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In Vitro Antiviral Effect of "Nanosilver" on Influenza Virus

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ABSTRACT

*Introduction***:** Influenza is a viral infectious disease with frequent seasonal epidemics causing world-wide economical and social effects. Due to antigenic shifts and drifts of influenza virus, long-lasting vaccine has not been developed so far. The current annual vaccines and effective antiviral drugs are not available sufficiently. Therefore in order to prevent spread of infectious agents including viruses, antiseptics are considered by world health authorities. Small particles of silver have a long history as general antiseptic and disinfectant. Silver does not induce resistance in microorganisms and this ability in Nano-size is stronger.

Materials and methods: The aim of this study was to determine antiviral effects of Nanosilver against influenza virus. $TCID_{50}$ (50% Tissue Culture Infectious Dose) of the virus as well as CC_{50} (50% Cytotoxic Concentration) of Nanosilver was obtained by MTT (3- [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide, Sigma) method. This compound was non-toxic to MDCK (Madin-Darbey Canin Kidney) cells at concentration up to 1 µg/ml. Effective minimal cytotoxic concentration and 100 $TCID_{50}$ of the virus were added to the confluent cells. Inhibitory effects of Nanosilver on the virus and its cytotoxicity were assessed at different temperatures using Hemagglutination (HA) assay, RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction), and DIF (Direct Immunofluorescent). RT-PCR and free band densitometry software were used to compare the volume of the PCR product bands on the gel.

Results and Discussion: In this study it was found that Nanosilver has destructive effect on the virus membrane glycoprotein knobs as well as the cells.

Keywords: Nanosilver, Influenza virus, MDCK, RT-PCR, DIF

INTRODUCTION

Influenza virus infection is an important cause of respiratory diseases worldwide. Due to antigenic shifts and drifts of the virus, it is hardly managed with antiviral drugs (1) and there is not suitable long-lasting vaccine available. Therefore, there is a great need for agents to be able to inhibit or kill the virus on the surface (2). Because of unavailability of sufficient effective drugs, antiseptics and disinfectants are considered by world health authorities.

Small silver particles with a long history as general antiseptic and disinfectant are able to interact with disulfide bonds (3-5) of protein contents of microorganisms such as viruses, bacteria and fungi. They can change three-dimensional structure of proteins containing S-S bonds and block the function of the microorganism. This ability is stronger in Nano-size (6) and effects of its different forms on surface glycoprotein knobs have been evaluated on gp_{120} membrane glycoprotein of HIV (Human Immunodeficiency Virus) in vitro (7).

Influenza virus surface glycoproteins are hemagglutinin (HA) and neuraminidase (NA). HA structure consists of HA_1 and HA_2 subunits linked by disulfide bond and due to the essential role of this bond to commence the function of the virus (8), effects of Nanosilver on this glycoprotein by biochemical and molecular techniques were evaluated.

MATERIAL AND METHODS

Viruses

Influenza A/New Caledonia/20/99 (H1N1); standard virus was obtained from National Institute for Biological Standards and Control

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(NIBSC) and used after several passages. It was propagated in MDCK cells in the presence of 2 µg/ml of Trypsin-TPCK (Tosylamide, Phenylethyl Chloromethyl Keton-treated Trypsin) (Sigma, St.Louis, MI) to help virus penetration by cleavage of the HA_0 to HA_1 and HA_2.

Cell culture

Continuous MDCK cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (ICN) contained 10% heat-inactivated Fetal Bovine Serum (FBS) (Gibco, Gaithersburg, MD), 100 Units/ml Penicillin G and 100 µg/ml Streptomycin (Sigma Co.) at 37°C in a humidified 5% CO_2 incubator.

Test compound

Nanosilver was obtained as a Clear Goldish liquid at 2000 ppm (particle/ml). It was used in different dilutions to assess its antiviral activity.

Colorimetric MTT assay

Colorimetric MTT assay was performed according to Raphael Levi, et al (9). Yellow MTT is reduced to purple formazan by mitochondrial succinate dehydrogenase of living cells. Stock MTT (10x), was prepared by dissolving tetrazolium in PBS (Phosphate Buffer Saline) at a concentration of 5 mg/ml and filtering through 0.45 µm filter. The medium of the confluent cells was removed, then 100 µl of 1x MTT was added to each well. Following incubation at 37°C with 5% CO_2 for 2 hrs, 100 µl of acidic isopropanol was added and mixed to release the color from the cells. Optical density was measured at 540 nm using ELISA reader (Stat Fax-200) to elevate live cells.

Determination of CC_{50} and effective minimal cytotoxic concentration of Nanosilver

Cytotoxicity of the compound against MDCK cells was evaluated in term of CC_{50} (50% cytotoxic concentration). MDCK cell culture was exposed to the compound at different concentrations. Following 1 hr of incubation at 37°C and washing with PBS, the TPCK-containing medium was added. After 48 hrs incubation under the same conditions, the viability of the cells was measured by MTT method. Effective minimal cytotoxic concentration was determined by statistical analyses (Spss ver. 11.0).

Virus inhibition assay

Confluent MDCK cell cultures were treated with Nanosilver during and after virus infection in three sets of experiments as follows:

1-100 $TCID_{50}$ of the virus was exposed with effective minimal cytotoxic concentration of Nanosilver for one hr at 37°C. Then 100 µl of the mixture was added to the cells cultured fluently in 96-well flat-bottom microtiter plate. Following one hr incubation at 37°C, the supernatants were removed and the cells were washed with PBS. Then 100 µl of the TPCK-containing medium was added to each well. (Pre-penetration exposure)

2-100 $TCID_{50}$ of the virus (100 µl/well) was added to each well. After one hr incubation at 37°C the unabsorbed viruses were removed and cells were washed and 100 µl of Nanosilver diluted with TPCK-containing medium was added to each well (Post-penetration exposure).

3-100 $TCID_{50}$ of the virus and effective minimal cytotoxic concentration of Nanosilver were mixed and incubated at 37°C for one hr. The mixture was added to the cells (100 µl/well) and after one hr of incubation at 37°C and washing the cells, 100 µl of the compound was diluted with TPCK-containing medium and was added to each well (Pre and post-penetration exposure).

All plates including MDCK cells and influenza virus inoculated MDCK cells were incubated at CO_2-incubator for 48 hrs and the virus titration was carried out by HA. The viability of the infected and non-infected cells was evaluated using absorbance values of formazan. The percent of protection was calculated as follows:

Percent protection =
[(ODT) V-(ODC) V] / [(ODC) M-(ODC) V] ×100

Where (ODT) V, (ODC) V and (ODC) M indicate absorbance of the sample, the virus-infected control (no compound) and mock-infected control (no virus and no compound), respectively (10).

Hemagglutination assay

In order to assess the presence of the virus in cell culture, serial dilutions of the cell culture media were added to 96-well U-shape microplates. Chicken red blood cells (cRBCs) (0.5%) were added to each well. Following incubation at least for one hr at room temperature, precipitation of the RBCs demonstrated absence of the virus while hemagglutination indicated presence of the virus.

RNA Extraction

Viral genomic RNA was extracted from 100 µl of the virus inoculated cell culture media using AccuPrep® Viral RNA Extraction Kit (Bioneer, Seoul, Korea). Using this kit, RNA was bound to glass fibers fixed in a column and finally was isolated and eluted with 50µl of the elution buffer. Since the influenza A virus has been used in this study, Influenza B virus was added to the samples as internal control (100 µl).

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RT-PCR

Complementary DNA was synthesized from 10 µl of viral RNA using Superscript™III First-Strand Synthesis System for RT-PCR Kit (Invitrogen, Carslbad, CA). Ten microliters of µl RNA sample along with random hexamer primers and dNTP mix were incubated at 56°C for 5 min and were added to a mixture of buffer, RNase out and Super Script III RT as instructed in the kit. PCR reaction was performed according to the previously described method (11). Primers used for this study were amplified NS gene of influenza B and H_1 gene of influenza A. Finally PCR products (amplicons) were run on 1.5% agarose gel and visualized by ethidium bromide staining. PCR products were measured semi-quantitavely by the band densitometry ratio using "Image Tool" software.

RESULTS

Cytopathic Effect (CPE) of the virus on MDCK cells

A serial 10-fold dilution of the virus was prepared. MDCK cells grown in 96-well plate were inoculated by the dilutions. $TCID_{50}$ of the virus was calculated using Hemagglutination assay and Karber formula (12).

Table 1. Cytotoxicity of Nanosilver on MDCK cells by MTT test

Sample(µg/ml)	Mean ±SD
4	0.003±0.001*
2	0.084±0.005*
1	0.280±0.014*
0.5	0.543±0.018
0.25	0.547±0.002
0.125	0.553±0.002
0.06	0.565±0.002
0.03	0.565±0.002
0	0.565±0.017

Different concentrations of Nanosilver were added to proper MDCK cell culture. Following MTT assay, OD at 540 nm was determined.Values are averages of four independent examinations.

*: Significantly different from values obtained for Nanosilver treated compared to untreated sample ($p<0.0001$).

CC_{50} and effective minimal cytotoxic concentration of Nanosilver

Viabilities of the MDCK cells were determined at 48 hrs after exposure to the compound by reading optical densities at 540 nm. CC_{50} of this compound was deducted from the results of the figure 2 or calculated by MTT results about 1µg/ml. After determination of CC_{50} of the Nanosilver, effective minimal cytotoxic concentration of Nanosilver with no meaningful difference with control and no serious cytopathic effect on the cells was obtained 0.5µg/ml (Table 1 & Figure 2).

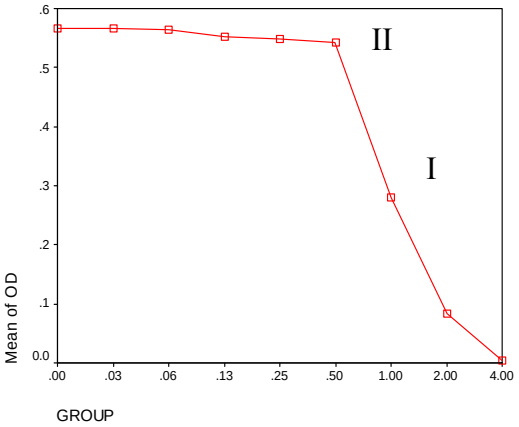

Figure 1. (I)CC_{50} and(II) effective minimal cytotoxic concentration of Nanosilver. Optical Density at 540 nm (Y axis) of different dilutions of the compound (X axis) was measured by MTT.

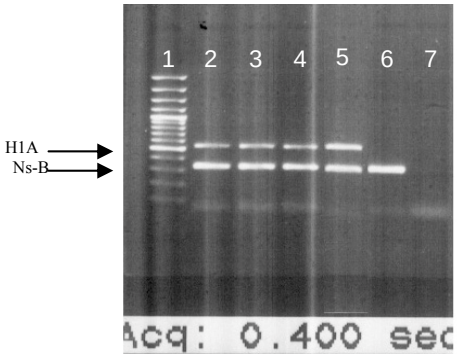

Figure2. RT-PCR products of Nanosilver-treated influenza viral RNA (Lanes 2-4) compared to untreated viruses (Lanes 5-6); Lane 1: DNA marker and Lane 7: blank.

Protection percentage

Two days after each experiment the viabilities of mock-infected and infected cells were evaluated by determination of absorbance of formazan at 540 nm wavelength. The percent of protection was calculated by the following formula:

Percent protection =
[(ODT) V-(ODC) V] / [(ODC) M-(ODC) V] ×100

Where (ODT) V, (ODC) V and (ODC) M indicates absorbance of the sample, the virus-infected control (no compound) and mock-infected control (no virus and no compound), respectively (10).

1-Percentage protection of the experiment I is 76.72%, 2-Percentage protection of the

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experiment II is 58.52%, 3-Percentage protection of the experiment 3 is 78.42%

Antiviral activity of Nanosilver against influenza virus A (H1N1)

Cell culture of the virus in the presence of the compound in different sets of experiments was assessed by hemagglutination assay. The inhibitory effect of the Nanosilver is shown by reducing HA titer (Table 2).

Table2: Hemagglutination assay of the virus cell cultures exposed to Nanosilver compared with the virus cell culture.

Group	Mean ± SD
V(10^{-1})	1.503±0.150
I	1.105±0.069*
II	1.453±0.097
III	0.987±0.111*

Values are averages of four independent examinations for HA assay calculated by Karber formula.

*: Significantly different from values obtained for Nanosilver treated compared to untreated sample ($p<0.0001$).

The results of RT-PCR

The effect of Nanosilver on the viral genome was shown by reduction in the content of the PCR product bands in gel electrophoresis (Figure 3). Quantitative analysis on PCR products using band densitometry software "Image Tool" and statistical analysis by Mann-Whitney U test showed statically meaningful decrease in genome content in direct exposure of Nanosilver to the virus (Figure 4).

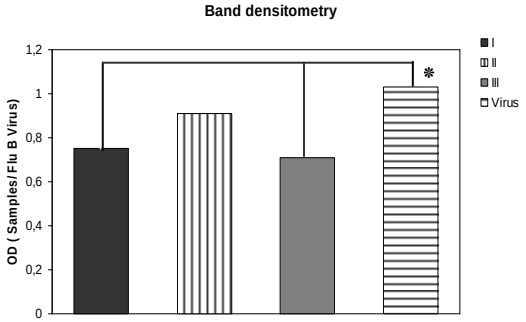

Figure3: Semi-quantitative analysis of the genome content in treated samples compared to untreated . The results showed meaningful difference ($p<0.05$) between samples with direct exposure of Nanosilver to virus (I: Pre-penetration exposure; II: Post-penetration exposure; III: Pre & post-penetration exposure) compared to untreated virus sample.

DISCUSSION

As long as influenza virus remains a serious cause of disease, there will be a need to identify and develop novel anti-influenza compounds with distinct mechanisms of action (13).The strong toxicity of silver in various chemical forms especially silver nitrate to a wide range of microorganisms is very well known and silver nanoparticles have shown to be a promising antimicrobial material (7).

The antiviral effect of Nanosilver for some viruses have been reported previously (14). In this study its inhibitory effect against influenza virus A/H_1N_1 was investigated. The capacity of silver nanoparticles to inhibit influenza infectivity was determined by MDCK cell culture of the virus. The CC_{50} value of this compound was found 1 µg/ml by MTT method and the effective minimal cytotoxic concentration with least cytopathic effects on the cells was found 0.5 µg/ml. Infection cycle of the virus involves fusion of viral and cellular membrane with subsequent transfer of viral genetic material into the cytoplasm. The virus envelope consisted of lipid bilayer interspersed with protruding glycoproteins: HA, NA and M_2. The glycoprotein involved in fusion is HA trimer, each consisted of two identical subunits: HA_1 and HA_2. HA_1 is exposed to the exterior and HA_2 transmembrane glycoprotein subunit spans the viral membrane and connects the exterior HA_1 with M_1 matrix protein. The main function of HA_1 is to bind with sialylated cell-receptor sites on host cells and are more exposed to the exterior and should be more accessible for potential Nanosilver interaction (15).

Each monomer of HA has two disulfide bonds, one of them links amino acids 14 (HA_1) and 137 (HA_2). These subunits undergo conformational changes by breaking disulfide bonds to make receptor binding sites available to interact with cell receptors (15). These exposed disulfide bonds would be the most attractive sites for Nanosilver to interact with the virus. The specificity of Nanosilver in its natural tendency to disulfide bonds plays an important role in the antiviral activity, therefore blocking the host receptor binding sites of the virus. This result can further extends previous findings that silver nanoparticles are useful for anti-HIV adsorption to the host cells (7). In this study inhibition of influenza virus adsorption to MDCK cells and chicken red blood cells by Nanosilver was investigated by MTT, HA and RT-PCR methods. High percent protection following exposure of Nanosilver to the virus cell culture by MTT method is in concordance with reduction of HA titer of the virus cell culture after incubation with Nanosilver. This inhibitory effect of Nanosilver was also observed in RT-PCR

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products by reducing the gel electrophoresis band content.

For all three kinds of treatments with Nanosilver at 0.5 µg/ml concentration, viral infectivity was reduced, while pre-penetration and pre and post-penetration were more effective ($p<0.05$) as it has been reported previously (16). The results show that Nanosilver may interfere whit viral membrane fusion by inhibition of penetration (17). However, its effect on the host cells that might be interference with fusion has also been suggested (18).

In order to analyze the target molecule to which Nanoailver interacts during the adsorption/penetration process, the inhibitory effect of Nanosilver against binding of antibodies to viral membrane glycoprotein knobs was also investigated. Result showed Nanosilver could inhibit interaction between glycoprotein knobs and antibodies to some extent. The decrease in the fluorescent was observed following addition of Nanosilver (data not shown).

Using biochemical and molecular methods to investigate the antiviral activity of Nanosilver, it is suggested that it could be a good candidate as disinfectant as well as antiseptic to prevent the viral infection.

Research on interaction of inorganic nonaparticles with biosystems has been started recently and its applications are going to be discovered. Knowing the exact Nanosilver effect on the virus provides the incentive for further research on the surface glycoprotein conformation following interaction with Nanosilver.

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