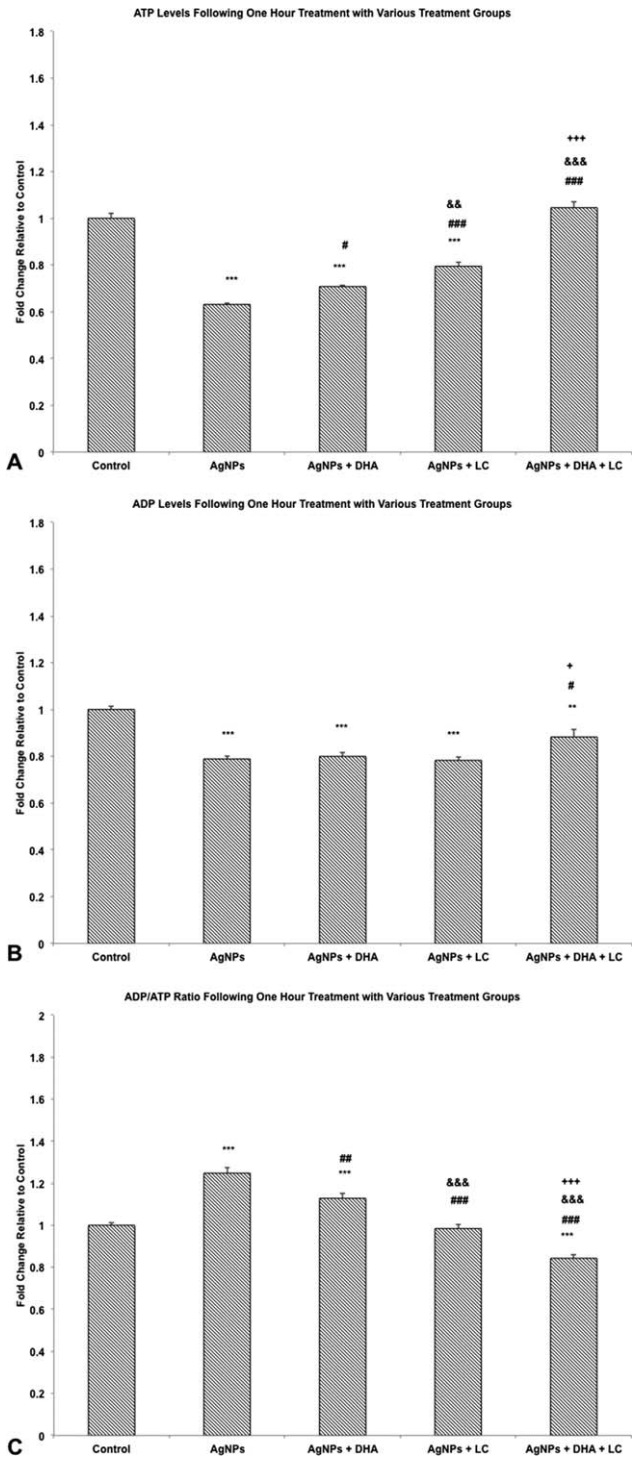


Fig. 1. (A) TEM micrograph of AgNPs used in the present study. AgNPs were found to be roughly spherical in shape, with an average diameter of 26 nm. Scale: 50 nm. (B) Cell viability of SH-SY5Y cells following one-hour incubation with various treatment groups-vehicle control; 78.7 μg/mL $AgNO_3$; 50 μg/mL AgNPs; 91.3 μg/mL AuNPs ($n = 3$ in each group). Each bar in the figure denotes mean + SEM. * Statistical significant differences in cell viability compared with controls, by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. $^{***}p < 0.001$. $^{\#}$ Statistical significant differences in cell viability compared with AgNPs, by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. $^{\#\#\#}p < 0.001$. $^{+}$ Statistical significant differences in cell viability compared with AuNPs, by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. $^{+++}p < 0.0001$. (C) Mitochondrial membrane potential following one-hour incubation of SH-SY5Y cells with negative controls, 50 μg/mL AgNPs, and 5 μM CCCP ($n = 4$ in each group). Each time point represents a 2-min interval. * Statistical significant changes in 590/535 ratio compared to negative controls by two-tail unpaired Student's *t* test. $^{***}p < 0.001$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

($p < 0.001$) compared to vehicle controls [Fig. 3(A)]. AuNPs-treated cells also showed a statistically significant increase in ATP levels compared to those treated with 50 μg/mL AgNPs ($p < 0.001$). Similar to previous findings, 50 μg/mL AgNPs treatment resulted in decrease in ATP levels to 0.47-folds ($p < 0.001$) compared to vehicle controls.



ADP Levels

One-hour incubation of SH-SY5Y cells with 91.3 μg/mL AuNPs did not show any significant difference in ADP levels ($p = 1.000$) compared to control cells [Fig. 3(B)]. However, AuNPs-treated cells showed a significant increase in ADP levels compared to those treated with 50 μg/mL AgNPs ($p < 0.001$). Treatment with 50 μg/mL AgNPs showed a significant decrease in ADP levels to 0.70-folds compared to controls ($p < 0.001$).

ADP/ATP Ratio

One-hour incubation of SH-SY5Y cells with 91.3 μg/mL AuNPs resulted in decrease in ADP/ATP ratio to 0.70-folds ($p = 0.004$) compared to vehicle controls [Fig. 3(C)]. AuNPs-treated cells resulted in significant decrease in ratio, compared to those treated with AgNPs ($p < 0.001$). Similar to previous findings, 50 μg/mL AgNPs treatment resulted in increase in ADP/ATP ratio to 1.46-folds ($p < 0.001$) compared to vehicle controls.

DISCUSSION

The present study examined the acute effects of AgNPs on SH-SY5Y mitochondria, and possible rescue of AgNPs-induced mitochondrial dysfunction by DHA and LC. Compared to vehicle controls, 92–94% of AgNPs- and AuNPs-treated cells were viable whereas only 0.99% of AgNO3-treated cells survived, after 1 hour treatment. This shows that AgNPs are much less toxic than silver ions in solution, and suggests that results obtained from AgNPs-treatment in the other two assays, mitochondrial membrane potential assay and ADP/ATP ratio assay, are mostly due to AgNPs. Since AgNO3 treatment resulted in death of almost all SH-SY5Y cells, it was not used in the remaining assays.

Fig. 2. (A) Fold change in ATP levels, (B) ADP levels, and (C) ADP/ATP ratio of SH-SY5Y cells following 1-h incubation with various treatment groups-vehicle controls; 50 μg/mL AgNPs; 50 μg/mL AgNPs + 10 μM DHA; 50 μg/mL AgNPs + 100 μM LC; 50 μg/mL AgNPs + 10 μM DHA + 100 μM LC ($n = 6$ in each group). Each bar in the figure denotes mean + SEM. *Statistical significant differences in fold change compared with controls, by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. $^{**}p < 0.01$, $^{***}p < 0.001$. $^{\#}$Statistical significant differences in fold change compared with AgNPs, by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$. $^{\&}$Statistical significant differences in fold change compared with AgNPs + DHA, by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$. $^{+}$Statistical significant differences in fold change compared with AgNPs + LC, by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. $^{+}p < 0.05$, $^{+++}p < 0.001$.

Mitochondrial membrane potential is the result of an electrochemical gradient across the inner mitochondrial membrane (Alberts et al., 2008; Voet et al., 2013). JC-1 dye accumulates in mitochondria as red fluorescent aggregates in healthy cells, but remains in its cytoplasmic green monomeric form when the mitochondrial membrane depolarizes (Ma et al., 2011; Guo et al., 2013). Acute exposure of AgNPs resulted in decreased red/green (590/535) ratio, similar to treatment with the positive control (CCCP), indicating adverse effect on the mitochondrial membrane. This is consistent with the results of previous studies, which showed AgNPs effects on mitochondria of non-neuronal cells, as determined by rhodamine 123 uptake (Hussain et al., 2005) or TPP^+—selective electrode (Teodoro et al., 2011). In this study, we showed decreased ATP levels in AgNPs-treated cells that could arise from damage to the mitochondrial respiratory chain (AshaRani et al., 2009; Costa et al., 2010). AgNPs could also cause oxidative stress and calcium dysregulation in cells (Haase et al., 2012) and trigger the Ca^{2+} ATPase pump or Na^+/Ca^{2+} exchanger to restore intracellular calcium levels. This could lead to increased intracellular sodium and activity of the Na^+/K^+ ATPase pump to maintain resting potential (Wang et al., 2003; Alberts et al., 2008). Use of these pumps could lead to decreased levels of ATP. A third reason that could result in lowered ATP is that AgNPs treatment causes SH-SY5Y cells to undergo lysis and release of ATP (Suszynski et al., 2008). This is, however unlikely in our context, since negligible cell death for AgNPs-treated cells was observed compared to vehicle controls in the trypan blue exclusion assay at one-hour post treatment. As ATP levels decrease, a corresponding increase in ADP levels is expected, since ATP is hydrolyzed to produce ADP (Alberts et al., 2008; Myhill et al., 2009; Voet et al., 2013). Nevertheless, decrease rather than increase in ADP level was observed. One possible explanation is that ADP may be utilized to produce ATP, via adenylate kinase (AK)—a phosphotransferase that catalyzes the conversion of ADP to ATP and AMP (Alberts et al., 2008; Voet et al., 2013). When ATP concentration decreases, AK compensates by utilizing two molecules of ADP to produce more ATP and in the process generates AMP. The latter can be degraded by AMP deaminases to produce inosine monophosphate (IMP), which prevents the recycling of AMP to ADP and ATP (Myhill et al., 2009). This reduces the adenine nucleotide source and depletes ADP and ATP stores.

Previous studies have shown protective actions of DHA and LC against mitochondrial dysfunction and neuronal injury (Horrocks et al., 2004; Alves et al., 2009; He et al., 2011). In this study, we attempted to prevent AgNPs-induced decrease in ATP and ADP levels, by introducing DHA and LC to SH-SY5Y cells. Co-treatment of cells with AgNPs and DHA resulted in greater ATP and ADP levels compared to cells treated with AgNPs only, suggesting that

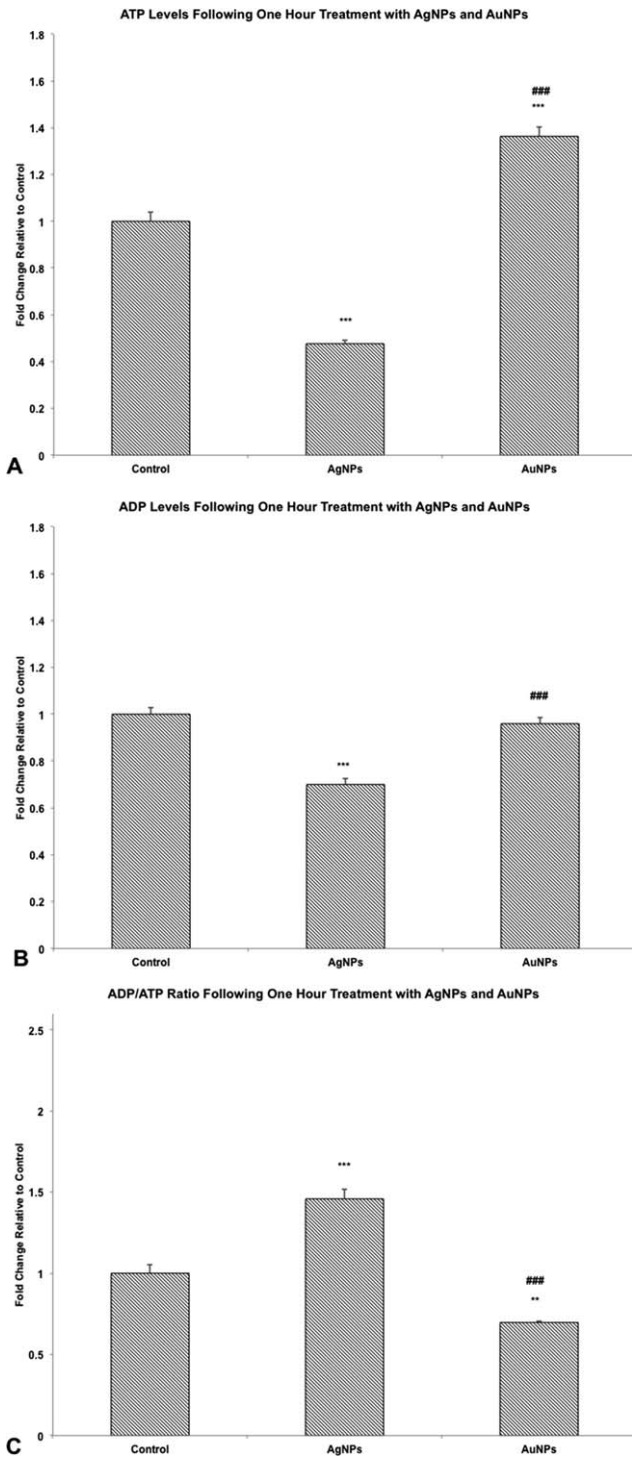


Fig. 3. (A) Fold change in ATP levels, (B) ADP levels, and (C) ADP/ATP ratio of SH-SY5Y cells following one-hour incubation with vehicle controls, 50 μg/mL AgNPs, and 91.3 μg/mL AuNPs ($n = 6$ in each group). Each bar in the figure denotes mean + SEM. *Statistical significant differences in fold change compared with controls, by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. $^{**}p < 0.01$, $^{***}p < 0.001$. $^{\#}$Statistical significant differences in fold change compared with AgNPs, by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. $^{\#\#\#}p < 0.001$.

DHA was capable of partially reversing the damage caused by acute exposure to AgNPs. DHA is incorporated in both cell (Reynolds et al., 2001) and mitochondrial membrane phospholipids (Ng et al., 2005), and could help repair AgNPs induced damage to these membranes. Furthermore, co-supplementation of DHA and LC was even more effective in preventing AgNPs induced decrease in ATP levels. Significant differences were observed between cells treated with AgNPs + DHA vs. AgNPs + DHA + LC; or AgNPs + LC vs. AgNPs + DHA + LC, suggesting synergistic effect of co-supplementation with DHA and LC. Although LC is used as a vehicle to bring fatty acids to mitochondria for energy production, it is also crucial for mitochondrial function (Hino et al., 2005). We hypothesize that the effectiveness of LC and DHA in attenuating toxic effects of AgNPs could be due to a role of LC in aiding the transfer of DHA to mitochondria for repair of damaged phospholipids, and that co-supplementation with DHA and LC might be useful for treatment of accidental *environmental* exposure to AgNPs or other engineered nanomaterials.

In contrast to AgNPs, treatment with AuNPs for one hour resulted in increased ATP level, no change in ADP level, and decreased ADP/ATP ratio, compared to vehicle controls. The mechanism is unknown, although it is possible that cellular processes may have been depressed during the one-hour incubation of SH-SY5Y cells with AuNPs, leading to decreased consumption of ATP. As nanoproducts become more commonplace, it is crucial to develop screening tests or indicators for their potential toxicity. Since neurons rely heavily on glucose for energy and are sensitive to perturbations of the cell membrane which require energy consuming ion pumps to maintain homeostasis, neuron-like cell lines such as SH-SY5Y cells may be a suitable and sensitive cell type for detecting changes in ATP, with AgNPs and AuNPs as two end points. In addition, since ATP is essential for many of the cell's metabolic processes, including repair from damage, ATP and ADP depletion could signal irreversible damage to cells, and hence accurately reflect actual impact of a nanomaterial to cells. The fact that results are obtained in 1 h could also be of practical importance, in circumventing issues related to NP solubility and ions in solution. As an *in vitro* test therefore, the ADP/ATP ratio assay on SH-SY5Y cells has multiple benefits, including low cost, easy interpretations, few ethical issues, reduced variability across samples and the ability to use human cells such as SH-SY5Y cells (Hartung and Daston, 2009; Takhar and Mahant, 2011), and could be useful as a screening test for nanoparticle toxicity.

CONCLUSION

In conclusion, acute exposure of AgNPs to SH-SY5Y cells caused mitochondrial dysfunction, in particular, disrupted mitochondrial membrane potential, and loss of both ATP and ADP, which could be rescued by co-supplementation with DHA and LC.

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