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# Inhibitory effect of silver nanomaterials on transmissible virus-induced host cell infections

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ABSTRACT

Coronaviruses belong to the family *Coronaviridae*, which primarily cause infection of the upper respiratory and gastrointestinal tract of hosts. Transmissible gastroenteritis virus (TGEV) is an economically significant coronavirus that can cause severe diarrhea in pigs. Silver nanomaterials (Ag NMs) have attracted great interests in recent years due to their excellent anti-microorganism properties. Herein, four representative Ag NMs including spherical Ag nanoparticles (Ag NPs, NM-300), two kinds of silver nanowires (XFJ011) and silver colloids (XFJ04) were selected to study their inhibitory effect on TGEV-induced host cell infection *in vitro*. Ag NPs were uniformly distributed, with particle sizes less than 20 nm by characterization of environmental scanning electron microscope and transmission electron microscope. Two types of silver nanowires were 60 nm and 400 nm in diameter, respectively. The average diameter of the silver colloids was approximately 10 nm. TGEV infection induced the occurring of apoptosis in swine testicle (ST) cells, down-regulated the expression of Bcl-2, up-regulated the expression of Bax, altered mitochondrial membrane potential, activated p38 MAPK signal pathway, and increased expression of p53 as evidenced by immunofluorescence assays, real-time PCR, flow cytometry and Western blot. Under non-toxic concentrations, Ag NPs and silver nanowires significantly diminished the infectivity of TGEV in ST cells. Moreover, further results showed that Ag NPs and silver nanowires decreased the number of apoptotic cells induced by TGEV through regulating p38/mitochondria-caspase-3 signaling pathway. Our data indicate that Ag NMs are effective in prevention of TGEV-mediated cell infection as a virucidal agent or as an inhibitor of viral entry and the present findings may provide new insights into antiviral therapy of coronaviruses.



## 1. Introduction

Metal nanomaterials have received considerable attention due to their attractive physicochemical properties. In the biomedical fields, many applications for metal nanomaterials have been explored including biosensors [1], labels for cells and biomolecules [2], and cancer diagnostics and therapeutics [3]. Although the antibacterial, antifungal and antiviral properties of silver ions and silver compounds have been extensively studied and used for centuries, silver nanoparticles have shown much more superior efficacy for their promising antimicrobial potential [4], which have been used for wound healing against bacteria [5]. Moreover, *in vitro* studies have demonstrated that spherical Ag nanoparticles (Ag NPs) can be used as antiviral agents against the human immunodeficiency virus (HIV) [6], respiratory syncytial virus [7], H1N1 influenza A virus [8], monkeypox virus [9] or hepatitis B virus [10]. However, the antiviral effects of silver nanomaterials (Ag NMs) against coronaviruses (CoVs) remain an undeveloped area.

CoVs have been very common throughout the world, as both human and animals are susceptible to them (http://www.cdc.gov/coronavirus/). Particularly some coronaviruses, such as severe acute respiratory syndrome coronavirus (SARS-CoV) and the recently identified Middle East respiratory syndrome coronavirus (MERS-CoV) may cause fatal infection in human. Transmissible gastroenteritis virus (TGEV), a porcine coronavirus, causes very high mortality in piglets and thus has tremendous impact of pig industry [11,12]. Vaccination has been extensively applied to prevent pigs from TGEV infection. Virulent or attenuated vaccines can

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be used to vaccinate sows and the resulting colostrum may provide protection to sucking piglets, however, the immune efficacy was not ideal and the potential dissemination as well as prevalence of infectious agent in piglets remain [13,14]. Therefore, development of preventive and therapeutic strategies is indispensible for control of TGEV infection. To date, the understanding and knowledge regarding effectiveness evaluation of chemical reagents on TGEV infection are limited. Although Ag NMs have been displaying appealing effect on antiviral research [15,16], their potential inhibitory effect on TGEV infection has not been studied and reported until now. Moreover, TGEV, like many other viruses, exerts much of its cell effect through the induction of apoptosis of its host cell [17–19]. Previous reports showed that the activation of caspases played a critical role in cell apoptosis triggered by TGEV [19–21]. However, whether and how TGEV-mediated apoptosis could be regulated by Ag NMs is unknown.

Therefore, in this study, we used TGEV as a model of CoVs and comparatively evaluated the inhibitory effect of four different Ag NMs (Ag NPs, silver nanowires 60 nm, silver nanowires 400 nm, and silver colloids) on TGEV-mediated cell infection and apoptosis. The underlying molecular mechanism was further explored. Mitochondrial membrane potential (MMP), p38 MAPK signal pathway activation, the expression of Bcl-2 family proteins and the cleavage of the precursor caspase-3 were measured.

## 2. Materials and methods

### 2.1. Preparation and characterization of Ag NMs

Four Ag NMs consisting of Ag NPs (NM-300), two kinds of silver nanowires (XFJ011) and silver colloids (XFJ04) were selected in this study. Ag NPs was supplied by the Institute for Health and Consumer Protection (IHCP, one Joint Research Centre of European Commission located in Italia) under coordination within the Project of European Commission 7th Framework Program. The others were purchased from Nanjing XFNANO Materials Tech Co., Ltd., (Nanjing, China). Their size and morphology were characterized by environmental scanning electronic microscopy, ESEM (Quanta 200 FEG) or transmission electron microscopy, TEM (Tecnai G2 20 S-TWIN). The particle size distribution of Ag NPs and silver colloids was further characterized by nanoparticle tracking analysis (NanoSight LM10-Base, Nano Sight Ltd., UK). All Ag NMs were dispersed in deionized water to 1 mg/mL and were then further diluted into fresh medium to the final concentrations as required.

### 2.2. Cell culture and virus

Swine testicle (ST) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37 °C with 5% $CO_2$. TGEV strain PUR46-MAD was propagated in ST Cells as previously described [11,13].

### 2.3. Cell viability assays

Toxic effects of the Ag NMs on ST cells were determined using the Mosmann-based 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [22]. Briefly, approximately $6 \times 10^3$ cells were plated onto 96-well plates (Corning, USA). After the cells became confluence, the cells were incubation with different concentrations of Ag NMs for 48 h at 37 °C. Then, MTT reagent was added to the cells according to the manufacturer protocol and the absorbance at 570 nm was recorded by an Infinite M200 microplate reader (Tecan, Durham, USA). Mock-treated cells served as control. Each experiment was performed in triplicate.

### 2.4. Antiviral activity of Ag NMs

The inhibitory effect of the Ag NMs on TGEV was determined using the MTT assay, as described previously [23–25]. Briefly, TGEV at an MOI (multiplicity of infection) of 0.5 was incubated with serially diluted Ag NMs in medium at 37 °C for 1 h. Then, ST cells cultured on 96-well plates were infected with viruses containing or not containing Ag NMs for another hour. Then the cells were washed three times with phosphate-buffered saline (PBS) and maintained in DMEM (200 μL/well). At 48 h post-infection (hpi), the cells were incubated with MTT reagent. $OD_{570}$ values of the cells were determined as above. Sole virus-infected and mock-treated cells served as infection and blank controls, respectively. Relative amount of survival cells (%) was calculated as follows: Percentage of viable cells = {[OD of drug treatment group−OD of infection control]/[OD of blank control-OD of infection control]} × 100%.

### 2.5. Real-time quantitative PCR

TGEVs at an MOI of 0.5 were incubated with the indicated dose of Ag NMs (From 3.125 μg/mL to 12.5 μg/mL) for 1 h. Then the viruses were used to infect ST cells grown in 6-well plates as above. At 48 hpi, the total RNA was extracted using TRIzol agent (Invitrogen, Carlsbad, CA, US), and 2 μg of each RNA sample was subjected to reverse transcription according to the manufacturer's instructions. The resulting cDNA samples were analyzed by quantitative real-time PCR (qRT-PCR) (Eppendorf, Germany) using SYBR green as fluorescence dye, as described previously [13,26]. Relative quantifications of mRNA expression of the genes of interest were calculated using the comparative threshold cycle number for each sample. The following genes were measured: S-X (a fragment comprising the 3′half of the S gene, the intervening sequence and the 3a gene), 3CLpro (a nonstructural gene encoding the 3CLprotease of TGEV), Bax, Bcl-2 and beta-actin. The primers for qRT-PCR in this study were shown in Table S1.

### 2.6. Western blot

The samples were treated as above and the cells were harvested and washed with ice-cold PBS followed by treatment with ice-cold RIPA lysis buffer with 1 mM phenylmethyl sulfonylfluoride (PMSF). Protein concentrations were measured using BCA Protein Assay Reagent (Pierce, Rockford, IL, US). Equivalent amounts of proteins were loaded and run on 8-12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Subsequently, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp, Atlanta, GA, US). The membranes were blocked with 5% non-fat dry milk at room temperature for 1 h, and then incubated with indicated primary antibodies overnight at 4 °C, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 h. Information on antibodies can be found in the supporting information section. Blots were developed using enhanced chemiluminescence, as described previously [26,27]. The tested proteins included Bax, Bcl-2, caspase-3, Phospho-p38 MAP Kinase (Pi-p38), p38 MAP Kinase (p38), p53, PARP and beta-actin.

### 2.7. Indirect immunofluorescence assays

Inhibitory effect of the Ag NMs on cell infection by TGEV was further evaluated by immunofluorescence. ST cells in 96-well plates were infected with TGEV at an MOI of 0.5, or TGEV pre-treated with Ag NPs, silver nanowires 60 nm, silver nanowires 400 nm or silver colloids respectively for 1 h. At 36 hpi, the cells were rinsed once with PBS and fixed in 4% formaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton X-100 for 5 min at room temperature and incubated with the anti-TGEV polyclonal antibody (1:200 dilution) for 2 h and fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (H+L) secondary antibody (1:200 dilution) for 1 h. Images were all collected using an IN cell analyzer 2000 (GE health). Nine image fields/well were captured at the same exposure time and at least 200 cells per image filed were measured for effective data output. Fluorescence density calculations were performed automatically by the IN cell analyzer software 3.7.1 (GE Healthcare, US) according to the manufacturer protocol.

### 2.8. Flow cytometry analysis

Cell apoptosis induced by TGEV was further analyzed with FITC Annexin V Apoptosis Detection kit (Becton Dickinson, Sunnyvale, CA) as previously described [13,28]. The above-mentioned cell samples were trypsinized and then centrifuged at 2000 rpm for 5 min. The cells were re-suspended with 500 μL of binding buffer at a density of $10^6$ cells/mL, after washing two times with PBS at 2000 rpm for 5 min. Then, 5 μL of FITC-conjugated Annexin V and 5 μL of propidium iodide (PI) were added to the suspension for another incubation at room temperature for 15 min in the dark. The samples were analyzed within 1 h post-staining.

Mitochondrial membrane potential was determined using Rhodamine 123 (Invitrogen, Carlsbad, CA, US) as previously described [29]. ST cells were cultured at a density of $6 \times 10^3$ cells per well in 96-well plates in medium and incubated for 24 h. Cell samples included mock-infected ST cells or ST cells infected with TGEV at different MOIs for 12 h. Then, the cells were stained with 10 μM of Rhodamine 123 in PBS solution with 0.4% glucose for 30 min. The cells in each dish were washed gently with PBS three times prior to Flow cytometry analysis (BD FACSCalibur™ Flow Cytometer).

## 3. Results and discussion

### 3.1. Characterization of various Ag NMs

Four types of Ag NMs were physically characterized by ESEM or TEM. As shown in Table 1, silver nanowires were 60 nm and 400 nm in diameter. Ag NPs were uniformly distributed, with particle sizes less than 20 nm. The average diameter of silver colloids was approximately 10 nm. The stabilizing agents of Ag NPs were comprised of 7% ammonium nitrate, 4% each of Polyoxyethylene