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Inhibitory effects of silver nanoparticles on H1N1 influenza A virus *in vitro*

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Silver nanoparticles have demonstrated efficient inhibitory activities against human immunodeficiency virus (HIV) and hepatitis B virus (HBV). However, the effects of silver nanoparticles against H1N1 influenza A virus remain unexplored. In this study, the interaction of silver nanoparticles with H1N1 influenza A virus was investigated. Silver nanoparticles with mean particle diameters of 10 nm were prepared for the hemagglutination inhibition test, the embryo inoculation assay, and the Mosmann-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, where these tests were used to determine the inhibitory activity of silver nanoparticles on H1N1 influenza A virus. MDCK cells were used as the infection model. Electron microscopy analysis and flow cytometry assay were used to determine whether silver nanoparticles could reduce H1N1 influenza A virus-induced apoptosis in MDCK cells. This study demonstrates that silver nanoparticles have anti-H1N1 influenza A virus activities. The inhibitory effects of silver nanoparticles on influenza A virus may be a novel clinical strategy for the prevention of influenza virus infection during the early dissemination stage of the virus.

1. Introduction

Influenza pandemics remain a serious public health problem worldwide. Influenza virus-associated illnesses cause an estimated 200,000–500,000 hospital admissions and hundreds of thousands of deaths annually (Simonsen et al., 2000; Suwannakarn et al., 2010; Cox and Subbarao, 2000; Webby and Webster, 2003; Wang et al., 2010; Chu et al., 2009). The emergence of variations in virus strains is one of the principal challenges facing prevention of infection with influenza virus. In response to rapid antigenic drift of influenza virus, extensive use of inactivated viral vaccines has been considered as the most effective approach (Bardiya and Bae, 2005). However, the production of vaccines may not be adequate to meet the increase in demand when an influenza pandemic occurs. Although the treatment of influenza produces rapid suppression of H1N1 influenza A virus infection in the short-term, this effect is not often sustainable due to the emergence of drug-resistant H1N1 strains. Therefore, it is important to develop new antiviral strategies to combat wild-type and mutant H1N1 influenza A virus infections. The development of new influenza antiviral drugs is urgent.

Nanotechnology offers the ability to explore again the properties of materials by manipulating their sizes (Bonnemann and Richards, 2001). The unique nature of nanoparticles has been exploited in the hope of developing novel diagnostic and antimicrobial agents (Percival et al., 2007; Shahverdi et al., 2007). Recently, metal nanoparticles have attracted increasing attentions for their important applications in a variety of areas, such as biosensors, labels for cells, biomolecules, and cancer treatment (Nam et al., 2003; Tkachenko et al., 2003; Hirsch et al., 2003). A notable application of nanoparticles lies in the antimicrobial properties of silver nanoparticles, which have received considerable attention because of their physicochemical properties. Silver nanoparticles have been shown to be a promising antimicrobial and are considered as an option for antiviral treatment (Sondi and Salopek-Sondi, 2004; Samuel and Guggenbichler, 2004; Yang et al., 2007). Currently, studies on the interaction of silver nanoparticles with viruses are limited. In the literature reviewed for this study, only six published papers on antiviral activities showed that silver nanoparticles can inhibit viral replication of viruses, such as HIV-1, hepatitis B virus, respiratory syncytial virus, herpes simplex virus type 1, and monkeypox virus (Sun et al., 2005; Elechiguerra et al., 2005; Lu et al., 2008; Sun et al., 2008; Pinto et al., 2009; Rogers et al., 2008). The studies found that silver nanoparticles can bind to HIV viral particles, thus inhibiting the virus from binding to host cells (Elechiguerra et al., 2005). In addition, silver nanoparticles can inhibit the DNA or RNA replication of the virus (Elechiguerra et al., 2005; Lu et al., 2008).

However, knowledge on whether silver nanoparticles have effective anti-H1N1 influenza A virus activity is limited. To understand the future role of silver nanoparticles for anti-H1N1 influenza A virus, a number of assays were used, including (i)

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hemagglutination inhibition (HAI) tests and embryo inoculation assays; (ii) a cytotoxicity assay of silver nanoparticles in MDCK cells; (iii) inhibition of silver nanoparticles of H1N1 influenza A virus; and (iv) transmission electron microscopy (TEM) analysis combined with a flow cytometry (FCM) assay. Initial findings of the investigation are presented. The data from these experiments confirm that silver nanoparticles can interact with H1N1 viral particles and exert efficient anti-H1N1 influenza A virus activity.

2. Materials and methods

2.1. Cell culture, silver nanoparticles, H1N1 influenza A virus

MDCK cells were cultured and maintained in Dulbecco's modified Eagle medium (DMEM, GIBCO™, USA), supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA), penicillin (50 μg/ml), streptomycin (100 μg/ml), kanamycin (50 μg/ml), sodium pyruvate (110 mg/L), L-glutamine, pyridoxine hydrochloride, low glucose, and without sodium bicarbonate. Cells were maintained at 37 °C in 5% CO_2 in a humidified atmosphere. MDCK cells were used to represent the common route of influenza virus exposure. Silver nanoparticles used in this study were prepared by our laboratory and placed at room temperature for 6 months to observe their stability. H1N1 influenza A virus (A/PR/8/34) was obtained from Wuhan Institute of Virology, Chinese Academy of Sciences.

2.2. HAI test

HAI was performed to detect the silver nanoparticles and their capacity of inhibiting the formation of hemagglutination caused by H1N1 influenza A virus. H1N1 influenza A virus was briefly treated with silver nanoparticles in 96 "V"-shaped wells containing the virus control for four times at 30-min intervals (at 30, 60, 90, and 120 min). Firstly, 100 μl of physiological saline (NS) was added into all of the experiment's wells. 100 μl of each sample was dispensed into the first well (column 1) and thoroughly mixed; subsequently, the 100 μl mixture in column 1 was transferred to column 2. Two-fold serial dilutions of the samples were performed, from column 2 to column 11. The last 100 μl of fluid was discarded, thus making column 12 the NS control. Then, 100 μl of 1% chicken RBC suspension was added and mixed to all of the wells. The viral HAI titers were evaluated after the plates had been placed at room temperature for 60 min.

2.3. Embryonate inoculation assay

Each virus sample was diluted for 2 h in 1.5-ml EP tubes with distilled water, solvent, and silver nanoparticles. Four 10-day-old fertile chicken embryos were separately inoculated with 100 μl of a sample containing antimicrobials by the chorioallantoic sac route. The eggs were inoculated for 72 h at 37 °C and then were placed in a 4 °C refrigerator for 12 h. The viral HAI titers of the chorioallantoic sac fluid were evaluated, as previously described.

2.4. Cytotoxicity assay

The toxic effects of silver nanoparticles on MDCK cells were determined using the Mosmann-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Denizot and Lang, 1986). Briefly, MDCK cells were cultured in 96-well plates at a density of 2×10^5 cells per well and grow to 80% confluence in the presence of 100 μl of DMEM with 10% FBS. Then the cells were washed twice with phosphate-buffered saline (PBS) and were exposed to silver-np with levels ranging from 6.25 μg/ml to 200 μg/ml (2-fold serial dilution) for 120 min. The cells were washed and added to 150 μl of DMEM with 2% FBS and incubated for 48 or 72 h. Cellular viability was detected on OD_{492} nm.

2.5. Inhibition of silver nanoparticles on H1N1 influenza A virus

The inhibitory effect of silver nanoparticles on H1N1 influenza A virus was measured using the MTT assay, as described previously. Briefly, MDCK cells were grown in 96-well plates and infected with a mixture containing 100 $TCID_{50}$ H1N1 influenza A virus that had been treated for 2 h with silver-np dosing levels ranging from 6.25 μg/ml to 200 μg/ml. The groups contained solvent and a virus control group (each dose $N = 6$). The cells were washed and added to 150 μl of DMEM medium (2% FBS) and then incubated for 48–96 h. Cellular viability was detected at OD_{492} nm and the cytopathic effect (CPE) was observed daily under an inverted microscope.

2.5.1. TEM analysis

MDCK cells were grown to 80% confluence in each of the 6-well plates and infected with H1N1 influenza A virus, which had been treated for 2 h with 50 μg/ml silver nanoparticles. Then, the cells were incubated until the virus control appeared to have significant lesions. The cells were digested and collected in a 1.5-ml tube, then centrifuged at 2000 rpm for 10 min. The cell pellet was fixed with 4% glutaraldehyde at 4 °C for 12 h and washed three times with PBS. Then, the pellet was fixed with osmium tetroxide for 1 h, stained with uranium acetate, and embedded into epoxide resin. After the cells were sectioned into ultra-thin slices, they were stained with osmic acid and examined under TEM (Hitachi, Tokyo, Japan).

2.6. FCM assay

It has been reported that the influenza virus can induce apoptosis in tissue culture cells (Takizawa et al., 1993). To determine if silver nanoparticles is an inhibitor of cell apoptosis when induced by H1N1 influenza A virus, FCM analysis was used to check early apoptotic activity of the MDCK cells Five groups were included in this assay: (1) normal cell control ($n = 6$); (2) virus control ($n = 6$); (3) virus treated with silver nanoparticles for 2 h ($n = 6$); (4) cells were treated with silver nps for 2 h following a treatment with the virus for 2 h ($n = 6$); (5) cells were treated with the virus for 2 h following a treatment with silver naps for 2 h ($n = 6$). A large number of cells were collected, washed twice with ice-cold PBS, mixed with RNase A, and incubated at 37 °C for 30 min. Propidium iodide (PI) was added and the mixture was incubated at 4 °C for 30 min in the dark. Finally, binding buffer was added to the cells and about 10,000 cells were analyzed with FCM (Becton Dickinson Co., San Jose, CA). All the experiments in this assay were performed three times.

2.7. Statistical Analysis

The data were expressed as means ± standard deviation (SD). All differences were evaluated by either the unpaired Student's *t*-test or ANOVA, as appropriate, where a P value of < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Characteristics of silver nanoparticles

There were many different methods of manufacturing silver nanoparticles with different shapes and sizes (Lu et al., 2008). A resultant golden yellow solution was obtained by our group, which reveals the formation of silver nanoparticles. These nanoparticles

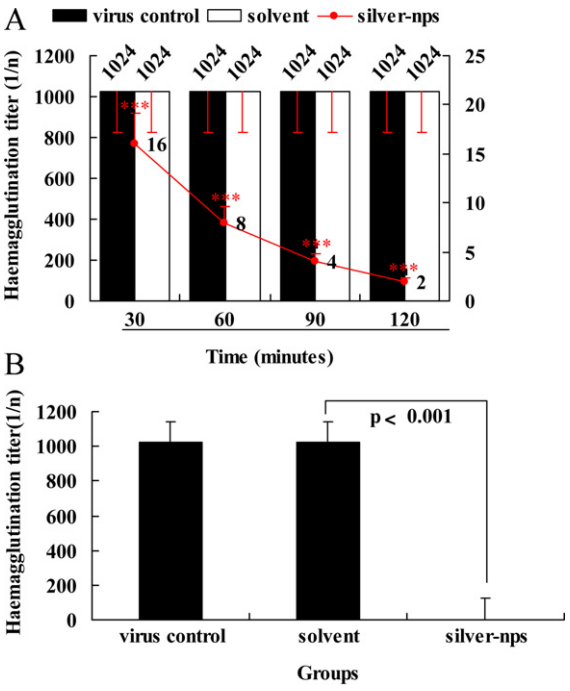

Fig. 1. The ability of silver-nps to inhibit hemagglutination caused by H1N1-IFV. HAI tests (A) and an embryonate inoculation assay (B). ***$P < 0.001$, the viral HAI titers in silver-nps group vs. virus control and solvent group. The viral titers of both virus control and solvent groups stabilized at 1:1024, whereas the viral titers of silver-naps group significantly decreased, which were 1:16, 1:8, 1:4 and less than 1:2 when determined at 30 min, 60 min, 90 min and 120 min respectively.

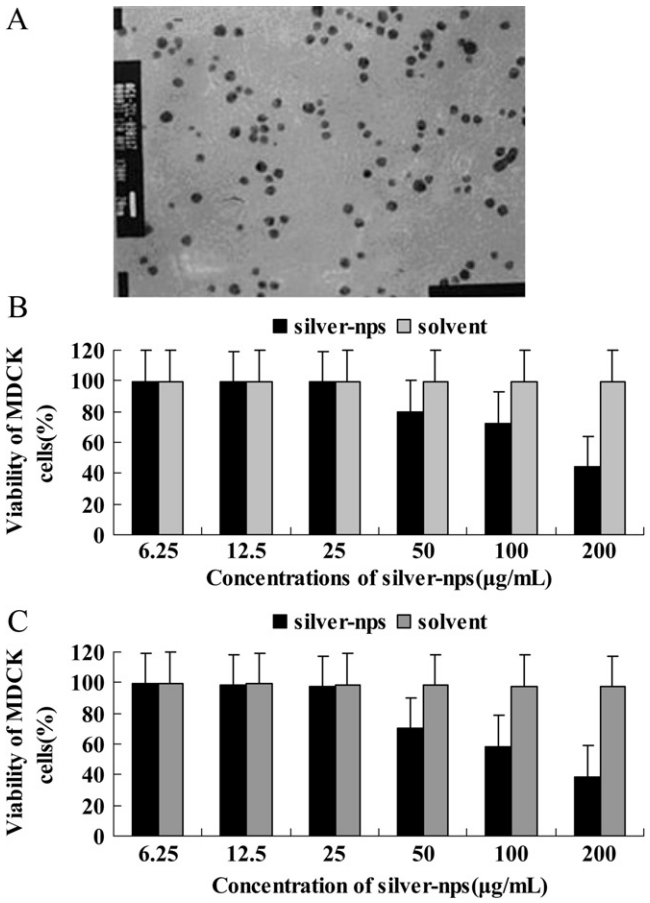

Fig. 2. The TEM images of silver-nps and toxicity of silver-nps against MDCK cells. The average particle size of silver-nps was 10 nm (A). Viability of MDCK cells in the presence of silver-nps and solvent at 24 h (B) and 48 h (C), which shows that the solvent has no toxicity to cells and that higher cytotoxicity can be caused by a higher concentration of silver-nps.

were uniformly distributed, with particle sizes varying from 5 to 20 nm (the average diameter was about 10 nm). TEM images for silver nanoparticles are shown in Fig. 2A. There was no obvious silver precipitation formation at room temperature after 6 months.

3.2. HAI test and embryonate inoculation assay

HAI is a test used to detect the presence of a certain hemagglutinating virus, based on whether the red blood cells in the sample lose their ability to aggregate or not after adding the antibody. H1N1 influenza A virus possesses the ability to agglutinate chicken erythrocytes. Antibodies or special factors against the viral protein responsible for hemagglutination can prevent the reaction. In the presence of silver nanoparticles, the ability of H1N1 influenza A virus to agglutinate the erythrocytes was either weakened or completely inhibited. A serial 10-dilution of H1N1 influenza A virus was performed, followed by the addition of 1% chicken RBCs. In four replications of this experiment, we found that silver nanoparticles can rapidly inhibit H1N1 influenza A virus hemagglutination of chicken RBCs. Compared with the titers 1:1024 of both the virus control and solvent group, the viral HAI titers in silver-np groups were 1:16 (30 min), 1:8 (60 min), 1:4 (90 min), and below 1:2 (120 min) (Fig. 1A). These results suggest that silver nanoparticles play a significant role in obstructing the binding of H1N1 influenza A virus to the RBCs. Furthermore, the results of viral HAI titers in embryonating eggs among diverse samples were statistically significant. Compared with the titers 1:1024 of both the virus control and solvent group, the viral HAI titers in silver nanoparticles groups were below 1:2 (Fig. 1B). The hemagglutination antigen of the influenza A virus is relevant to virus adsorption and virus neutralization; the ability of H1N1 influenza A virus to agglutinate the erythrocytes was inhibited in the presence of silver nanoparticles.

3.3. The cytotoxicity of silver nanoparticles toward MDCK cells and its anti-H1N1 influenza A virus activity

The toxic results of each concentration of silver nanoparticles on MDCK cells were shown at 24 h and 48 h (Fig. 2B and C), which clearly shows that over 98% of cell viability was observed at the concentrations of 6.25, 12.5, and 25 μg/ml at 24 and 48 h. When the concentration of silver nanoparticles increased, the viability of the cells decreased. Whereas 100 μg/ml of silver nanoparticles was highly toxic to MDCK cells, 50 μg/ml resulted in less cytotoxicity *in vitro*. It is possible that cytotoxicity can be caused by high concentrations of silver nanoparticles. This can explain the severe cytotoxicity found using 200 μg/ml of silver nanoparticles when comparing the lack of toxicity found in the solvent group cells (Fig. 2B and C).

To observe the anti- H1N1 influenza A virus activity of silver nanoparticles, MDCK cells were separately treated with a mixture combined H1N1 influenza A virus with silver nanoparticles at a concentration of 6.25–200 μg/ml. Compared with the virus control, silver nanoparticles which were at a concentration of 12.5–100 μg/ml were found to exhibit potential anti-H1N1 influenza A virus activity at both 72 and 96 h (Fig. 3A and B). The survival percentages of MDCK cells at a concentration of 12.5 μg/ml were 47.97% (72 h) and 49.87% (96 h); the percentages at 25 μg/ml of silver nanoparticles were 82.4% (72 h) and 86.5% (96 h); the rate percentages at 50 μg/ml were 96.77% (72 h) and 97.47% (96 h); and the percentages at 100 μg/ml were 68.46% (72 h) and 50.26%

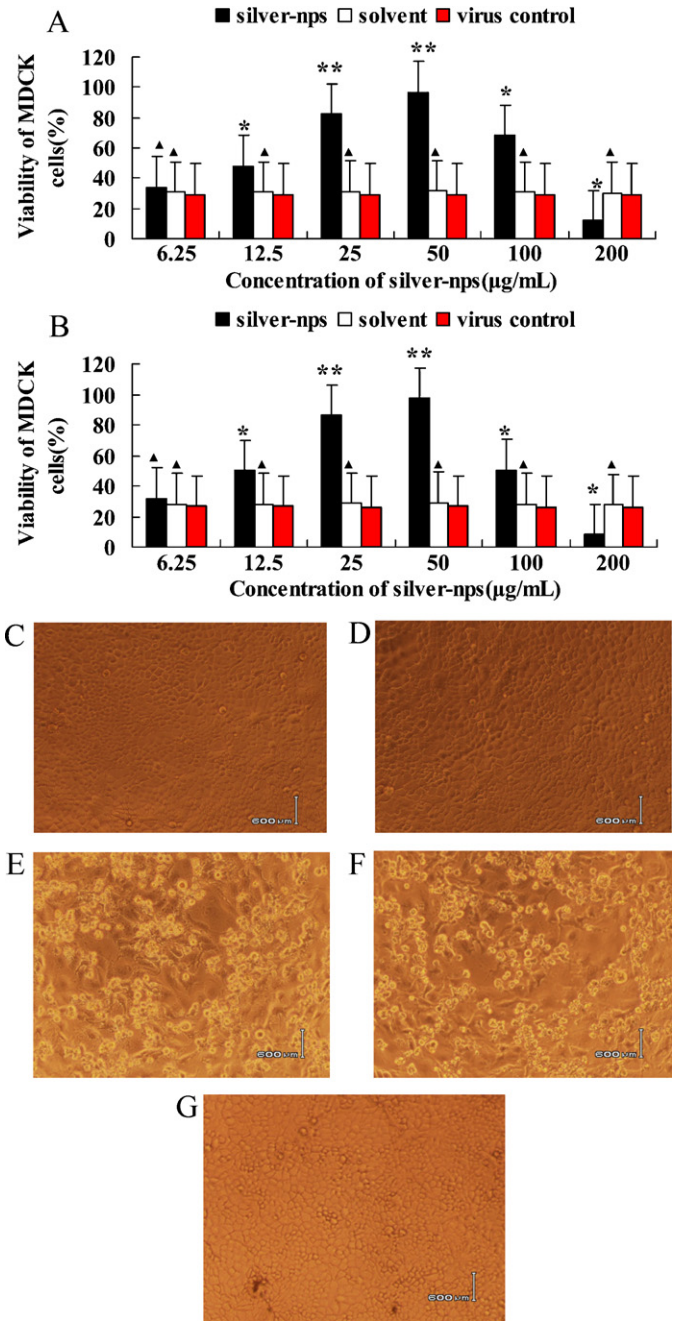

Fig. 3. Inhibition of silver-nps against H1N1-IFV in MTT assay and the CPE method. The survival percentages of MDCK cells in solvent group, virus control group as well as in silver-nps groups when the silver-naps concentration was 6.25 μg/ml, 12.5 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml and 200 μg/ml were investigated at both 72 h (Fig. 3A) and 96 h (Fig. 3B). ▲$P > 0.05$, *$P < 0.05$ and **$P < 0.01$ vs. virus control. Both normal cells (C) and cells treated with solvent (D) expressed virtually no CPE (−). Significant CPEs (++++) were observed in the virus control (E) and the virus-treated group with solvent (F), which suggests that cells died and had broken off. CPE was rarely observed when the virus was treated with silver-nps (G) (+/−). (−) represents no cell infection, while (+), (++), (+++) and (++++) represent the cell infection percentages were 25%, 50%, 75% and 100%, respectively.

(96 h). The virus control group survival rates were 29.24% (72 h) and 26.94% (96 h). There was not much difference between the cellular viability of the solvent and the virus control (Fig. 3A and B). The concentration-dependent anti-H1N1 influenza A virus effects of silver nanoparticles at 72 h were examined as an example. The maximum inhibitory effect was found at a concentration of 50 μg/ml of silver nanoparticles and the antiviral activity was shown to continue with prolonged incubation (96 h) (Fig. 3B).

Using an inverted microscope, no significant CPE was observed in the normal cells (Fig. 3C) nor in the solvent group (Fig. 3D); this indicates that the solvent of silver nanoparticles is not toxic to the MDCK cells. Typical CPEs can be found in the virus control (Fig. 3E); this includes gaps between cells becoming larger, cells becoming round, swollen, died, and broken off. The same CPEs found in the virus control were found when the solvent was mixed with the virus for 2 h and then added to the cells (Fig. 3F), thus showing that the solvent of silver nanoparticles has no antiviral effect. No significant CPE expressed using 50 μg/ml of silver nanoparticles had a strong inhibitory effect toward H1N1 influenza A virus (Fig. 3G).

3.4. TEM analysis and FCM assay

The purpose of these assays was to determine whether silver nanoparticles can inhibit apoptosis in MDCK cells induced by H1N1 influenza A virus. The morphological changes and ultrastructural features of cells treated with H1N1 influenza A virus and silver nanoparticles were detected under TEM. Normal cells (Fig. 4A) and cells infected with the silver-np-treated virus for 2 h previously (Fig. 4B) revealed normal silhouettes, suggesting that silver nanoparticles can inhibit H1N1 influenza A virus infectivity *in vitro*. In contrast, cells infected with the virus control displayed characteristics of apoptosis, including nuclear condensation, chromatin aggregation, and apoptotic bodies in some cells (Fig. 4C and D).

Furthermore, the experimental MDCK cells mentioned above were stained with PI and analyzed by FCM. The apoptosis rate in normal cells was 5.58 ± 2.20% (Fig. 4E) and the rate in cells infected by the virus control was 23.37 ± 2.50% (Fig. 4F); the rate in cells treated with the combined mixture of virus and 50 μg/ml of silver nanoparticles was 9.77 ± 1.59% (Fig. 4G); the rate in cells first treated with virus and silver nanoparticles later was 14.66 ± 1.80% (Fig. 4H); the rate in cells first treated with silver nanoparticles and virus later was 18.48 ± 0.98% (Fig. 4I). These results suggest that silver nanoparticles are able to reduce apoptosis which has been induced by H1N1 influenza A virus in MDCK cells.

Apoptosis is a highly conserved and evolutionary biological process requiring the regulated activation of several signals, resulting in typical biochemical and morphological alterations of the cell (Xiang et al., 2008). There are many mechanisms through which apoptosis can be induced in cells. The apoptotic stimuli comprise extrinsic signals, such as the binding of death-inducing ligands to cell surface receptors (called death receptors). Apoptosis can also be initiated by intrinsic signals which are produced following cellular stress, which may result from viral infections. Past studies have shown that apoptosis is a host defense response which reduces virus propagation, and that the apoptosis phenomenon is essential for virus mRNA synthesis and replication (Zhang et al., 2010; Stasakova et al., 2005; Kurokawa et al., 1999; Stray and Air, 2001). Typical alterations of apoptosis include chromatin condensation, condensation of the cell and the nuclei, generation of evoluted membrane segments (zeiosis), cellular shrinkage, formation of apoptotic bodies, and disintegration of mitochondria (Xiang et al., 2008; Gulbins et al., 2000; Chen et al., 2005). It has been shown that H1N1 influenza A virus can lead to apoptosis in numerous cells *in vitro* (Morris et al., 1999, 2005). Using the TEM and the FCM assay, the results verified that silver nanoparticles are capable of reducing apoptosis induced by H1N1 influenza A virus in MDCK cells.

This study presents evidence for the antiviral properties of silver nanoparticles. The soluble silver nanoparticles were active against H1N1 influenza A virus which is responsible commonly for infection. This finding suggests that silver nanoparticles can provide enhanced protection against influenza virus infection without the risk of cell toxicity. The molecular mechanism underlying the antiviral activity of silver nanoparticles still remains unknown. However, the virus is presumed to be dependent on the

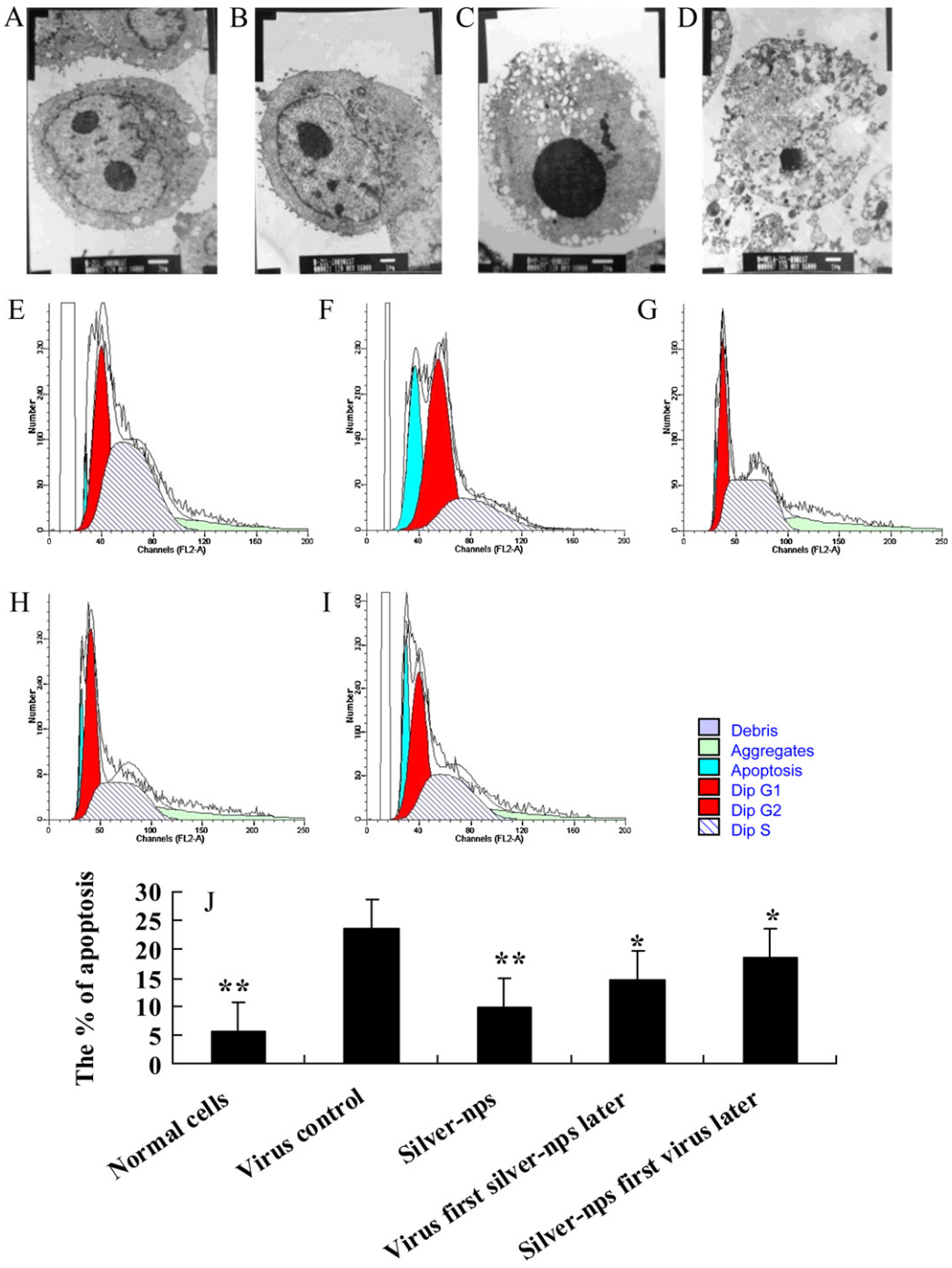

Fig. 4. Silver-nps reduce apoptosis induced by H1N1-IFVs in MDCK cells. Silver-nps reduce apoptosis in MDCK cells visualized by TEM. Normal cells (A) and virus treated with silver-nps (B) expressed that the nuclear shapes of cells are unbroken. The cells infected by the virus (C and D) expressed condensed chromatins that were shrunken and aggregated along the inside of the nuclear membrane, and that revealed the apoptotic bodies (5000×). Cells were collected and the percentage of cells undergoing apoptosis was analyzed under the flow cytometry assay. Normal cells (E) and virus control group (F). Cells treated with a combined mixture of virus and silver-nps (G). Cells first treated with the virus and then silver-nps (H). Cells first treated with silver-nps and then the virus (I). These data were calculated from the apoptosis percentage of cells, as shown in diagrams from E to I (every group $N = 6$) (J). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. virus control. TEM magnification, 5000×. Bar = 2 nm.

soluble Ag^{+} ions, which inhibit strongly the growth of pathogens by suppression of respiratory enzymes and electron transport components and interference with DNA functions (Li et al., 1997, 2006; Guggenbichler et al., 1999). More extensive microscopic analysis of the silver nanoparticles, after coating and acting with the virus at different times, may be useful to test the mechanism for antiviral activity in the future.

In similar experiments, direct DNA sequencing of RT-PCR products amplified from HA viral gene segments (after the virus was treated with silver nanoparticles at different times) was performed; both the virus control and the solvent control groups appeared as significant bright bands in the 1700 bp area, whereas the silver nanoparticles group did not. This result led to the hypothesis that silver nanoparticles inhibiting the HA gene might, in turn,

reduce formation of the HA protein, subsequently decreasing the number of extracellular virions. In addition, both the immunofluorescence assay and the neuraminidase inhibition assay show that silver nanoparticles exerts a strong inhibitory influence on H1N1 influenza A virus, although this needs to be investigated further.

In summary, the findings provide evidence that silver nanoparticles have efficient inhibitory activity on H1N1 influenza A virus, which can inhibit rapidly H1N1 influenza A virus hemagglutination of chicken RBCs in HAI tests and an embryonate inoculation assay. Silver nanoparticles can also reduce H1N1 influenza A virus-induced apoptosis toward MDCK cells according to TEM analysis and FCM assays. Further investigations are needed to clarify how silver nanoparticles inhibit H1N1 influenza A virus infectivity. The application of silver nanoparticles as an effective drug against influenza should be considered.

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