Inhibitory Activity and Mechanism of Silver Nanoparticles Against Herpes Simplex Virus Type 1

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Abstract

Herpes simplex virus type 1 (HSV-1) is a common pathogen that infects 50-90% of the world's population and causes a variety of diseases, some of which can be life-threatening. Silver nanoparticles (AgNPs) have been proven to have broad spectrum antiviral activity. In this study, we investigated the activity of AgNPs against HSV-1 by means of some antiviral tests and transmission electron microscopy (TEM). The results of antiviral tests demonstrated that AgNPs effectively inhibited the plaque formation and progeny production of HSV-1, reduced the genomic load and interfered with the mRNA expression and the protein synthesis of HSV-1. TEM observed that AgNPs could interact with HSV-1 and deform the HSV-1 particles. Furthermore, AgNPs could affect HSV-1's entry into cells, HSV-1's release and cell-to-cell spread. AgNPs could also down-regulate the expression of pro-inflammatory cytokines upon HSV-1 infection. In addition, the combined treatment of AgNPs and Acyclovir (ACV) confirmed that AgNPs could significantly enhance the inhibitory effect of ACV against HSV-1. Our findings offer a new insight into clarifying the mechanism of AgNPs against HSV-1, thus providing a theoretical basis for its clinical application.

1 Introduction

Herpes simplex virus type 1 (HSV-1) is a double-stranded DNA-enveloped virus which belongs to the herpes simplex virus genus of the Herpesviridae family [1]. The genome of HSV-1 contains approximately 150k nucleotides and encodes about 80 proteins [2, 3]. When infecting the host cell, HSV-1's genome is released into the the cell's nucleus to start the expression of viral genes, which is subjected to a precise regulation process, proceeding in a cascade manner. The HSV-1 genes generally include three types: immediate early genes (IE or α gene), early genes (E or β gene) and late genes (L or γ gene) [4]. The IE genes (ICP0, ICP4, ICP22, ICP27 and ICP47) are the first to be expressed, whose products are used to promote and regulate the efficient expression of the early and late genes [5]. Promoted by IE genes' products, E genes begin to express around 2-8 h after HSV-1 infection. The products of E genes such as UL5, UL8, UL29, UL30, UL42 and UL52 are necessary for viral DNA replication and synthesis [6]. Likewise, regulated by the IE genes' proteins, L genes start expressing the mainly viral structural proteins, such as gB, gD, gH, VP16, VP26, etc. [2]. The entire replication process of HSV is about 18-20 h, including adsorption, penetration, uncoating, biosynthesis, release and other processes, ultimately forming mature virus particles and releasing from the host cells[3].

HSV-1 is one of the most common viruses in the world, infecting about 50%-90% of the population [7]. In the adults, the seroprevalence of HSV-1 is approximately 90% [8]. About 3.7 billion people are infected with HSV-1 in the world, which is equivalent to 61% of the world's population [9]. The primary infection of HSV-1 can cause some clinical symptoms, such as oral and facial herpes, keratitis and peripheral nervous system disorders, and even some life-threatening symptoms like acute necrotizing encephalitis [10–12]. After the primary infection, HSV-1 can reach sensory ganglia such as the trigeminal ganglion through the peripheral nerves, establishing latent infection [13–15]. When the body is locally stimulated or the immunity is impaired, the latent viruses can be activated, resulting in recurrent local herpes [16, 17]. In addition to latent infection, recent studies have shown that HSV infection can suppress the host's

immune attack and thus achieve immune evasion [18–20], which is an important reason why it is not easily cleared by the body. Currently, drugs for anti-HSV commonly used in clinical practice are nucleoside analogs such as acyclovir (ACV), penciclovir, famciclovir, etc. [21, 22], among which, acyclovir is considered to be the gold standard for the treatment of HSV-1 infection [23, 24]. Phosphorylated by viral thymidine kinase and cellular kinase, ACV can selectively inhibit the virus' DNA polymerase to prevent its replication, and ultimately block the production of HSV-1 progeny [25, 26]. However, there are growing evidences that excessive use of nucleoside analogs including ACV can spur the emergence of resistant mutants of HSV-1[27]. It has been revealed that the mutations, insertions or deletions in the HSV-1 genome could induce viral resistance, which was extremely unfavorable to traditional antiviral drugs [28]. Increasing common drug-resistant HSV-1 strains have created a huge obstacle to the successful cure of HSV-1 infection [29]. In addition, there are some reports showing that long-term treatment of ACV could cause many side effects, such as nausea, vomiting, abdominal pain, diarrhea, skin rash, nerve poisoning, etc. [30, 31]. With the growing resistance of HSV-1 to traditional drugs and HSV vaccine has currently been unvailable for global market, research and development of new and efficient anti-HSV-1 drugs become very urgent.

Nanotechnology has been employed to exploit both advanced and functional nanoscale materials, and the its continuous progression in recent years has brought unique opportunities for nanoparticle drug development [32, 33]. Nanoparticles have aroused great interest and shown potential application value in medical diagnosis and treatment [34, 35]. AgNPs are metallic silver element with diameters at nanometer scale, which exhibit broad spectrum and high efficiency of antimicrobial properties due to their excellent physical, chemical and optical properties [36]. At present, a variety of metal nanoparticles, such as copper [37] and gold nanoparticles [38], have been studied as antimicrobial agents, but AgNPs display the most efficient antimicrobial effects [39]. Current studies on AgNPs have confirmed AgNPs' characteristics of anti-bacterium [40, 41], anti-fungus [42, 43], anti-virus[44, 45], anti- protozoon[46], anti-arthropod[47] and anti-tumor[48, 49]. Notably, in addition to broad spectrum and high antiviral activity[50–53], AgNPs exert strong inhibitory effects on human life-threatening viruses such as HIV [54] and HBV [55]. Although AgNPs have been proven to be an excellent antiviral agent, the potential antiviral mechanism remains to be clarified with more extensive and in-depth research, which is the aim of our present exploration.

2 Materials And Methods

2.1 Cells and virus

Hela cells were propagated in Dulbecco's modifified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin, supplied by Gibco, Grand Island, NY. HSV-1 strain F was originally obtained as a gift from Zhongnan Hospital of Wuhan University (China), and then propagated with HeLa cells. The amplified HSV-1 was collected and stored at −80°C for further use.

HSV-1 virus titer was measured by 50% tissue culture infective dose (TCID50) [56]. Briefly, monolayer HeLa cells grown in 96-well plates were infected with 10-fold series of HSV-1 and incubated at 37°C for 2

h. Next, the viral inocula was maintained in DMEM cell solution. The above cell culture plates were incubated for 72 h and the virus titer was assigned as TCID50/mL according to Spearman-Karber method [57].

The plaque assay was also performed to determine the potency of HSV-1. Briefly, monolayer HeLa cells grown in 12-well plates were infected with 10-fold series of HSV-1. Next, the viral inoculum was preserved in DMEM with 0.3% agarose. Following cultivation for 3 days, the cells were fixed in 4% paraformaldehyde for 2 h and stained with 1% crystal violet for 30 min. Finally, plaques were counted and plaque forming units (PFU/mL) were calculated.

2.2 Reagents and drugs

The AgNPs used in this study was synthesized by chemical REDOX method with a typical absorption peak at 407.9 nm under uV-vis spectrophotometer. Under TEM, AgNPs appeared nearly spherical, evenly dispersed and uniform in size. Dynamic light scattering (DLS) showed that the nanoparticles were distributed between 5-20 nm, most of which were around 5-10 nm in diameter [58]. The antiviral agent of ACV was purchased from Sigma (St Louis, Mo, USA) and dissolved in dimethyl sulfoxide (DMSO) (Sigma, St Louis, Mo, USA). ACV was diluted with cell culture medium, and the final concentration of DMSO was less than 0.1%.

2.3 Cytotoxicity assay

CCK-8 kit was used to measure the relative safe concentration of AgNPs for HeLa cells according to the experimental procedure in reference [59]. Briefly, the cells in 96-well plates were exposed to different concentrations of AgNPs for 72 h. CCK-8 reagent was then added to each cell culture well, and the plates were further maintained at 37°C for 4 h. After thoroughly shaking the plates, Polarstar Optima Microplate Reader (BMG Labtech, Offenberg, Germany) was used to detect the absorbance value at 450 nm (A_{450}). Cell survival rate = (Experimental group A_{450}/Control group A_{450})×100%.

2.4 Plaque reduction assay

Plaque reduction assay was performed with reference to previous literature [60]. Briefly, Monolayer HeLa cells grown in 12-well plates were infected with HSV-1 (100 PFU/well), and then the cell layer was covered with 0.3% agarose-DMEM culture medium containing different concentrations of AgNPs and subsequently treated as described in the plaque assay. The plaque counts of three repeated wells were averaged, and then the plaque inhibition rate was calculated. Inhibition rate of plaque (%) = [1-(number of plaques)$_T$/(number of plaques)$_V$]×100%, where T and V respectively refer to the number of plaques in the treated group and the untreated virus control.

2.5 Virus yield reduction assay

The virus yield reduction assay was carried out to assess the antiviral effect of AgNPs in accordance with the reference [60]. Briefly, HeLa cells cultured in 12-well plates were infected with HSV-1 (MOI=0.1) and then exposed to different concentrations of AgNPs. After 24 h of treatment, freeze-thaw cycle was

repeated for three times between -80℃ and 37℃ and the yielded viruses were determined by plaque assay.

2.6 Time-of-addition and time-of-removal assays

Time-of-addition and time-of-removal assays were conducted to determine at which stage AgNPs inhibit HSV-1 replication [61, 62]. For the former, HeLa cells were infected with HSV-1 (MOI=0.1), and then treated with AgNPs (3.13 μg/mL) at 0, 3, 6, 9, 12, 18 and 24 h post infection. After cultured for 24 h and repeated freeze-thaw for 3 times, the virus yield was determined by plaque assay. For the latter, HeLa cells were initially infected with HSV-1 (MOI=0.1) and then treated with 3.13 μg/mL AgNPs. Next, cell media containing AgNPs were removed at 0, 3, 6, 9, 12, 18 and 24 h post treatment. After cultured for 24 h and repeated freeze-thaw for three times, the virus collection was analyzed by plaque assay.

2.7 CPE Inhibition assay

CPE Inhibition assay was carried out in line with the instruction [63] to explore the protective effect of AgNPs on HSV-1-induced cytopathological changes. Briefly, HeLa cells grown in 12-well plates were infected with HSV-1 (200 TCID50), and then dealt with 3.13 μg/mL AgNPs and 8μmol/L ACV for 72 h. When obvious cytopathic changes occurred in the the virus control group, the cell morphology was observed and photographed under the microscope.

2.8 Quantitative PCR(Q-PCR)

The effect of AgNPs on HSV-1 DNA load was examined according to the previous study [64]. Hela cells were infected with HSV-1 (MOI=0.1) and then treated with 3.13 μg/mL AgNPs. Total DNA was extracted by Treef Animal Genomic DNA Kit (Tsingke Biotech, Beijing, China) at 6, 12 and 24 h post AgNPs treatment, respectively. The specific primers were designed for HSV-1 US4 coding region, and the DNA copy number of HSV-1 was determined by Q-PCR [65]. The procedure of Q-PCR with a total of 40 amplification cycles was as follows: pretreatment at 95℃ for 2 min; denaturation at 95℃ for 5 s;annealing at 60℃ for 10 s and extension at 72℃ for 15 s. The relative copy number of the viral genome was normalized to β-actin in the corresponding sample. Primer sequences were shown in Table 1.

Table 1
Primer sequences of related genes

Gene target	Sequence(5’-3’)
ICP4-F	CTATATGAGCCCGAGGACGC
ICP4-R	CGTCTGACGGTCTGTCTCTG
ICP22-F	GCCCGGAGTGTGATCTTAGT
ICP22-R	AAACTCATCCTCCAGACGCA
ICP27-F	TCTGGCGGACATTAAGGACA
ICP27-R	GGTGCGTGTCTAGGATTTCG
ICP47-F	GGAAATGGCGGACACCTTC
ICP47-R	CGGTTCTTCGATGTGCCAC
ICP8-F	TTACCGAGGGCTTCAAGGAG
ICP8-R	ACGGAAGCGGGTAGGTAAAA
UL12-F	CCGTGGTTCTGGAATTCGAC
UL12-R	TCACCTTTATCTTGCTGCGC
UL30-F	ACATTCCGCTATCTCCTGGG
UL30-R	CGGCTAGTATATGGGAGGGC
UL42-F	CTCGGGGTTAACGGCAAATT
UL42-R	CAGAGAAGGTGCGATGGGTA
gB-F	CCACCGCTACTCCCAGTTTA
gB-R	GATGCAGTTTACCGTCGTCC
gD-F	TTTGTCGTCATAGTGGGCCT
gD-R	GGCGTAGTAAACCGTGATCG
gH-F	CGCCCACTGTTCCTAACCTA
gH-R	TGTGTTGTCCGTATCCACCA
VP16-F	TGTTTGACTGCCTCTGTTGC
VP16-R	CGAATGTGGTTTAGCTCCCG
US4-F	CGTGCCGTTGTTCCCATTAT
US4-R	TCCTCCTCCTCCAGTCCAAT
GADPH-F	AAATTCCATGGCACCGTCAA

Gene target	Sequence(5’-3’)
GADPH-R	CAGGAGGCATTGCTGATGAT
β-actin-F	AGGATTCCTATGTGGGCGAC
β-actin-R	ATAGCACAGCCTGGATAGCAA
NFκB-p50-F	AGTATTTCAACCACAGATGGCACT
NFκB-p50-R	CCCACATAGTTGCAGATTTTGACC
TNF-α-F	TCTCCTTCCTGATCGTGGCA
TNF-α-R	CAGCTTGAGGGTTTGCTACAAC
IL-1β-F	TGATGGCTTATTACAGTGGCA
IL-1β-R	GGTCGGAGATTCGTAGCTGG
IL-6-F	ATGAACTCCTTCTCCACAAGCG
IL-6-R	CATGTTACTCTTGTTACATGTCTCC
IL-8-F	ACTCCAAACCTTTCCACCCC
IL-8-R	ATGAATTCTCAGCCCTCTTCAA

2.9 Gel mobility shift assay

Gel mobility shift assay was carried out on the basis of the reference with appropriate modification [55]. Viral DNA from HSV-1 (1×10^7 PFU) was extracted with the Purelink ™ Viral RNA/DNA Mini Kit (Omega Bio-Tek, GA, USA) according to the manufacturer's instructions. After the concentration and purity of the DNA were detected by microspectrophotometer, each 500 ng of DNA extract was mixed with AgNPs at a series of concentration of 0.78, 1.56, 3.13 and 6.25 μg/mL and ACV at a concentration of 8 μmol/L. The mixture was then incubated at room temperature for 20 min and then separated by 3% agarose gel electrophoresis DNA bands measurement.

2.10 Reverse Transcription Q-PCR (RT-qPCR)

The effect of AgNPs on HSV-1 mRNA was detected with reference to the literature [64–66]. HeLa cells were infected with HSV-1 (MOI=0.1), and then treated with 3.13μg/mL AgNPs for 24 h. Total RNA was extracted according to the instructions of the SteadyPure Universal RNA Extraction Kit (Accurate Biotech, Hunan, China). The EVO-MLV RT Kit with GDNA Clean for qPCR II (Accurate Biotech, Hunan, China) was used to synthesize cDNA from RNA reverse transcription. SYBR® Greenpremix Pro Taq HS qPCR Kit (Accurate Biotech, Hunan, China) was applied to detect IE genes (ICP4, ICP22, ICP27 and ICP47), E genes (ICP8, UL30, UL12 and UL42) and L genes (gB, gD, gH and VP16). The procedure of Q-PCR was carried out in detail as described in 2.8. The results were analyzed using the $2^{-\Delta\Delta CT}$ threshold cycle method[67].

2.11 Western blot analysis

The effect of AgNPs on expression of HSV-1's proteins was evaluated with respect to a previous research [68]. After infected with HSV-1 (MOI=0.1) and treated with 3.13 μg/mL AgNPs for 24 h, HeLa cells were lysed by RIPA Lysis Buffer (Beyotime Biotech, Shanghai, China). The total protein extract was centrifuged at 12000×g at 4℃ for 5 min, and then the protein concentration was determined. After that, the proteins were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a prebalanced PVDF membrane, which was then sealed in 5% bull serum albumin (BSA) for 2 h and kept overnight at 4℃with ICP4 mouse monoclonal antibody (Santa Cruz Biotech, CA, USA), ICP8 mouse monoclonal antibody (Santa Cruz Biotech, CA, USA), gB mouse monoclonal antibody (Santa Cruz Biotech, CA, USA) and GAPDH mouse monoclonal antibody (Proteintech Group, IL, USA). After washing with 0.1% tween-20-PBS, HRP goat anti-mouse IgG (Proteintech Group, IL, USA) were added and incubated at room temperature for 2 h. Finally, the ECL Western Blot Detection Kit (Millipore Corp, MA, USA) was conducted for visualization.

2.12 Direct interaction between AgNPs and HSV-1

The direct interaction between AgNPs and HSV-1 was observed by the method in literature [69, 70]. On one hand, 10^5 PFU/mL HSV-1 was mixed with AgNPs in gradient concentrations of 0, 0.78, 1.56, 3.13 and 6.25 μg/mL and then incubated at 37℃ for 2 h. On the other hand, 10^5 PFU/mL HSV-1 was mixed with 3.13 μg/mL AgNPs and then incubated at 37℃ for 0.5, 1, 2, 3 and 4 h. The above mixture was diluted 100 times with cell maintenance solution and subsequently treated as described previously for plaque assay. Finally, the plaques were analyzed and quantified.

The TEM observation was designed based on previous studies [71]. Briefly, HSV-1 and AgNPs were mixed to the final concentration 10^7 PFU/mL HSV-1 and 6.25 μg/mL AgNPs and kept at room temperature for 2 h. The carbon-coated copper mesh was immersed in the mixture for 30 min, and then taken out and immersed in 2% phosphotungstic acid solution with pH=6.5 for 10 min. After washing the copper mesh with distilled water and drying for 30 min, the virus morphology was scoped under a Hitachi S-3400N TEM (Hitachi, Japan).

2.13 AgNPs affect HSV-1's entry into host cells

The test of AgNPs' influence on the adsorption of HSV-1 was carried out in relation to previous researchs [62, 72, 73]. Briefly, following precooled at 4℃ for 1 h, HeLa cells were treated with 100 PFU HSV-1 and different concentrations of AgNPs. The cell culture were incubated at 4℃ for 2 h for the virus absorption and entry into the cells. After washing the cells three times with PBS (pH=7.4), the plaque was assayed as described above.

The influence of AgNPs on the penetration of HSV-1 was investigated in respect to the methods in literatures [74–76]. Briefly, HeLa cells precooled at 4℃ for 1 h were infected with 100 PFU HSV-1 and then maintained at 4℃ for 2 h, followed by AgNPs addition and 37℃ incubation for another 2 h. After that, the host cells were treated with PBS (pH=3.0) for 1min to inactivate the unpenetrated virus, and then washed three times with PBS before plaque assay as described above. Plaque inhibition rate (%)=[1-(plaque number)$_T$/(plaque number)$_V$]×100%, where T and V refer to the plaque counts of AgNPs in the treated group and the virus control group, respectively.

2.14 AgNPs inhibit the release and cell-to-cell spread of HSV-1

The influence of AgNPs on the release of HSV-1 was explored regarding to previous method [75]. Briefly, HeLa cells were infected with HSV-1 (MOI=0.2), and then exposed to different concentrations of AgNPs for 24 h. Then, the supernatant was separated, and the cells were repeated three freeze-thaw cycles between -80℃ and 37℃. Finally, the extracellular and intracellular fractions of the virus were titrated, and the release ratio of HSV-1 was calculated: Release%=100V_{ex}/(V_{ex}+V_{in})%, where V_{ex} and V_{in} represent extracellular and intracellular viral titers, respectively.

To investigate the effect of AgNPs on cell-to-cell spread of HSV-1, the viral plaque size reduction assay was exerted on the basis of previous studies [67, 76–78]. Hela cells grown into monolayer in the 6-well plates were infected with HSV-1 (200 PFU/well) and then incubated for 72 h with 0.3% agarose-DMEM culture medium containing different concentrations of AgNPs and 10 mg/mL HSV-1 gB neutralizing antibody, to ensure plaque formation was due to the virus' cell-to-cell spread. BenQ SZW3300V scanner (BenQ; Suzhou, Jiangsu, China) was performed to capture 30 discrete and uniform plaque images in each group. Size of the plaques was measured with Image Pro Plus 6.0 software and the average was calculated.

2.15 AgNPs down-regulate the level of pro-inflammatory factors activated by HSV-1.

HeLa cells were infected with HSV-1 (MOI=0.5) and then 3.13 μg/mL AgNPs were added. Meanwhile, groups of normal cell, AgNPs-treated alone and HSV-1 infected in absence of AgNPs were introduced as controls. Total RNA was isolated following 24 h treatment and the expression levels of NFkB-p50, TNF-α, IL-1β, IL-6 and IL-8 were detected by RT-qPCR. The experimental procedures were carried out in details as described above in 2.10.

2.16 AgNPs enhance the antiviral effect of ACV against HSV-1

To understand the anti-HSV-1 efficacy of ACV in vitro, cytotoxicity assay, plaque reduction assay, and virus yield reduction assay were performed as described above in 2.3, 2.4 and 2.5. The synergistic anti-HSV-1 effects of AgNPs and ACV were designed regarding to previous studies [79, 80]. The experiments groups were divided into virus control (VC), AgNPs (1.56 μg/mL) treated group, ACV (1 μmol/L) treated group and AgNPs (1.56μg/mL) coupled with ACV(1μmol/L) treated group. The plaque reduction assay and the virus yield reduction assay were performed to assess the overall synergistic effect, followed by Q-PCR (2.8) to evaluate their effect on HSV-1 genomic load. Then, RT-qPCR (2.10) and Western blotting (2.11) were employed to analyze the expression of gB gene.

2.17 Statistical analysis

The data were processed using GraphPad Prism 8 and expressed as the mean ± standard deviation from at least three separate experiments. Statistical differences between two experimental groups were analysed by Student's t-test and one way analysis of variance (ANOVA) was used to compared the means between multiple groups. $p<0.05$ was considered statistically signifificant between different groups.

3 Results

3.1 Inhibitory effect of AgNPs on HSV-1 in vitro

To determine the relatively safe concentration of AgNPs on the viability of HeLa cells, the CCK8 assay was firstly conducted. The results showed that high concentration of AgNPs had certain effects on the cell viability, while low concentration was relatively safe (Fig. 1A). The antiviral efficacy of AgNPs assessed by plaque reduction assay showed that AgNPs could inhibit the formation of HSV-1 plaque at low concentrations; the plaque inhibition rate significantly increased with the elevation of AgNPs concentrations (Fig. 1B). Virus yield reduction assay found that the harvest of progeny virus treated with AgNPs was dramatically shrunk in a dose-dependent manner (Fig. 1C).

Assays time-of-addition and time-of-removal revealed that the longer treatment of AgNPs, the lower yield of the progeny viruses was. Moreover, in the late stage of virus replication, such as 18 h post viral infection, AgNPs could still significantly suppress the production of live HSV-1 in progeny, suggesting that AgNPs might have blocking effects on various stages of HSV-1 replication. Observation of morphological changes further confirmed the protective effect of AgNPs on HSV-1-infected cells (Fig. 1E). The morphology of the cells treated with ACV or AgNPs was well protected compared to the virus control group, where cells showed serious destruction such as death, shedding and fusion. Taken together, these results indicated that AgNPs were a relatively safe and effective anti-HSV-1 preparation in vitro.

3.2 Anti-HSV-1 mechanism of AgNPs

3.2.1 The effect of AgNPs on HSV-1 genome

It was speculated from the results of 3.1 that AgNPs might interfere with the replication process of HSV-1. In order to determine whether AgNPs have impact on HSV-1 genic load, Q-PCR was performed to test the HSV-1 DNA burden after AgNPs treatment. Compared to the control group, the DNA copy number of the AgNPs treated group was significantly reduced at 6, 12 and 24 h post treatment (Fig. 3A), suggesting that AgNPs could inhibit the load of HSV-1 genome. Results of gel mobility shift assay demonstrated that DNA samples in AgNP-treated group exhibited 'shifting up' effects compared with the virus control (Fig. 3B). Higher the proportion of AgNPs, more significant the tailing effect was, and even at low proportion of AgNPs, the shifting-up effect was still observable. However there was no 'shifting up' effect in the ACV treated group. These results suggest that the DNA of HSV-1 may substantially bind to AgNPs, which lead to enlarge the size and viscosity of DNA and thus affect DNA motility.

3.2.2 Effect of AgNPs on HSV-1 gene expression

RT-qPCR detection of the mRNA levels of IE, E and L genes proved that AgNPs could decrease the mRNA expression levels of IE genes (ICP4, ICP22, ICP27 and ICP47), E genes (ICP8, UL12, UL30, UL42) and L genes (gB, gD, gH, VP16) (Fig. 3A-C). As shown in Fig. 3D-E, Western blotting confirmed that compared with the virus control, AgNPs significantly down-regulated the protein levels of ICP4, ICP8 and gB, indicating that AgNPs participate in suppressing the gene expression of HSV-1.

3.2.3 Interaction between AgNPs and HSV-1

Plaque titration experiments disclosed that the infectivity of HSV-1 decreased with increased concentration of AgNPs (Fig. 4A). Plaque assay performed at different durations revealed that the infectivity of HSV-1 significantly decreased with the extension of the interaction time between AgNPs and HSV-1 in vitro (Fig. 4B). Images of co-incubated AgNPs and HSV-1 particles captured by TEM showed that the normal HSV-1 was a complete hexahedron-shape with the diameter of about 80-100 nm (Fig. 4C,I). Moreover, AgNPs could not only directly interact with the surface of HSV-1, but also could make HSV-1 virions morphologically irregular and even disruptive (Fig. 4C, II-VI). These results strongly suggest that AgNPs have a direct influence on the HSV-1infectivity in vitro.

3.2.4 AgNPs inhibit HSV-1’s entry into host cells

Exploration of AgNPs on viral attachment and penetration discovered that the plaque formation of HSV-1 was significantly inhibited by AgNPs in the virus adsorption stage (Fig. 5A-B); explosion to AgNPs at the HSV-1 penetration stage significantly blocked the plaque formation (Fig. 7C-D). All in all, AgNPs showed outstanding inhibitory effects on the attachment and penetration of HSV-1.

3.2.5 AgNPs affect the release and cell-to-cell spread of HSV-1

As shown in Fig. 6A, with AgNPs treatment, both intracellular and extracellular viral titers decreased in a dose-dependent manner; compared with the virus control, the release ratio of HSV-1 significantly decreased after the AgNPs treatment (Fig. 6B), suggesting that AgNPs could inhibit the release of progeny viruses from the host cells. Comparison of the size of viral plaques between AgNP-treated group

and the virus control revealed that compared with control, the plaque area formed by HSV-1 was significantly diminished with AgNPs treatment (Fig. 6C, 6D), suggesting that AgNPs could hinder HSV-1 transmission.

3.3 AgNPs down-regulate the level of pro-inflammatory factors activated by HSV-1

Some studies have shown that the infection of HSV could activate related inflammatory factors in NF-kB and MAPK signaling pathways[81, 82], and the activation of NF-kB signaling pathway has proven to be involved in the process of maintaining the replication of HSV and escaping the immune attack from the host cells [83–86]. Our experiment of RT-qPCR determined the expression of the transcription factor of NFκB-p50 and the major pro-inflammatory cytokines (TNF-α, IL-1, IL-6 and IL-8), which showed in Fig. 7A that under normal circumstances, NFκB-p50 was slightly expressed in normal cells, and the expression decreased with AgNPs treatment; whereas in HSV-1-infected cells, the expression of NFκB-p50 was distinctly enhanced; interestingly, when HSV-1-infected cells were treated with AgNPs, the expression level of NFκB-p50 was overwhelmly decreased. Similar experimental results were also observed in expression of TNF-α (Fig. 7B), IL-1β (Fig. 7C), IL-6 (Fig. 7D) and IL-8 (Fig. 7E), suggesting that HSV-1 could activate the expression of pro-inflammatory while AgNPs may down-regulate the activation of these cytokines induced by HSV-1.

3.4 AgNPs enhance the antiviral effect of ACV against HSV-1

3.4.1 Evaluation of anti-HSV-1 effect and screening for synergistic concentration of ACV

CCK8 assay results showed that the toxicity was not significant when ACV was at a usual level in the cells, wheras when the concentration reached very high, the cells' viability was apparent to be compromised (Fig. 8A). The evaluation of antiviral effect of ACV by plaque reduction assay found that low level of ACV could inhibit plaque formation caused by HSV-1, and the inhibitory effect was amplified with the elevation of ACV level (Fig. 8B). Virus yield reduction assay proved that after ACV treatment, the progeny virus yield was obviouly shrunk in a concentration-dependent manner. According to the above experimental results, 1 μmol/L ACV was selected for subsequent collaborative antiviral studies.

3.4.2 AgNPs enhance the anti-HSV-1 effect of ACV

Plaque reduction assay showed that the combined management of AgNPs and ACV achieved a higher plaque inhibition rate, compared to the treatment of HSV-1 with either AgNPs or ACV alone (Fig. 9A). The anti-HSV-1 effect of the combined treatment was further tested by the virus yield reduction assay, which found that either AgNPs or ACV could reduce the titer of active HSV-1 in progeny compared to the virus control. However, combination of the two showed a better inhibitory effect (Fig. 9B), suggesting that the combination therapy was more conducive in inhibiting the generation of active HSV-1 in progeny.

Furthermore, q-PCR measurement for the genomic load post 24 h HSV-1 infection demonstrated that the combination treatment could reduce viral genomic load more than the non-combined treatment (Fig. 9C). Consistent with the inhibition on viral genome, the combination therapy was better able to inhibit the mRNA and protein levels of gB than treated by AgNPs or ACV alone (Fig. 11D-F), suggesting that the combination therapy might be more beneficial to inhibit the expression of genes involved in HSV-1. The above results together indicated that AgNPs could enhance the antiviral effect of ACV against HSV-1.

4 Discussion

The infection of HSV-1 can not only induce skin herpes, but also lead to fatal diseases such as gingival stomatitis and encephalitis in severe cases [87–89]. Viral replication cycle is a complex process, including entry, uncoating, genomic amplification and expression, assembly and release, etc.[90]. Inhibition of any of these steps can effectively interfere with viral infection. AgNPs have a broad spectrum of antiviral effects against a variety of viruses. However, the specific inhibitory mechanism of AgNPs against each type of viruses has not been fully clarified [91, 92]. In this study, we confirmed that AgNPs could directly or indirectly inhibit HSV-1 through a variety of ways, and the combined management of AgNPs and ACV (a clinical anti-herpes drug) could enhance the lethality of HSV-1, indicating that AgNPs might be a potential anti-herpes material.

It has been found that AgNPs could reduce plaque formation caused by monkeypox virus[93], and inhibit the production of progeny viruses of Tacaribe virus [94]. In this study, we proved that AgNPs could restrain the plaque formation of HSV-1 and production of the progeny viruses, exerting good inhibitory effect on the cytopathic changes caused by HSV-1. Taken together, these findings suggest that the inhibitory effect of AgNPs against different viruses might be universal in vitro. The inhibition of AgNPs on plaque formation and virus development might be attributed to the capacity that it could directly affect the viral invasion by reducing the production of progeny viruses in different ways, or indirectly interfere with the viral damage to cells by oppressing the activity of progeny viruses.

When HSV-1 infects host cells, the genome will undergo biological processes such as replication, transcription and translation. AgNPs could enter cells through endocytosis [95], and the DNA of the cells would lose the ability to replicate after receiving Ag^+ treatment [96, 97]. Our results verified that AgNPs could downregulate the viral genomic load, indicating that AgNPs might directly interact with the viral DNA to interfere with the replication process, or disturbing the enzymes or proteins associated with viral DNA replication to inhibit genomic load. Furthermore, we found that AgNPs had the ability to bind directly to viral DNA. It has been reported that AgNPs could also interact with the double-stranded DNA of hepatitis B virus [55], which is consistent with the results of this study. We speculate that AgNPs are prone to adsorb negatively charged nucleic acids due to the large amount of positive charges on their surface. In addition, structures like large or small furrows in DNA molecules might also be potential binding sites for AgNPs.

The genes of HSV-1 are mainly composed of three main groups: IE, E, and L, which are sequentially controlled and expressed in a cascading manner [98]. The expression of IE genes plays an important role in initiating the subsequent transcription of HSV-1. Regulated by protiens of IE genes, E genes begin to be successively expressed, whose products are involved in the replication of HSV-1 genome. The L genes are expressed in the late stage of viral infection, whose products are mainly structural proteins of HSV-1[2]. We discovered that AgNPs could restrain the genetic transcription of these stages, including IE gene (ICP4, ICP22, ICP27, ICP47), E gene (ICP8, UL12, UL30, UL42), and L gene (gB, gD, gH, VP16). It has been revealed that AgNPs could also reduce viral replication by interfering with RNA polymerase dependent on Tacaribe virus and prevent viral unencapsulation in the endosome [94]. Combined with the findings of this study, it is reasonable to believe that AgNPs might interfere with the process of gene expression of different viruses in host cells. We assume that AgNPs could down-regulate the mRNA level of the corresponding genes of HSV-1, due to the abilities that AgNPs could disturb the DNA synthesis of the virus, thus indirectly affecting the transcription level of the related genes, and that AgNPs could hinder the action of the enzymes of viral genome transcription, thus affecting the mRNA production. In addition, AgNPs might also directly interact with the RNA formed by transcription or interfere with the environment of viral genome transcription, ultimately reducing the expression of viral mRNA.

ICP4, a transcriptional agonist, is an important regulatory protein expressed by IE gene, which is necessary to activate the expression of HSV-1's E gene and L gene [99]. ICP8 is a single-stranded DNA binding protein expressed by the E gene (UL29), involved in regulating the replication of HSV-1's genome and the transcription of L gene [100]. gB is an enveloped glycoprotein expressed by HSV-1 L gene (UL27), which can recognize receptors on host cells and enable HSV-1 to adsorb to membranes of the host cells. gB is also important to mediate the penetration and the diffusion between cells [101]. In the present experiments t investigating the expression levels of these three proteins after AgNPs treatment, all the levelsl were found to be descended. We believe that AgNPs could be highly likely to indirectly affect the expression level of downstream proteins by down-regulating the genomic load of HSV-1 and the transcription level of the corresponding genes. Meanwhile, the presence of AgNPs might also interfere with the enzymes and regulatory factors required for HSV-1 related protein synthesis, thus directly impacting the formation of viral proteins. In the study of AgNPs against foodborne viruses, it has been found that AgNPs at 10 nm could reduce viral titers in a dose-dependent manner and significantly inhibit the expression of viral capsid proteins detected by Western bloting [102]. Therefore, the effect of AgNPs against the synthesis of viral protein might be a key step for its broad-spectrum inhibition of viral replication.

In the study of curcumin-modified AgNPs (cAgNPs) against respiratory syncytial virus (RSV), it was found that cAgNPs could directly interact with the virions to prevent RSV from infecting host cells [103]. Likewise, in the research of naked AgNPs against influenza virus, AgNPs could destroy influenza virus envelope glycoprotein, thereby preventing viral infection [104]. Electron microscopic observation has revealed that AgNPs could absorb to the surface of the virion of Peste des petits ruminants virus and interact with the virus core [105]. Under the observation of TEM, AgNPs could also destroy the structure of adenosine virus type 3 in vitro [106]. We observed the direct interaction between AgNPs and HSV-1, and

found that after their interaction, the virus infectivity and morphology were significantly changed. Tanken together, these results indicat that AgNPs can directly bind to different types of virions to inhibit the infectivity of the viruses, which is probably an important reason for the broad-spectrum antiviral properties of AgNPs. The explaination to AgNPs' interacting with different viruses may be that AgNPs can nonspecifically adsorb to the cell surface, and cause oxidative damage on the capsids and envelope proteins, therefore, destroying the normal form of the viruses and suppressing the viral activity.

Previous studies have shown that AgNPs with a diameter of 1-10 nm could interact with HIV gp120 to inhibit viral adsorption to the host cells [107]. Moreover, AgNPs with a diameter of about 10 nm also could effectively inhibit the binding and penetration of monkeypox virus [93]. The results of this study also confirmed that AgNPs could inhibit the absorption and penetration of HSV-1. The infection of virus depends on the appropriate combination between the viruses and the host cells, and is closely related to the integrity of the viral particles [2]. Based on our results, we speculate that the effect of AgNPs on the adsorption and penetration of HSV-1 could be attributed to the destruction of the envelope glycoprotein by AgNPs, thereby breaching the stable binding of HSV-1 to the membrane of host cells, and expediting competition between AgNPs and viruses for receptors on cell surface, resulting in reduction of the entry of HSV-1 into the cells.

By exploring the influence of AgNPs on viral release and cell-to-cell spread in the late stage of HSV-1 infection, we found that the release ratio and plaque size of HSV-1 were significantly reduced after the treatment of AgNPs, indicating that AgNPs could restrict HSV-1 transmission. The products of L genes, such as gB, gD, gH and VP16 ect., not only construct the structural proteins of HSV-1, but also participate in the assembly and the cell-to-cell transmission of progeny viruses [108]. As observed in this study, reduced expression of gB, gD, gH and VP16 might contribute to AgNPs' indirect inhibition of the release and cell-to-cell spread of HSV-1. In addition, AgNPs could directly interact with the HSV-1 virions in vitro, causing less infectivity of the virus; AgNPs can also bind to the mature virions that have been assembled in the host cells to prevent the spread and transmission of the virus.

It has been shown that MAPK signaling pathway is involved in regulating viral replication and cytokine production [109], and the activation of NF-κB and MAPK signaling pathways in host cells plays a key role in HSV replication [110, 111]. After infecting host cells, HSV can activate the expression of NF-κB, a key transcription factor in the inflammatory pathway, and induce the production of downstream pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α [81, 82], which can not only promote the replication of HSV itself, but also cause excessive inflammatory response and further damage to host cells. Previous Studies have reported that AgNPs could inhibit the production of pro-inflammatory cytokines [112–115]. In particular, curcumin-coated AgNPs could significantly reduce HIV replication by inhibiting the activity of NFκB-p50 and the downstream expression of IL-1β, TNF-α and IL-6 [116]. Our results also revealed that AgNPs could reduce the expression levels of TNF-α, IL-1β, IL-6 and IL-8 induced by HSV-1. Since TNF-α, IL-1β, IL-6 and IL-8 are the hallmarks of cell chemotaxis and pro-inflammatory response, it is suggested that AgNPs could not only alleviate the host cell damage caused by the inflammatory response by inhibiting the production of pro-inflammatory cytokines, but also indirectly hinder viral replication by blocking HSV-

1-activated cytokines. It has been disclosed that AgNPs could impose broad-spectrum and highly effective killing effect on common pathogenic bacteria and a variety of fungi, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* [117]. Moreover, AgNPs could exert anti-inflammatory activity by controlling the production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 in vivo and in vitro [114, 118]. Therefore, compared with the traditional nucleoside antiviral drugs, AgNPs might not only have the effect of anti-HSV-1, but also play roles in sterilization, antipruritis and anti-inflammatory effects in the lesions, which suggested that development of AgNPs as antiviral agents have higher clinical value.

Acyclovir is the first clinically effective drug discovered and used worldwide for the treatment of HSV and VZV infections [119], which requires activation of viral thymidine kinases and cellular kinases, and ultimately inhibits viral DNA synthesis [23]. However, the therapeutic resistance of some HSV and VZV strains to ACV became an urgent problem for the clinic [120, 121]. Although the specific mechanism of the inhibition remains to be further studied, our experiments give evidences that AgNPs can inhibit HSV-1 transcription and DNA synthesis and the effect of AgNPs do not depend on the activity of viral kinase, which is different from action of ACV. AgNPs may escape the resistance of HSV-1 and hopefully become a stable anti-herpes agent. In this study, we also found that AgNPs treatment coupled with ACV were more effective than either treatment alone againt HSV-1 infection. Although the underlying mechanism requires further investigation, combination therapy might lessen the clinical dose of ACV to HSV-1, thereby reducing the adverse effects of ACV.

In conclusion, this study suggests that AgNPs could directly or indirectly restrict the generation, formation and development of viral plaque in vitro, thus showing effective anti-HSV-1 infection activity, which might be attributed to the mechanism that AgNPs could inhibit genetic replication and expression of HSV-1, block viral entry and transmission between cells, and directly interact with the virus particles to disrupt the normal structure of the viruses. We also found that AgNPs could inhibit the inflammatory response activated by HSV-1 infection and enhance the anti-HSV-1 activity of ACV. These results not only confirmed that AgNPs were effective and broad-spectrum antiviral agents, but also provided experimental and theoretical basis for further study of AgNPs as antiviral agents. Further studies should focus on the in-depth and specific molecular mechanism of AgNPs against HSV-1 and the development of safer and more efficient AgNPs products. In addition, the pharmacodynamics and toxicology in animal models also deserve further exploration to determine the safety parameters of AgNPs as well as to investigate how AgNPs effectively inhibit HSV-1 infection in vivo.

5 Conclusion

AgNPs show outstanding inhibitory activity against HSV-1 and can enhance the anti-HSV-1 effect of ACV. The related inhibitory pathways include that AgNPs can reduce the genetic load and expression of HSV-1, affect HSV-1’s entry into cells, interfere with the release and transmission of HSV-1, and down-regulate the expression of inflammatory cytokines activated by HSV-1 (Fig. 10).

Declarations

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Author contributions

LC and **LW** developed the research hypothesis and designed the experiments. **XP** performed the main experiments and wrote the main manuscript. **YZ , SY,and YZ** carried out literature search, data acquisition and manuscript editing. **CX** provided assistance for data acquisition, data analysis and statistical analysis.All authors contributed to drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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Figures

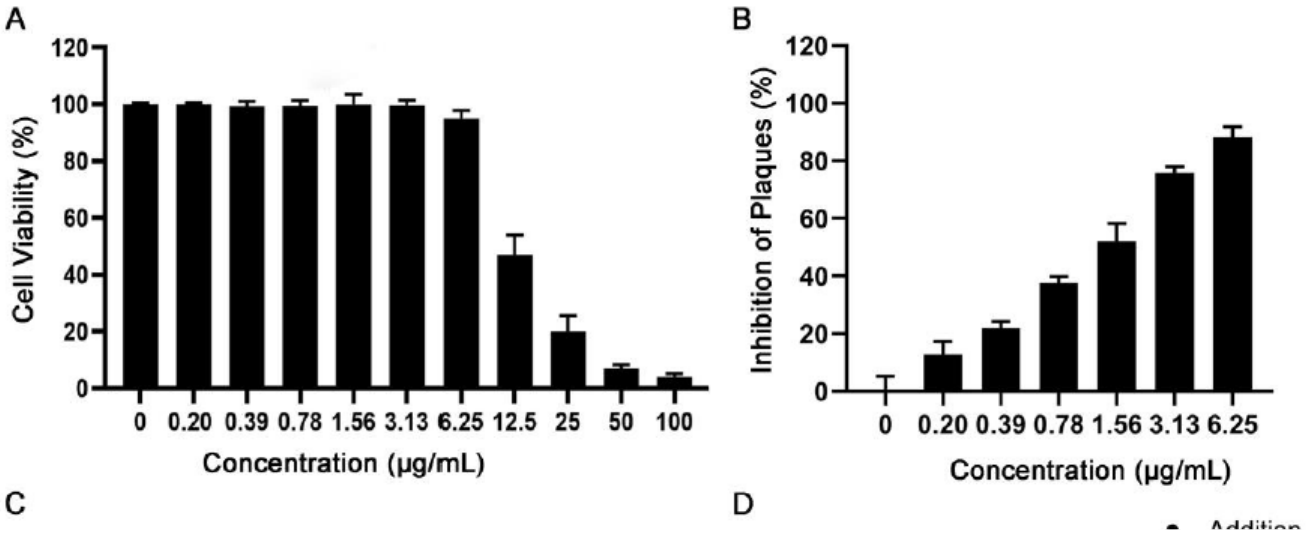

Figure 1

Inhibitory effect of AgNPs on HSV-1 in vitro. (A) Cytotoxicity Assay. HeLa cells were exposed to different concentrations of AgNPs (2×dilution). After 72 h of incubation, the cell viability was measured with the CCK8 kit in comparison with the cell control. (B) Plaque reduction assay. HeLa cells were infected with HSV-1 (100 PFU/well) and then treated with 0.3% agarose-DMEM medium containing different concentrations of AgNPs. After 72 h exposion, plaques were counted and compared to the virus control.

(C) Virus yield reduction assay. HeLa cells were infected with HSV-1 (MOI=0.1), and then treated with different concentrations of AgNPs for 24 h. Active HSV-1 in progeny were fully released from lysed cells and titrated by plaque assay. (D) Time-of-addition assay: AgNPs (3.13 μg/mL) were added at different time points after infecting HeLa cells with HSV-1 (MOI=0.1). Active HSV-1 in progeny were titrated by plaque assay post 24 h infection. Time-of-removal assay: AgNPs (3.13 μg/mL) were added to HeLa cells following HSV-1 (MOI=0.1) infection. AgNPs were removed at different time points and active HSV-1 in progeny were titrated by plaque assay post 24 h infection. (E) CPE inhibition assay. HSV-1-infected HeLa cells (200 TCID50) were treated with AgNPs (3.13 μg/mL) or ACV(8 μmol/L) for 72 h. Morphological changes of the cells were recorded by phase contrast microscopy. CC, Cell Control; VC, Virus Control; ACV, Acyclovir.

Figure 2

Effects of AgNPs on HSV-1 genome. (A) HeLa cells were infected with HSV-1 (MOI=0.1), followed by AgNPs (3.13 μg/mL) treatment. Total DNA was extracted at 6, 12 and 24 h post treatment. The specific primers were designed based on HSV-1 US4 coding region and the DNA copy number of HSV-1 was determined by qPCR. The relative copy number of the viral genome was normalized to β-actin in the corresponding samples. (B) Gel mobility shift assay was used to analyze the interaction between AgNPs and HSV-1 genomes. Genomic DNA of HSV-1 was extracted, and 500ng viral DNA was interacted with different concentrations of AgNPs (0.78, 1.56, 3.13 and 6.25 μg/mL) or 8 μmol/L ACV, and then separated by 3% agarose gel electrophoresis. VC, Viral Control; ACV, Acyclovir. (*** $P \leq 0.001$)

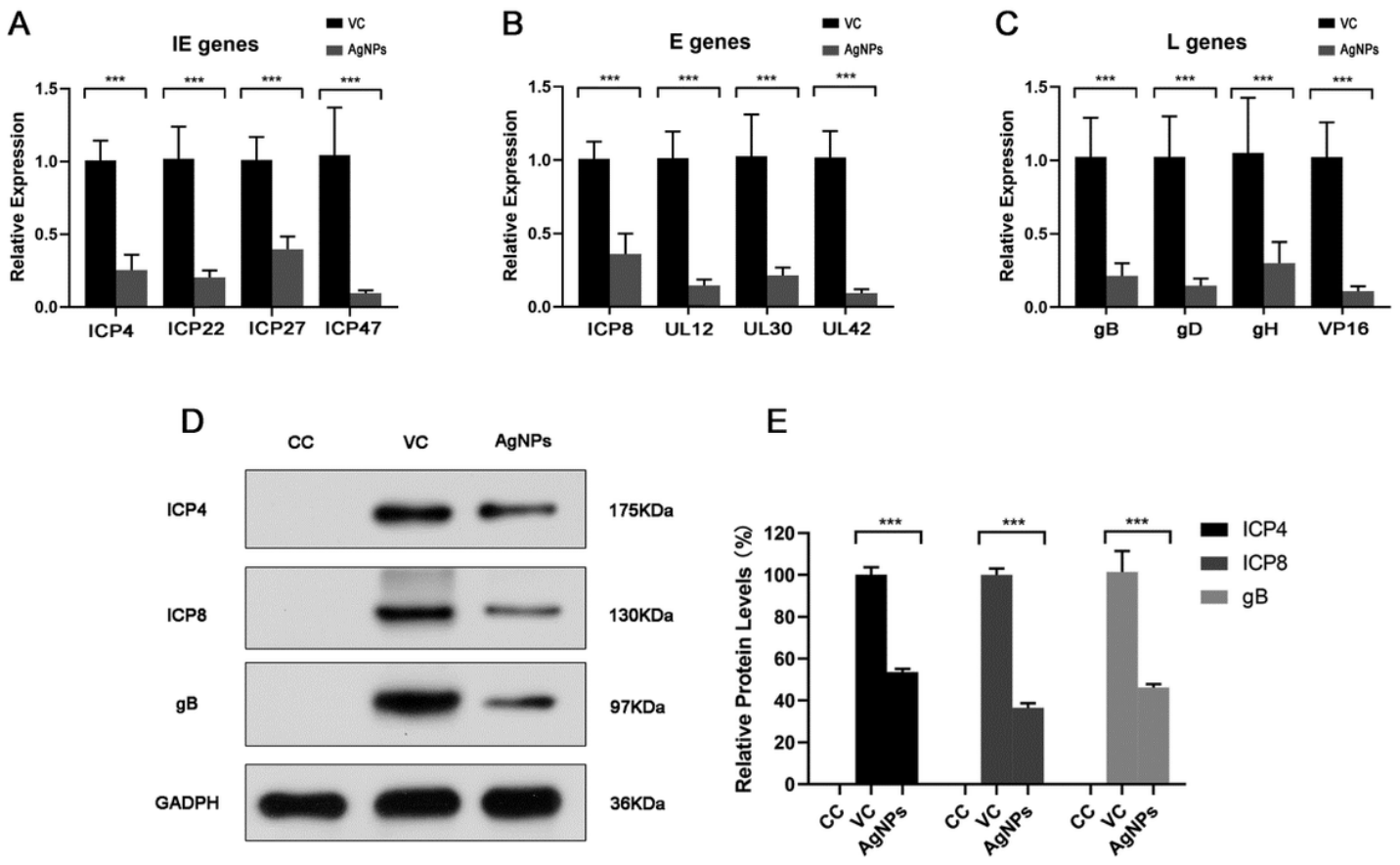

Figure 3

Effect of AgNPs on HSV-1 gene expression. (A-C) HeLa cells were infected with HSV-1(MOI=0.1) and then treated with AgNPs (3.13 μg/mL). After treatment for 24 h, mRNA levels of IE genes (ICP4, ICP22, ICP27 and ICP47), E genes (ICP8, UL12, UL30 and UL42), and L genes (gB, gD, gH and VP16) were detected by RT-qPCR. The mRNA levels were first normalized to GAPDH and then compared with the virus control. (D-E) HeLa cells were infected with HSV-1 (MOI=0.1) and then treated with AgNPs (3.13 μg/mL). The protein levels of the genes were analyzed by Western blotting (D). The grayscale values of ICP4, ICP8 and gB were quantified with Quantity One professional grayscale analysis software, which were first normalized to GAPDH and then compared with the virus control (E). CC, Cell Control; VC, Viral Control. (*** $P \leq 0.001$)

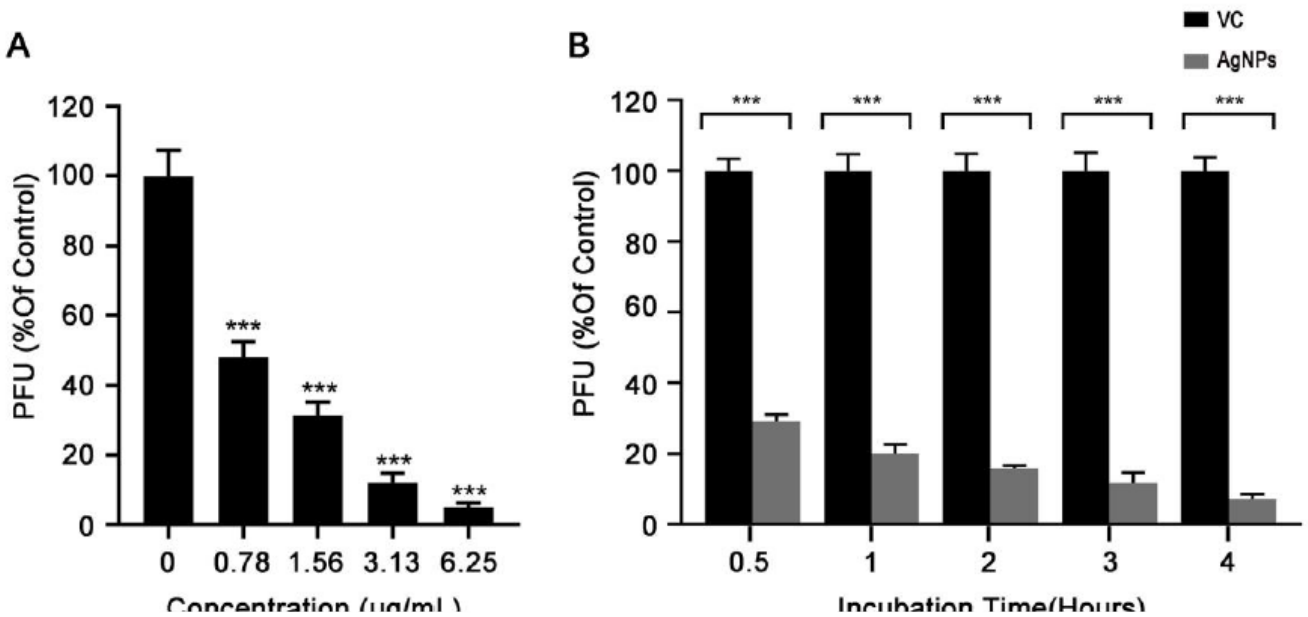

Figure 4

Effect of AgNPs on the infectivity of HSV-1. (A) HSV-1 was mixed with different concentration of AgNPs (the final concentration of AgNPs was 0, 0.78, 1.56, 3.13 and 6.25 μg/mL, and HSV-1 was 105 PFU/mL), and incubated at 37°C for 2 h. Then, the mixture was diluted 100 times with cell maintenance solution followed by plaque assay and the results were compared with the virus control. (B) HSV-1 was mixed with AgNPs (the final concentration of AgNPs and HSV-1 were 6.25 μg/mL and 105 PFU/mL, respectively),

and incubated at 37℃ for 0.5, 1, 2, 3, and 4 h. The mixture was diluted 100 times at each time points followed by plaque assay and the results were compared with the virus control. (C) The direct interaction between AgNPs and HSV-1 was observed by TEM and some of the silver nanoparticles are marked by white arrows. (I) Control samples. (II-VI) 2 h in vitro interaction between HSV-1 and AgNPs (6.25 μg/mL). VC, Viral Control. (*** $P \leq 0.001$)

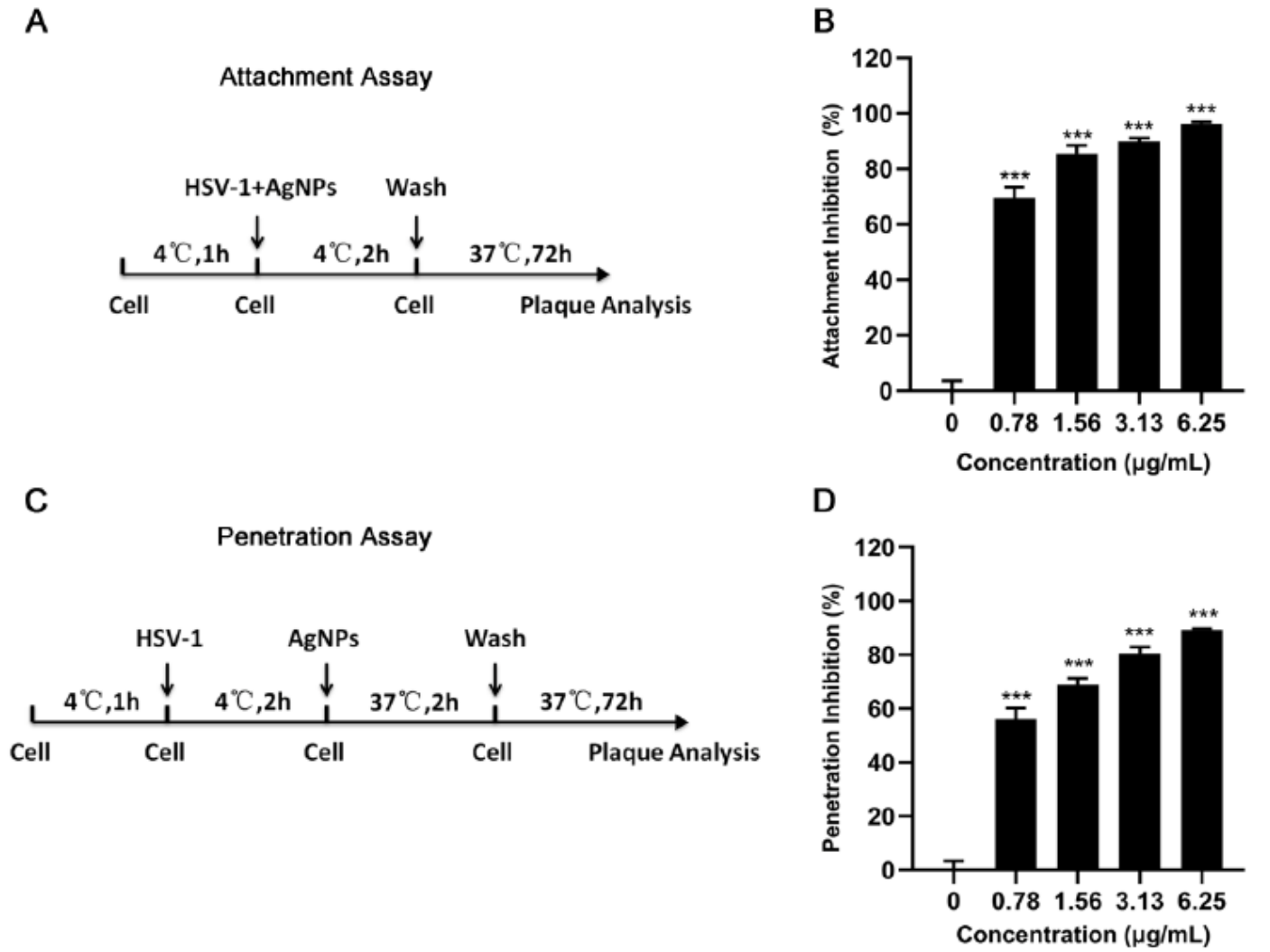

Figure 5

Effects of AgNPs on the attachment and penetration of HSV-1. (A) Schematic diagram of AgNPs affecting HSV-1 attachment. HeLa cells precooled at 4℃ for 1 h were simultaneously treated with HSV-1 (100PFU) and AgNPs, and then incubated at 4℃ for 2 h to let HSV-1 fully adsorb to the cells. The cells were then washed three times with PBS (pH=7.4) and incubated with 0.3% agarose-DMEM culture medium for 72 h, followed by plaque analysis. (B) is the result of comparing the plaque of experiment A with the virus control. (C) Schematic diagram of AgNPs affecting HSV-1 penetration. HeLa cells precooled at 4℃ for 1 h were infected with HSV-1 (100 PFU), and then incubated at 4℃ for 2 h to make HSV-1 fully attach to the cells, which were subsequently treated with AgNPs and incubated at 37℃ for another 2 h. The cells were treated with PBS (pH=3) for 1 min to inactivate the unpenetrated virus and

then washed 3 times with PBS (pH=7.4), and then kept in 0.3% agarose-DMEM culture medium for 72 h, followed by plaque analysis. (D) is the result of comparing the plaque of experiment C with the virus control. (*** $P \leq 0.001$)

Figure 6

Influence of AgNPs on the release and cell-to-cell spread of HSV-1. (A-B) HeLa cells were infected with HSV-1(MOI=0.2), and then treated with different concentrations of AgNPs. After kept at 37℃ for 24 h, the supernatant and cells were collected separately. The cells were repeatedly frozen and thawed cycles three times to fully release the inside viruses. The virus titers (A) of extracellular and intracellular fractions were titrated by plaque assay and the release ratio of HSV-1 was calculated by the release formula (B). (C-D) HeLa cells grown in 6-well plates were infected with HSV-1 (200 PFU/well) and then cultured with 0.3% agarose-DMEM medium containing different concentrations of AgNPs. After 72 h, the scattered and uniform plaques were selected (n=30), of which the average size were measured (C). Representative plaques at different concentrations of AgNPs were also recorded (D). CC, Cell Control; VC, Viral Control. (*** $P \leq 0.001$)

Figure 7

AgNPs downregulate HSV-1-activated pro-inflammatory cytokines. (A-E) HeLa cells were infected with HSV-1(MOI=0.5) and then treated with AgNPs (3.13 μg/mL). After 24 h, total RNA was extracted and the mRNA expression of NFκB-p50 (A), TNF-α (B), IL-1β (C), IL-6 (D) and IL-8 (E) were detected by RT-qPCR. The mRNA levels were first normalized to GAPDH and then compared with the cell control. CC, Cell Control. (*** $P \leq 0.001$)

Figure 8

Inhibitory effect of ACV on HSV-1 in vitro. (A) Cytotoxicity Assay. HeLa cells were treated with multifold dilution (5×dilution) of ACV and cell viability was detected by CCK8 kit after incubation for 72 h in comparison with the cell control. (B) Plaque reduction assay. HeLa cells were infected with HSV-1 (100 PFU/well) and then maintained in 0.3% agarose-DMEM medium containing different concentrations of ACV. After 72 h, plaques were counted and compared with the virus control. (C) Virus yield reduction assay. HeLa cells were infected with HSV-1 (MOI=0.1), and then treated with different concentrations of ACV for 24 h. The active HSV-1 in progeny were fully released from lysed cells and then titrated by plaque assay.

Figure 9

AgNPs enhance the anti-HSV-1 effect of ACV. (A) HeLa cells were infected with HSV-1 (100 PFU/well) and treated with 0.3% agrose-DMEM medium containing AgNPs (1.56 μg/mL), ACV(1 μmol/L) and AgNPs (1.56 μg/mL) coupled with ACV (1 μmol/L), respectively. After 72 h, plaques were analyzed in comparison with the virus control. (B) HeLa cells were infected with HSV-1 (MOI=0.1), and then treated with AgNPs (1.56 μg/mL), ACV (1 μmol/L) and AgNPs (1.56 μg/mL) coupled with ACV (1 μmol/L) for 24 h, respectively. The active HSV-1 in progeny were fully released from lysed cells and titrated by plaque assay. (C-F) HeLa cells were infected with HSV-1 (MOI=0.1), and then treated with AgNPs (1.56 μg/mL), ACV(1 μmol/L), and AgNPs (1.56 μg/mL) coupled with ACV(1 μmol/L), respectively. After treatment for 24 h, the genomic level of HSV-1 was measured by qPCR, and the relative copy number of the viral genome was normalized to β-actin in the corresponding samples (C). The mRNA level of gB was detected by RT-qPCR and the expression level was first normalized to GAPDH and then compared to the virus control (D). The protein level of gB was analyzed by Western blotting (E). The grayscale values of gB were quantified with Quantity One professional grayscale analysis software, which was first normalized to GAPDH and then compared to the control group (F). CC, Cell Control; VC, Viral Control. (* * $P \leq 0.01$, *** $P \leq 0.001$)

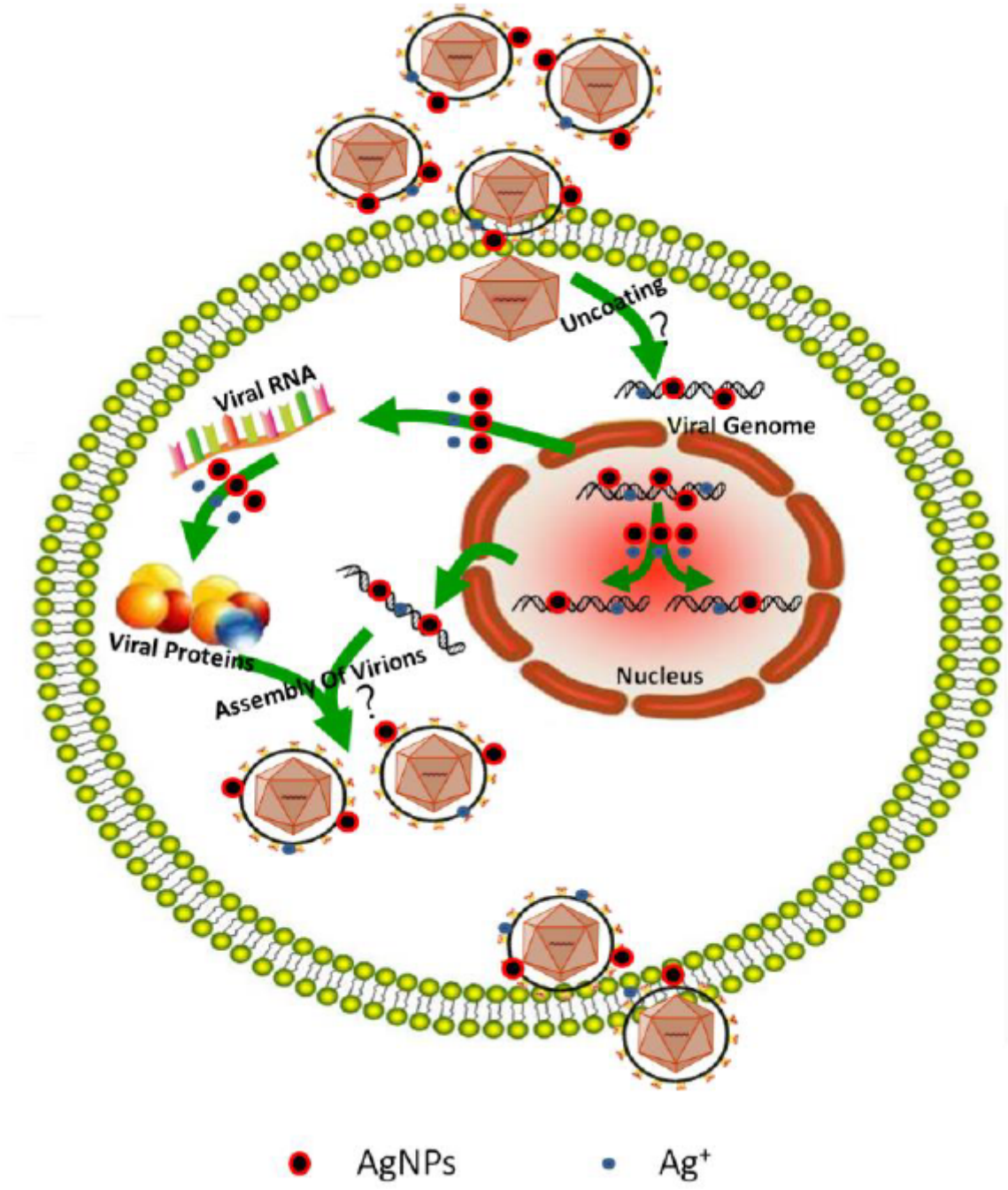

Figure 10

Model diagram of AgNPs against HSV-1. AgNPs affect the infection and invasion of HSV-1, including interfering with HSV-1's entry into cells, affecting the load and expression of HSV-1 genes, and inhibiting the release and transmission of HSV-1.