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Antiviral activity of silver nanoparticles against H1N1 influenza virus

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Abstract

Background Influenza virus is a significant cause of annual global respiratory disease and death. According to the limited availability of effective drugs and vaccines, innovative antivirals are currently being investigated as possible strategies to contain the spread of infectious agents. Among the various types of nanoparticles, silver nanoparticles (Ag-NPs) have attracted great interest due to their exceptional physicochemical properties. This study aims to investigate the antiviral activity of Ag-NPs against the influenza A virus (IAV)/H1N1.

Methods The MTT assay was used to determine the possible cytotoxicity of the Ag-NPs. Madin-Darby canine kidney (MDCK) cells were exposed to Ag-NPs extract in conjunction with 100 cell culture infectious dose 50% (CCID50) of virus administered at time intervals during the infection process. The antiviral activity of the extract was evaluated under pre-treatment, post-treatment, and simultaneous assay. Viral titer reduction was assayed using hemagglutination (HA) and CCID50 assays. Viral RNA relative quantification by real-time Polymerase Chain Reaction approach was performed in each experimental condition.

Results The study yielded significant findings regarding the inhibitory effects of Ag-NPs on the IAV/H1N1. Silver nanoparticles showed dose-dependent inhibition of the virus, with the strongest effect observed when administered simultaneously with the virus which the virus titer exhibited a substantial decrease from 5 Log10 in the control group to 1 Log10 in the initial samples, further reducing to 2 Log10 per milliliter at lower concentrations. Notably, Ag-NPs demonstrated a greater reduction in virus titer during the simultaneous stage, showing a statistically significant difference ($P < 0.05$) between the control and experimental groups). The reduction in viral titer was also evident in both pre- and post-inoculation stages, although the effects were different.

Conclusion Silver nanoparticles possess inhibitory effects on the growth of the IAV/H1N1, with a significant reduction in virus titer. These findings suggest the potential of Ag-NPs as effective antiviral agents and highlight opportunities for further research and potential clinical applications in combating IAV (H1N1) infections.

Keywords Influenza virus, Silver nanoparticles, Hemagglutination, Real-time PCR, MTT, CCID50, Anti-viral, Treatment

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Introduction

Influenza is a highly transmissible viral infection that affects the respiratory tract. Influenza viruses belong to the family *Orthomyxoviridae* and possess an ability to propagate rapidly, resulting in recurrent seasonal outbreaks and sporadic global pandemics with significant consequences [1, 2]. The public health impact of this virus is profound, manifesting in a substantial disease burden, increased hospitalizations, and, in severe cases, even mortality. The inherent property of the influenza virus lies in its propensity for frequent mutations affecting its surface proteins, specifically hemagglutinin (HA) and neuraminidase (NA). These changes referred to as antigenic shift, lead to novel viral strains that can evade the immunity conferred by previous infections or vaccinations. Consequently, the general population remains vulnerable to infections caused by these new strains, contributing to the recurrent cyclical occurrences of influenza outbreaks [3–5].

Influenza infections present a diverse spectrum of symptoms, ranging from mild respiratory unease to more severe illness. The virus predominantly targets the upper and lower respiratory passages, giving rise to symptoms encompassing fever, cough, sore throat, muscular discomfort, and fatigue. Although the majority of influenza cases demonstrate a tendency to improve with time and rest, the possibility of complications exists, particularly among susceptible groups such as infants, the elderly, pregnant women, and those with underlying medical conditions [6–8].

The influenza virus is constantly evolving, and new antigenic variants give rise to epidemics and pandemics. This virus is unique among respiratory viruses due to its considerable antigenic variations. These mutations make it exceedingly challenging to develop effective vaccines and drugs against the virus [9]. Therefore, it is imperative to use modern approaches to inhibit viral activity [10].

The term nana is derived from the Greek word meaning "dwarf." As a prefix, it implies a factor of 10^{-9}. A nanometer (nm) is a billionth of a meter, or roughly the length of three atoms side by side. Metal nanoparticles include silver, gold, alumina, copper, magnesium, titanium, and zinc [11]. Nanoparticles are particulate dispersions or solid particles with a size in the range of 10–100 nm. Among noble-metal nanomaterials, silver nanoparticles (Ag-NPs) have received considerable attention due to their attractive physicochemical properties for inhibiting viruses. Nanoparticles with a size in the range of 1–100 nm are most commonly utilized. Nowadays, viral infections constitute one of the major health problems. Chemically synthesized antiviral drugs have been associated with adverse side effects and other health complications [12, 13].

The ongoing exploration of influenza antivirals, with a focus on innovative solutions like Ag-NPs, plays a pivotal role in proactively addressing the ever-changing nature of the virus. These studies contribute to our understanding, the development of intervention, and ultimately the creation of a more resilient global health landscape.

In the present investigation, we examined the antiviral activity of the Ag-NPs on the influenza A virus (IAV)/H1N1. Our study uniquely evaluates Ag-NPs across three different treatment strategies—pre-treatment, post-treatment, and simultaneous treatment—while also comparing their efficacy to oseltamivir.

Materials and methods

Material preparation

Silver nanoparticle preparation

In this research, we utilized Ag-NPs with a remarkable purity of 99.99%. These nanoparticles, measuring 20 nm in diameter, were supplied in powder form with a total weight of one gram. The Silver Nano powder (metal basis) was sourced from US Research Nanomaterials, Inc. (Stock: US1038, CAS#: 7440-22-4). To facilitate their use, a solution with a concentration of 20 mg/ml was prepared in a DMEM (Dulbecco's Modified Eagle Medium). Considering the potential for contamination, sterilization was conducted using 0.22-micron filters.

Cell line and virus strain

The Madin-Darby canine kidney (MDCK-SIAT) cell line [obtained from National Influenza, center (NIC) Tehran, Iran] cultured in a mixture of DMEM, 10% heat-inactivated fetal bovine serum (FBS),100 µg/ml streptomycin, and 100 U/ml penicillin at 37 °C in a humidified atmosphere with 5% CO2. IAV (H1N1), 2009(H1N1)/California/07 strain was obtained from NIC. The IAV/H1N1 virus was propagated in MDCK cells in the presence of 0.5 mL of TPCK-trypsin (Sigma-Aldrich) (Tosylamide, Phenylethyl Chloromethyl Keton-treated Trypsin) stock solution containing 2 mg/mL, per mL of complete DMEM (without serum), resulting in a final concentration of 2 µg/mL for this study. The viral titer was standardized to 10^5 CCID50, ensuring consistency across experiments.

Cytotoxicity assay of Ag-NPs by MTT

The efficacy of Ag Nanoparticles against MDCK cells was measured using MTT (Sigma-Aldrich) (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) powder. MDCK cells were seeded at a density of 1×10^4 cells per well in a 96-well plate and incubated for 26 h. The medium was then replaced with DMEM containing various concentrations of Ag-NPs. After incubating the cells at 37 °C for 48 h, 100 µL of Roswell Park Memorial

Institute medium 1640 (RPMI 1640) (without phenol red) with 10 µL of MTT solution (5 mg/mL in phosphate-buffered saline) was added to each well, and the cells were incubated for 4 h. Then the supernatant was removed, and 50 µL of DMSO was added to each well and incubated for 30 min. The absorbance was recorded at a wavelength of 540 nm using a microplate reader.

Inoculation of virus preceding oseltamivir by 1 Hour

Oseltamivir tablets (Roche) were prepared into a powder and then converted into a liquid form using a DMEM medium containing antibiotics and trypsin without serum. To improve the solubility of the drug a concentration of 1.0% DMSO was also used. To sterile this solution, 0.22-micron filters were used, and the sterilized solution was stored in sterile glass containers at a temperature of 4 °C.

MDCK cells were cultured at 37 °C in a CO2 incubator for 48 h. Then, DMEM was aspirated from the cells, and washed twice with PBS. Subsequently, 100 µL of medium containing IAV/H1N1 was added at a multiplicity of infection (MOI) of 0.1. After a 1-h incubation, the medium was removed, and the cells were washed twice with PBS. Next, 100 µL of medium containing different concentrations (0.005, 0.01, 0.05, 0.1, and 0.5 mg/mL) of the synthetic antiviral drug oseltamivir was added. The cells were then maintained in a 37 °C incubator with 5% CO2 for 48–72 h, and cytopathic effects (CPE) were examined daily using an inverted light microscope. The medium was then collected from the cells for virus titer determination experiments and stored at −70 °C in a freezer for preservation.

IAV (H1N1) and Ag-NPs inoculation

DMEM medium with trypsin and antibiotics without serum was used to investigate the antiviral effect of Ag-NPs against IAV/H1N1. Three experimental groups were designed as pre-treatment, post-treatment, and co-treatment tests. For antiviral experiments, IAV/H1N1 was used with an MOI of 0.1. All experiments performed in triplicate.

Inoculation of IAV (H1N1) preceding Ag-NPs by 1 hour (pre-treatment)

MDCK cells were cultured in 24-well plates at 37 °C in a CO2 incubator with 5% CO2 using DMEM medium. The confluent monolayer of MDCK cells was inoculated with 100 µL of IAV/H1N1 containing medium (MOI = 0.1). After 1 h, the viral medium was removed, and then 100 µL of medium containing different concentrations of Ag-NPs (0.2, 0.5, 1, and 2 mg/mL) were added. The cells were incubated at 37 °C in a CO2 incubator for 48–72 h, and CPE was examined daily using a light microscope.

Inoculation of IAV (H1N1) following Ag-NPs by 1 hour (post-treatment)

100 µL of medium containing different concentrations (0.2, 0.5, 1, and 2 mg/mL) of Ag-NPs was added to MDCK cells. After 1 h, the medium was removed, and the cells were washed twice with PBS. Then, 100 µL of IAV/H1N1 containing medium (MOI = 0.1) was added. The cells were maintained in a 37 °C incubator with CO2 for 48–72 h, and CPE was examined daily using an inverted light microscope.

Simultaneous inoculation of IAV (H1N1) and Ag-NPs (Co-treatment)

100 µL of IAV/H1N1 containing medium (MOI = 0.1) was combined with 100 µL of various concentrations (0.2, 0.5, 1, and 2 mg/mL) of Ag-NPs, which had been pre-incubated at 4 °C for 1 h. This mixture was added to the cell culture and incubated for 48–72 h at 37 °C in a CO2 incubator with 5% CO2. Cytopathic effects (CPE) were assessed daily using an inverted light microscope.

Hemagglutination assay (HA)

After observing CPE (cell rounding and plaque formation), the cell culture supernatant was used for HA assay. For this purpose, 96-well plates with V-shaped wells were used. First, 50 µL of PBS was added to each well. Then 50 µL of each of the 5 different concentrations of cell culture supernatant inoculated with IAV/H1N1 were added to the first well. After mixing, 50 µL was transferred from the first well to the second well, and this dilution process was continued to the last well, where 50 µL was discarded. A solution of 0.5% chicken red blood cells (RBCs) in PBS buffer was added to the wells. After 60 min of incubation, the results were observed. This assay was performed separately to determine the virus titer in each of the 5 concentrations of Ag-NPs in three experimental groups at different time points.

CCID50 assay

This test was separately conducted to assess the virus titer at each of the 4 concentrations of Ag-NPs within three experimental groups at different time points. All the collected supernatants were tenfold serially diluted and added to 96-well plates with 80% confluent cells in triplicate. Three days following infection, the plates were scored for cytopathic effects. The Spearman-Karber statistical method was utilized for calculating the virus titer in this test. Determination of 50% endpoint titer using a simple formula regarding the following research [14].

RNA extraction and real-time PCR

RNA extraction from the cell culture supernatant was performed from 200 µL samples using a High Pure Viral Nucleic acid kit (Roche, Germany) according to the manufacturer's instructions. Nucleic acid was eluted in 50 µL of supplied elution buffer. Quantitative real-time PCR was used to determine the potential Ag-NPs effect on genome replication. Real-time PCR was employed using the RNA extracted from the cell culture supernatant samples obtained after inoculating various concentrations of Ag-NPs into the cell culture containing IAV (H1N1). The reaction mixture was prepared according to the kit's instructions (Invitrogen, USA) and was subjected to Real-time PCR using the Corbett 6000 Rotor-Gene instrument. Briefly, 4 µL of template RNA (0.01pg-1 µg) was added to the 10 µL Master Mix (10X), 0.4 µL each primer (40µm), 0.4 µL probe (10µm), 0.4 µL Taq Mix III RT/Platinum Super Script, 5.4 µL nuclease-free water. The thermal cycling for real-time PCR program is outlined in Table 1. Specific Primers and probes for RT-qPCR reaction were designed for the gene encoding the M protein of influenza A virus (Table 2). A threshold cycle (CT) value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit (10 times the standard deviation of the baseline). A plasmid containing the M gene was constructed from the PBR322 vector for positive control. A standard graph of the CT values obtained from serially diluted M gene-PBR322 was created. The CT values from clinical specimens were plotted on the standard curve, and the copy number was calculated. Each sample was tested in triplicate, and the mean of the three values was shown as the viral genome copy number. A sample was defined as negative if its CT value exceeded 40 cycles.

Table 1 The thermal cycling program specifications used in real-time PCR

Cycle count	Time	Temperature (°C)	Real-time PCR steps
1	30 min	50	cDNA synthesis
	2 min	95	Initial denaturation
45	15 s	95	Denaturation
	30 s	58	Primer annealing
	30 s	72	Extension

Statistical analysis

All statistical analyses were performed using GraphPad Prism software. For analyzing the differences between groups, a One-way Analysis of Variance (ANOVA) was employed. The significance level was set at $P < 0.05$. Each experiment was repeated at least three times independently to ensure reproducibility.

Results

Cell viability assay analysis

Using the MTT metabolic assay, Ag-NPs exhibit a cell viability higher than 90% starting from a concentration of 2 µg/ml. Figure 1 illustrates the impact of varying concentrations of Ag-NPs on cell viability.

Effects of oseltamivir on IAV (H1N1)

No evidence of virus growth was observed in the treated samples, and all of these samples were reported with a log10 value of zero. Considering the pronounced contrast with the control group, which has a CCID50 difference, it can be inferred that oseltamivir exerts a definitive inhibitory effect on the growth of IAV (H1N1) (Fig. 2a).

Based on the results obtained from the post-inoculation of oseltamivir after viral inoculation into cell culture, the IAV/H1N1 titer decreased from 53 HAU/50 µL in the control group to zero. This reduction was consistently maintained at zero in the subsequent samples, indicating the inhibitory effect of oseltamivir on the growth of IAV (H1N1) (Fig. 2b).

Furthermore, the results of the real-time PCR test at this stage are shown in Fig. 2c. The number of viral genome copies was higher in the control group compared to the experimental group. Oseltamivir exhibited a reduction in the titer of IAV/H1N1 at all concentrations used.

Inhibitory effects of Ag-NPs on IAV (H1N1)

Inhibitory effect of Ag-NPs one hour before inoculation of IAV (H1N1) on cell culture

The results of the virus titer assessment using the CCID50 test before virus inoculation revealed that the utilized nanoparticles led to a decrease in virus titer from

Table 2 Primers and probe used for Influenza-A virus detection in the study

Primers and probes	Sequence (5′−−>3′)	Concentration	Product size
InfA forward	GAC CRA TCC TGT CAC CTC TGA C	40 Mµ	175bp
InfA reverse	AGG GCA TTY TGG ACA AAK CGT CTA	40 Mµ	
InfA probe	TGC AGT CCT CGC TCA CTG GGC ACG	40 Mµ	

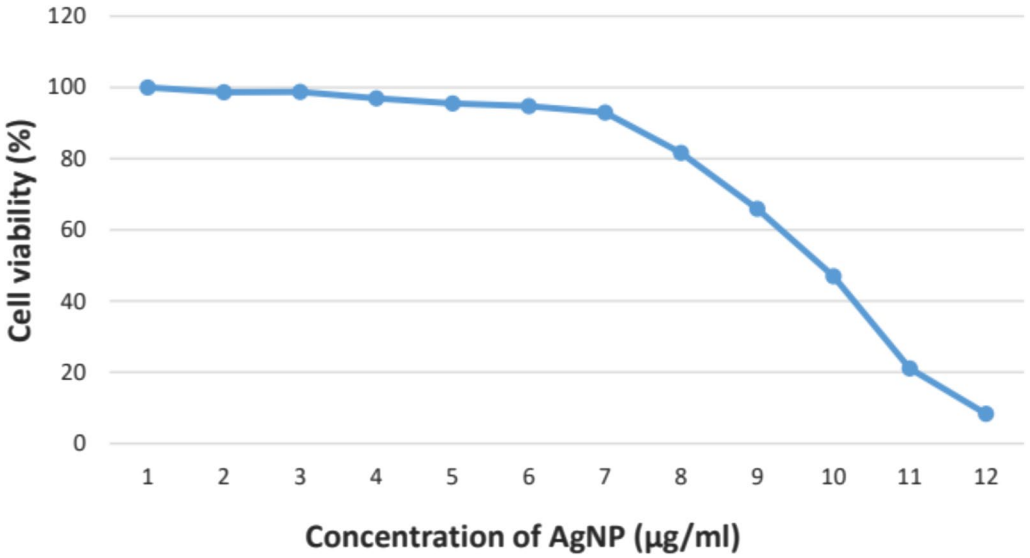

Fig. 1 This curve illustrates the impact of varying concentrations of silver nanoparticles (Ag-NPs) on cell viability, assessed using the MTT assay. The data clearly demonstrate that at the concentrations tested in our study, cell viability remains high

5 Log10 in the control group to 0.5 Log10 in the initial samples and further to 4 Log10 per milliliter at lower concentrations. The most significant reduction in titer was observed at concentrations of 2 µg/ml (Fig. 3a). After observing the CPE of the cell culture supernatant was utilized for the HA test. In the HA test, the virus titer, calculated based on HAU/50 µL, decreased from 298 in the control group to 18 HAU/50 µL at the utilized concentrations. Hence, it can be concluded that the initial concentrations of Ag-NPs led to a reduction in virus titer at this stage (Fig. 3b). Silver nanoparticles caused a reduction in virus titer from 5.421 log10 in the control group to 1.146 log10 at the initial concentrations. Interestingly, the number of viral genome copies was higher in the control group compared to the experimental group. Therefore, according to the obtained results, Ag-NPs at this experimental stage led to a decrease in virus titer (Fig. 3c).

Inhibitory effect of Ag-NPs one hour after the inoculation of IAV (H1N1) on cell culture

In this test, the virus titer decreased from 5 log10 in the control group to 1 log10 in the initial samples and further to 4 log10 per milliliter at lower concentrations. The results indicated that Ag-NPs did not have a significant impact on reducing the virus titer at this stage. Based on HA results obtained from the inoculation of Ag-NPs into the cell culture following the virus, there was no significant reduction in virus titer. Specifically, the virus titer in the control group changed from HAU 298 to HAU 85 per microliter at the initial concentration of nanoparticles and to HAU 213 at the final concentrations used (Fig. 4a, b). Based on real-time PCR, Ag-NPs caused a reduction in virus titer from 5.421 log10 genomic copies in the control group to 3.648 log10 at the initial concentrations. Moreover, at the final concentrations, the virus titer reached 4.120 log10 genomic copies. Based on the obtained results from this test, the use of nanoparticles at this stage did not lead to a very significant reduction in virus titer (Fig. 4c).

Inhibitory effect of simultaneous inoculation of IAV (H1N1) and Ag-NPs

The virus titer decreased from 5 Log10 in the control group to 0.5 Log10 in the initial samples and further to 2.5 Log10 per milliliter at lower concentrations. The results of the virus titer assessment in the CCID50 test during the simultaneous stage demonstrated that Ag-NPs at this stage led to a greater reduction in virus titer at the utilized concentrations. Additionally, a statistically significant difference ($P < 0.05$) exists between the control and experimental groups (Fig. 5a).

In the context of HA, the virus titer decreased from HAU/µL 298 to 9 per microliter at the initial concentrations. The results of the HA test at this stage indicated a reduction in virus titer by Ag-NPs, with the average virus titer in the control group significantly higher than the experimental groups (Fig. 5b).

As for real-time PCR analysis, Ag-NPs led to a reduction in virus titer, such that the number of virus genomes decreased from 5.421 log10 in the control group to zero at the initial concentrations, and to 3.033 log10 genomic copies at the final concentrations. The number of virus

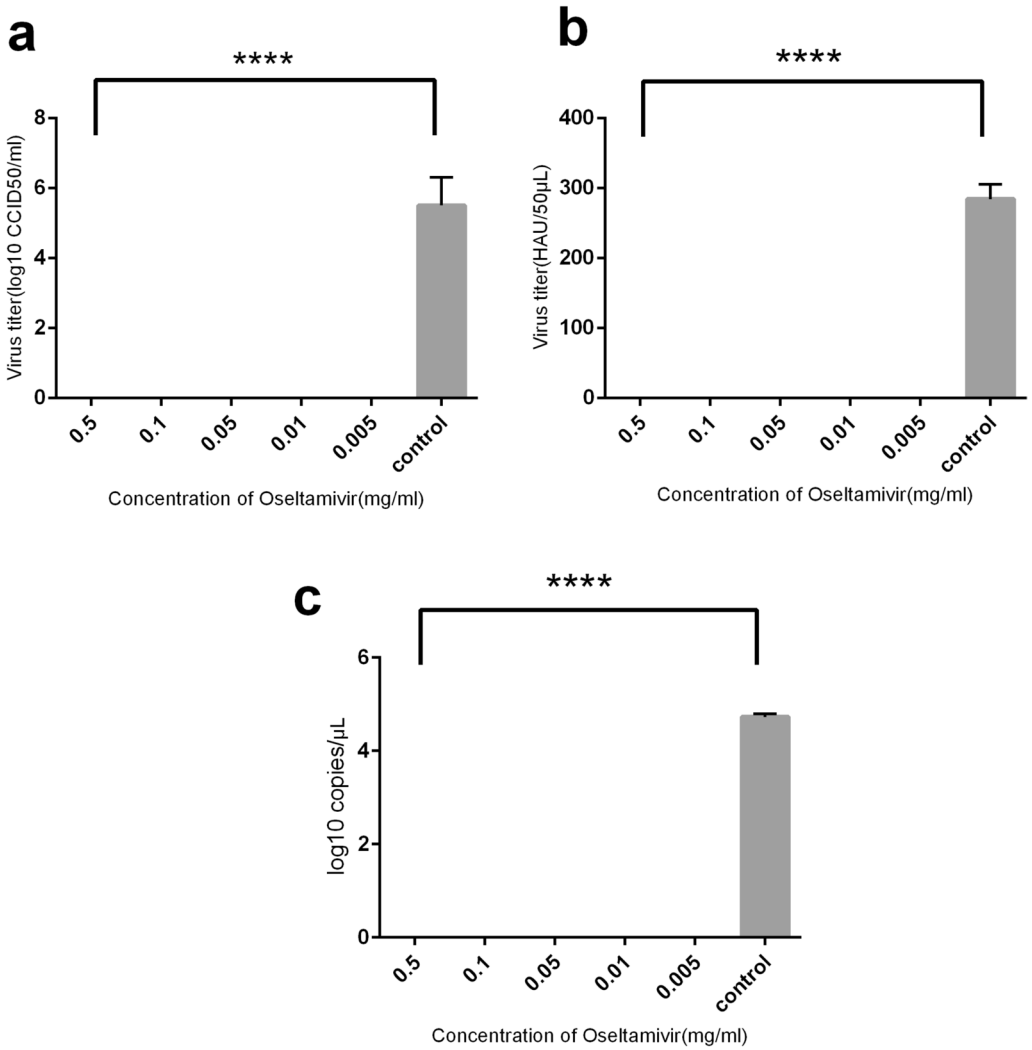

Fig. 2 **a** MDCK cells infected with influenza A virus/H1N1 at an MOI of 0.1 were treated in triplicate with different concentrations (0.005, 0.01, 0.05, 0.1, 0.5 mg/mL) of oseltamivir. Oseltamivir demonstrated a significant inhibitory effect on influenza A virus/H1N1 replication compared to the control group, as indicated by differences in CCID50. **b** Impact of oseltamivir at different concentrations (0.005, 0.01, 0.05, 0.1, 0.5 mg/mL) on the growth of influenza A/H1N1 virus following virus inoculation (MOI: 0.1) into MDCK cells. The influenza A virus (H1N1) titer decreased from 53 HAU/50 µL in the control group to zero, consistently maintained at zero in subsequent samples, demonstrating oseltamivir's inhibitory effect on virus growth. **c** MDCK cells infected with influenza A virus/H1N1 at an MOI of 0.1 were treated in triplicate with different concentrations (0.005, 0.01, 0.05, 0.1, 0.5 mg/mL) of oseltamivir. Real-Time PCR analysis revealed higher viral genome copies in the control group compared to the experimental group. Oseltamivir reduced influenza A virus (H1N1) titers at all tested concentrations. Statistical significance was determined at $p < 0.05$ for all tests

genome copies in the control group was higher than in the experimental groups (Fig. 5c).

Discussion

Given that the influenza virus remains a significant contributor to respiratory infections, there is a continued need for the discovery and development of innovative anti-influenza agents that act through distinct mechanisms. The powerful antimicrobial properties of silver, particularly in various chemical forms including silver nitrate, are well-established. Silver nanoparticles in particular have proven to be a promising material for combating microorganisms [15]. While previous research has highlighted the antiviral potential of nanoparticle of silver against certain viruses, this study looks at its inhibitory effects against the IAV (H1N1) [16]. The investigation focused on assessing the ability of Ag-NPs to contain influenza infectivity using MDCK cell culture of the virus. Notably, the inhibitory effect of Ag-NPs was also evident in real-time PCR. The study found that all three

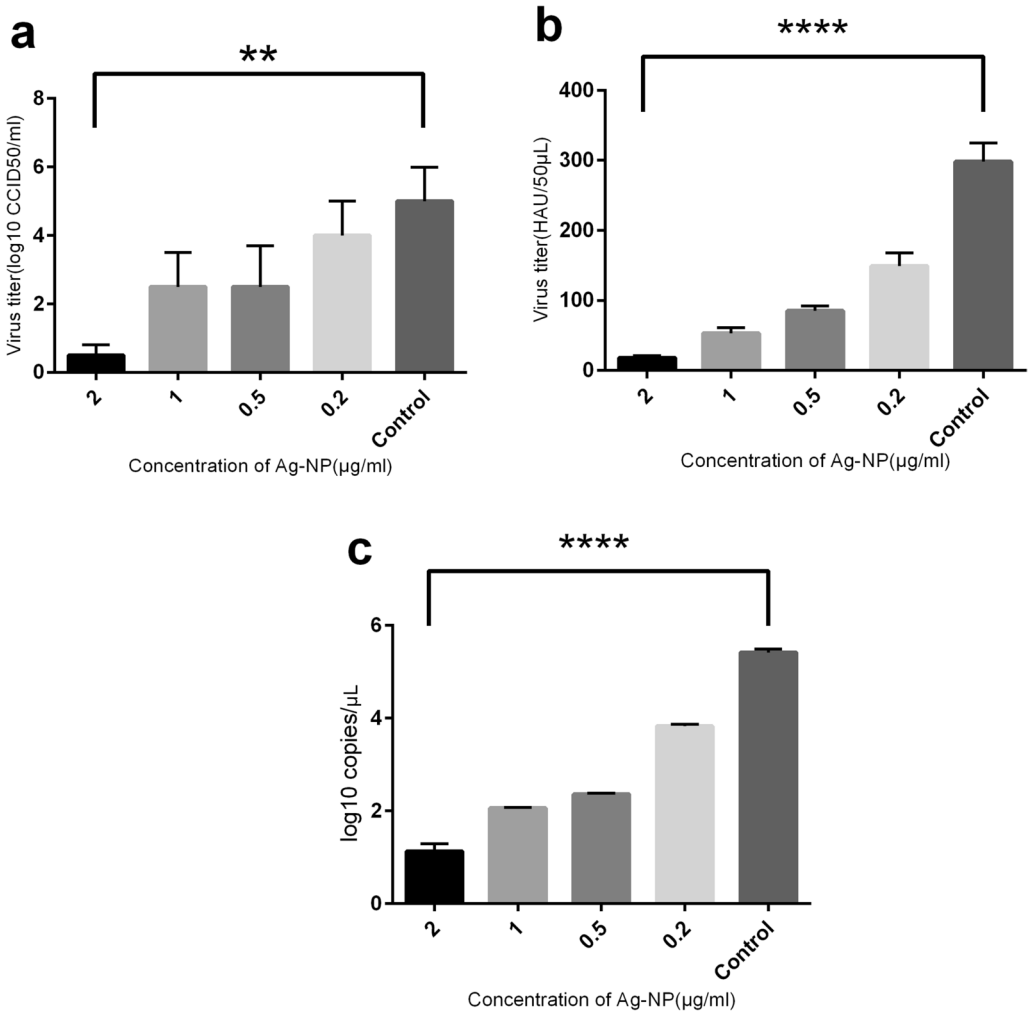

Fig. 3 **a** Effect of silver nanoparticles one hour before virus inoculation into cell culture assessed by CCID50 assay. Ag-NPs led to a reduction in virus titers compared to the control group, with the most significant reduction observed at concentrations of 1 and 2 µg/ml. **b** Impact of Ag-NPs one hour before virus inoculation into cell culture assessed by HA assay. The virus titer, calculated based on HAU/50 µL, decreased significantly compared to the control at the utilized concentrations. **c** Impact of silver nanoparticles one hour before virus inoculation into cell culture assessed by Real-Time PCR analysis conducted in triplicate. Ag-NPs showed a notable reduction in virus titers. Statistical significance was determined at $p < 0.05$ for all tests

levels of Ag-NPs treatment (at concentrations of 0.2, 0.5, 1 and 2 µg/ml) resulted in reduced viral infectivity. Notably, treatments applied before and after viral penetration showed a more pronounced effect (with a significance level of $p < 0.05$). The intriguing molecular basis of the antiviral influence of Ag-NPs on influenza virus warrants further exploration and provides a compelling approach for future investigations. There are studies related to Ag-NPs and materials, that report effectiveness against various viral infections [17, 18]. The most commonly recognized mechanisms that account for the antiviral effects of AgNPs involve the destruction of viral particles (virucidal activity), prevention of virus entry, and suppression of viral replication within the host cell [19]. When Ag-NPs are administered prior to viral exposure, they may stimulate the host cells to activate antiviral pathways. Moreover, Ag-NPs may directly interact with the influenza virus particles, potentially disrupting their structure or function. This interaction could inhibit the virus's ability to attach to host cells or penetrate them effectively. AgNPs have been employed as adjuvants in various vaccine formulations, such as intrapulmonary flu vaccines, highlighting their potential to improve adaptive immune responses [20–22].

Recent advances in nanotechnology have generated great interest in the potential applications of Ag-NPs as

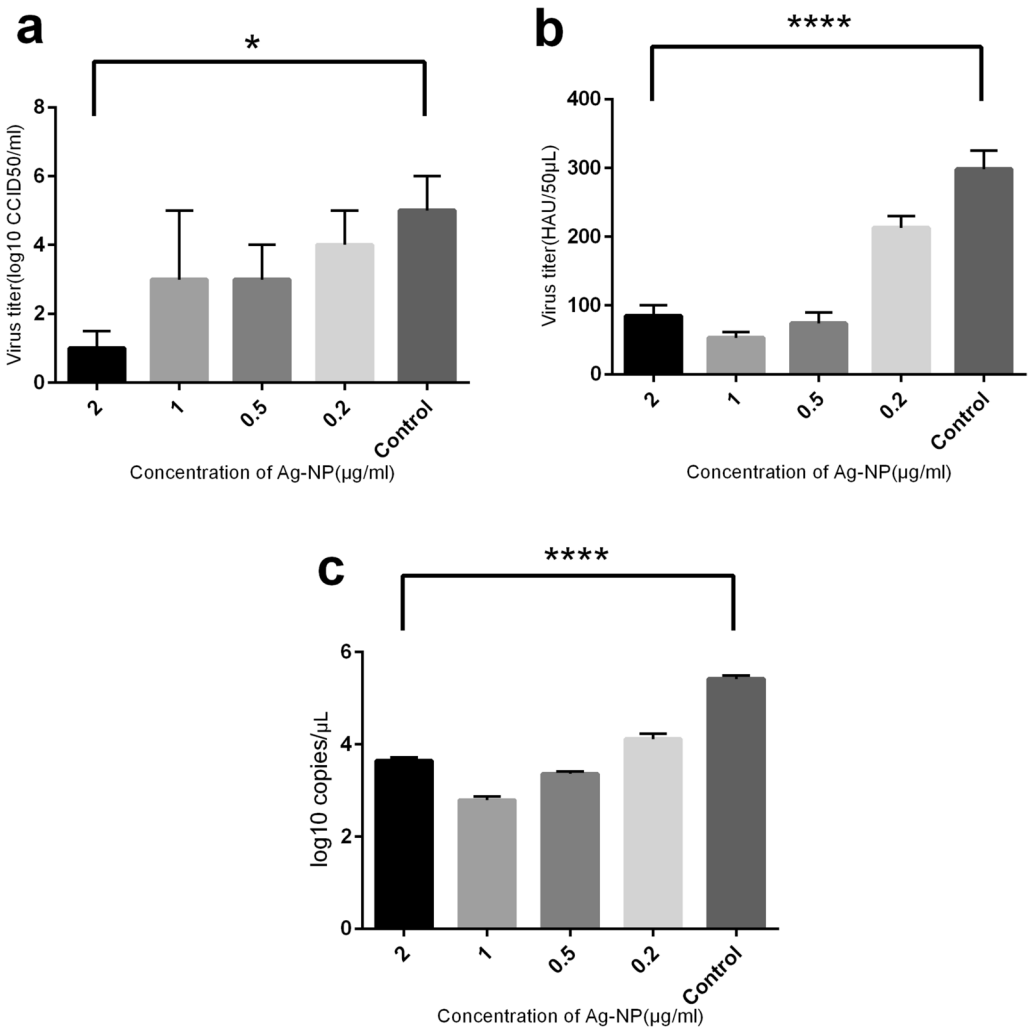

Fig. 4 **a** Effect of silver nanoparticles one-hour post-virus inoculation into cell culture assessed by CCID50 assay. Virus titers decreased from 5 Log10 in the control group to 3.25 Log10 initially and to 4 Log10 per milliliter at lower concentrations. At this stage, Ag-NPs did not significantly reduce virus titers. **b** Impact of silver nanoparticles one-hour post-virus inoculation into cell culture assessed by HA assay. HA results showed no significant reduction in virus titers following Ag-NPs treatment. Specifically, virus titers changed from 298 to 85 HAU per microliter at initial nanoparticle concentrations and to 213 HAU at final concentrations. **c** Impact of silver nanoparticles one-hour post-virus inoculation into cell culture assessed by Real-Time PCR analysis conducted in triplicate. Ag-NPs showed minimal impact on virus titers. Statistical significance was determined at $p < 0.05$ for all tests

antiviral agents. Adenoviruses, known to cause respiratory, ocular, and gastrointestinal diseases, pose a challenge due to the lack of specific treatment options [23]. Our findings agree with a study aimed to investigate the impact of Ag-NPs on Ad3. The results indicated that HeLa cells infected with Ad3 and treated with Ag-NPs exhibited enhanced cell viability and reduced cytopathic effects. Differences in fluorescence intensity further supported the inhibitory effect, with transmission electron microscopy revealing direct damage to Ad3 particle structures. The study suggested that Ag-NPs could potentially serve as an antiviral against Ad3 infections and expand their repertoire beyond known viruses [24]. Silver nanoparticles have demonstrated efficacy against human immunodeficiency virus (HIV) and hepatitis B virus (HBV). In a novel investigation, researchers explored the interaction between Ag-NPs and the IAV (H1N1). Using various tests, including HI and embryo inoculation, they found that Ag-NPs with a diameter of 10 nm exhibited an inhibitory effect against H1N1. Electron microscopy and flow cytometric analysis indicated reduced apoptosis induced by the influenza virus in MDCK cells. This study suggests that Ag-NPs hold promise as an early prevention

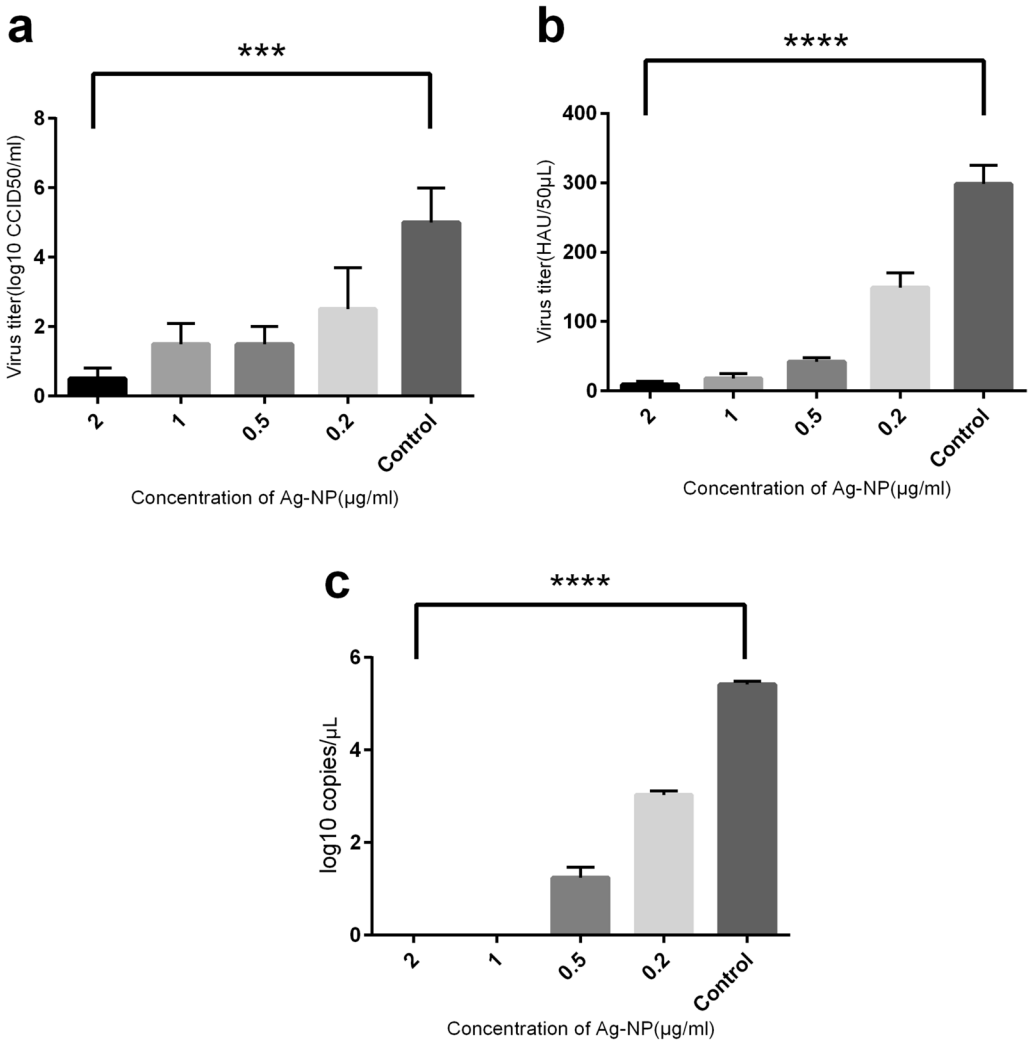

Fig. 5 Effect of silver nanoparticles on H1N1 influenza virus replication across different assays. **a** CCID50 assay: Ag-NPs significantly reduced virus titers from 5 Log10 in the control group to 1 Log10 in initial samples and further to 2 Log10/ml at lower concentrations ($P < 0.05$). **b** HA assay: Virus titers decreased from 298 HA Units/µL in the control to 9.3 HA Units/µL initially upon treatment with Ag-NPs, showing a significant reduction compared to the control ($P < 0.05$). **c** Real-time PCR analysis showed that Ag-NPs reduced virus genome levels to undetectable levels initially in the control group and to lower levels at reduced concentrations. The control group exhibited significantly higher virus genome levels compared to the experimental groups ($P < 0.05$). All experiments were conducted in triplicate

strategy against IAV (H1N1) infection [25]. The emerging field of nanomedicine offers potential solutions to the increasing threat of viral infections. Ag-NPs have garnered attention due to their antimicrobial properties. While designing antiviral drugs that target viruses without harming host cells is complex, Ag-NPs have shown potential in this regard. A comprehensive review highlights the diverse characteristics of Ag-NPs, including their antiviral activity, characterization methods, mechanisms, applications, and toxicity. The review underscores the potential of Ag-NPs as antiviral agents, shedding light on their role in combating viral infections while minimizing harm to host cells [19]. Taking a unique approach, a study delved into copper metabolism as an antiviral target using Ag-NPs. By reducing holo-ceruloplasmin levels, Ag-NPs exhibited potential antiviral effects. Different treatment regimens with Ag-NPs were tested in a mouse model of influenza virus A infection [26]. Notably, the study by Irina V. Kiseleva et al., showed that intranasal administration of Ag-NPs before influenza virus infection provided the most significant anti-influenza effects in mice. Intraperitoneal administration of Ag-NPs before infection also showed antiviral benefits but to a lesser degree than

the intranasal pre-treatment. Their study proposed the indirect antiviral potential of nano silver, opening new avenues for antiviral drug development [26]. Amidst the ongoing battle against coronavirus infections, silver nanomaterials (Ag NMs) have gained attention for their antimicrobial properties. In a study focusing on the transmissible gastroenteritis virus (TGEV), a significant coronavirus causing severe diarrhea in pigs, various Ag NMs were evaluated for their inhibitory effects. The results showed that Ag-NPs and silver nanowires significantly reduced TGEV-induced apoptosis in swine testicle cells through the regulation of signaling pathways. These findings suggest the potential of Ag NMs as effective agents in preventing cell infection and offer insights into novel antiviral therapies against coronaviruses [27]. As current antiviral drugs reach their limitations, Ag-NPs have emerged as potential alternatives due to their antibacterial and antiviral properties. One study investigated the inhibitory effect of Ag-NPs on bovine herpesvirus-1 (BoHV-1) replication. Safe concentrations of Ag-NPs were determined, and their protective effects against BoHV-1 infection were demonstrated. Notably, Ag-NPs were found to inhibit BoHV-1 when administered before viral infection. This study suggested that Ag-NPs could be used to protect cellular cultures from viral replications, offering a new avenue for antiviral interventions [28].

In the ever-evolving landscape of antiviral research, Ag-NPs have emerged as versatile and promising candidates for combating a range of viral infections. From adenoviruses to influenza A and coronaviruses, Ag-NPs have showcased their ability to inhibit viral replication, attenuate cytopathic effects, and potentially act as virucidal agents. These nanoparticles, with their distinct mechanisms of action, offer a novel avenue for antiviral intervention that extends beyond the limitations of current treatments. The introduction of Ag-NPs into the respiratory system may elicit an inflammatory response. While some inflammation is a natural part of the immune response to infection, excessive inflammation can lead to tissue damage and exacerbate respiratory conditions. The balance between effective antiviral action and inflammatory response is crucial [29]. Although studies have shown that Ag-NPs can maintain cell viability at certain concentrations, there is a potential risk of cytotoxicity at higher doses [30]. Further investigations into their mechanisms of action, dose-dependent effects, and potential clinical applications will likely uncover new insights into their role as powerful antiviral tools. With the ongoing challenges posed by viral infections, the remarkable properties of silver-associated materials offer a beacon of hope in the ongoing quest to combat these formidable adversaries.

Conclusion

In conclusion, this study evaluated the impact of Ag-NPs on the growth of IAV (H1N1) in MDCK cell culture, utilizing CCID50, HA, and real-time PCR tests. The Ag-NPs demonstrated its effects at various stages of virus replication. Therefore, in this study, the nanoparticles were introduced into the cell culture. Based on the results obtained, the use of nanoparticles in the post-virus inoculation stage did not have a significant impact on reducing the virus titer. Furthermore, the study revealed that in the pre-inoculation stage, these nanoparticles could effectively reduce the virus titer at initial concentrations. Simultaneous inoculation of the virus and nanoparticles in cell culture showed a more significant reduction in the influenza virus titer compared to the other two stages. In this experimental group, a reduction in virus titer was observed using all three measurement methods (CCID50, HA, real-time PCR). Therefore, considering the results of this study, nanoparticles had a more pronounced effect in the pre-inoculation stage and simultaneous inoculation stage, while their impact in the post-inoculation stage was not substantial.

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Author contributions
M.S, A.L, F.E: methodology, investigation, writing original draft. N.P, S.SM: review and editing, validation, investigation. A.M: supervision, conceptualization, review and editing.

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Availability of data and materials
Data will be available on reasonable request from corresponding author.

Declarations

Ethics approval and consent to participate
Ethical approval was obtained from Shiraz University of Medical Sciences (IR.SUMS.REC.1393.S6967). A consent letter was not needed due to this experiment was only cell culture-based and no human subject have participated in this research.

Consent to participate
No human subjects have participated in the study.

Competing interests
The authors declare no competing interests.

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