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In vitro virucidal activity of nebulized citrate-complexed silver nanoparticles against equine herpesvirus-1 and murine norovirus

Thibault Frippiat [a,b,*], Lorène Dams [c], Constance Wielick [c], Catherine Delguste [d], Louisa F. Ludwig-Begall [c], Tatiana Art [a], Etienne Thiry [c]

[a] Equine Sports Medicine Centre, Faculty of Veterinary Medicine, University of Liege, Belgium
[b] Sportpaardenarts – Equine Sports Medicine, Laren, the Netherlands
[c] Veterinary Virology and Animal Viral Diseases, Department of Infectious and Parasitic Diseases, FARAH Research Centre, Faculty of Veterinary Medicine, University of Liege, Belgium
[d] General Services, FARAH Research Centre, Faculty of Veterinary Medicine, University of Liege, Belgium

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ABSTRACT

Viruses can be involved in respiratory disorders in horses, with limited therapeutic options. Citrate-complexed silver nanoparticles (C–AgNP) have shown bactericidal properties after in vitro nebulization. The aim of the present study was to assess the virucidal activity of C–AgNP after in vitro instillation or nebulization on equine herpesvirus-1 (EHV-1) and murine norovirus (MNV), the latter used as surrogate for small non-enveloped viruses. Both viruses were instilled or nebulized with C–AgNP of increasing concentrations, and titres were determined via $TCID_{50}$ method. We demonstrated efficient inactivation of enveloped EHV-1 following instillation and nebulization of C–AgNP (infectivity losses of ≥ three orders of magnitude). While tenacious MNV was inactivated via 2000 ppm C–AgNP instillation, nebulized C–AgNP did not lead to reduction in MNV titres. Nebulization of C–AgNP may represent a novel virucidal therapeutic approach in horses. Further investigations are needed to assess its safety and effective concentrations for in vivo use.

1. Introduction

Respiratory diseases have been identified as the second most important cause of lost training days and poor performance in athletic horses (Bailey et al., 1997; Fraipont et al., 2011; Morris and Seeherman, 1991). A variety of pathogenic and commensal viruses are known to be involved in respiratory disorders in horses. Among these, enveloped equine influenza virus (EIV) and equine herpesviruses (EHV) as well as non-enveloped equine rhinitis A and B viruses (ERAV, ERBV) are frequently implicated. Other equine viruses, which are less frequently involved and/or only transiently replicate within the equine airways, include equine arteritis virus, equine adenoviruses, Hendra virus, and African horse sickness virus (Gilkerson et al., 2015). Equine influenza virus, EHV-1 and EHV-4 show the highest prevalence in horses with acute respiratory disease (Broux et al., 2016; Pusterla et al., 2022a). Pathogenic EHV-1 and EHV-4 as well as commensal EHV-2 and EHV-5 have been found in nasal secretions of healthy horses during routine dental care, after transport, or at a show (Pusterla et al., 2020, 2022b; Smith et al., 2018). While their precise implication in the aetiology of equine asthma remains unclear (Couetil et al., 2021; Newton et al., 2003), exposure to respiratory viruses has been suggested to be associated with mild to moderate forms of the disease (Doubli-Bounoua et al., 2016; Houtsma et al., 2015; Wood et al., 2005). Furthermore, EHV-5 is associated with equine multinodular pulmonary fibrosis (Marenzoni et al., 2011; Williams et al., 2007).

Most equine respiratory viral infections are self-limiting and resolve without intervention within a few weeks from the onset of clinical signs. In general, treatment involves medical support and monitoring of diseased horses, and preventive measures to limit the spread to healthy ones. Supportive medication, including anti-inflammatory drugs and bronchodilators, can be administrated to reduce morbidity in affected horses. Antiviral treatment has been proposed to protect adult horses from EHV-1-associated disease via nucleoside analogues, *e.g.* acyclovir or its prodrug valacyclovir (Friday et al., 2000; Garré et al., 2009; Maxwell et al., 2017), and ganciclovir or its prodrug valganciclovir (Thieulent et al., 2019, 2022). Against EHV-5 infection however, valacyclovir has

* Corresponding author. Equine Sports Medicine Centre, Faculty of Veterinary Medicine, University of Liege, Quartier Vallée 2 - B42, Avenue de Cureghem 7A-7D, 4000, Liège, Belgium.
E-mail address: thibault.frippiat@uliege.be (T. Frippiat).

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been shown to be ineffective as a short-term antiviral treatment (Easton-Jones et al., 2018). Prophylactic protection by means of vaccination against EIV and EHV-1 represents currently one of the best approaches for controlling viral propagation within the equine population.

While silver has been used as an antimicrobial agent for millennia (Alexander, 2009), the advent of antibiotics in the early 20th century led to a deceleration of its development. However, owing to the increasing prevalence of antimicrobial resistance in recent years, compounds containing metal nanoparticles have been studied with renewed interest for the treatment and prevention of microbial infections, and silver has resurfaced as a therapeutic option (Sim et al., 2018). Silver is known for its pre- and post-entry inhibitory virucidal activities, inactivating enveloped viruses through charge-based interactions with their outer lipid envelope, and non-enveloped viruses through formation of bonds with sulphur groups on key proteins (Chen et al., 2016). Silver nanoparticles (AgNP) (typically 1 to 100 nm diameter) enhance this physicochemical antimicrobial action through a high surface to volume ratio and can further gain access within cells to exert intracellular antiviral activity via interactions with viral genomes (DNA or RNA) (Galdiero et al., 2011; Greulich et al., 2011; Morris et al., 2019). Silver nanoparticles are currently used in human medical care, *e.g.* in wound dressings, surgical sutures, cardiovascular implants, catheters, or bone cement (Alt et al., 2004; Andara et al., 2006; Gallo et al., 2016; Lackner et al., 2008; Leaper, 2006; Lu et al., 2008). In vitro studies have shown that AgNP can inhibit the replication of human respiratory viruses, *e.g.* H1N1 influenza A virus, respiratory syncytial virus, herpes simplex virus type 1, adenovirus 3, and potentially SARS-CoV-2 (Baram-Pinto et al., 2009; Chen et al., 2013; Hong et al., 2015; Jeremiah et al., 2020; Park et al., 2018).

Aerosol therapy is characterized by the administration of aerosols through inhalation and constitutes an efficient means of delivering topical drugs directly into the airways. Commonly used in the treatment of patients with pulmonary disease in human medicine, it has garnered increasing interest in (equine) veterinary practice. The development of portable, silent, and animal-adapted devices has contributed to this trend. Drugs commonly aerosolized for use in horses include corticosteroids, bronchodilators and antibiotics (Arroyo et al., 2016; Cha and Costa, 2017; Fultz et al., 2015; Mainguy-Seers et al., 2019; McKenzie and Murray, 2000; Wieder et al., 2015). Aerosol therapy with AgNP could represent an innovative way for the treatment of respiratory infections in horses.

Various capping or stabilizing agents are used to control particle size and thus ensure the stability of AgNP suspensions (Omelyanchik et al., 2021; Tolaymat et al., 2010). Among these, organic acids such as citric acid are commonly used. Citric acid, a cheap, biologically compatible, and environmentally friendly acidifying agent, is a well-known disinfectant additive. It has been shown to exert virucidal effects on various enveloped viruses (Chae et al., 2018; Ionidis et al., 2016; Mileto et al., 2021; Poli et al., 1979; Sato et al., 2020; Zinn and Bockmühl, 2020). Citric acid has further been shown to alter the morphology of non-enveloped norovirus particles (Koromyslova et al., 2015), and to block their binding to antigens (Hansman et al., 2012).

Recently, we demonstrated in vitro bactericidal activity of commercially available citrate-complexed AgNP (C–AgNP; SilvaPlex™) against *Streptococcus equi* subsp. *zooepidemicus* and *Actinobacillus equuli* subsp. *equuli* after instillation and nebulization (Frippiat et al., 2021). SilvaPlex™ is not sold as a veterinary drug, but it is used by veterinary practitioners and equestrians as a general support of the equine respiratory function (based on presumed bactericidal, virucidal, fungicidal and immunomodulatory properties of C–AgNP). To our knowledge, detailed data on the in vitro virucidal efficacy of the instilled or nebulized compound is yet missing. To investigate the potential of C–AgNP inhalation as an innovative therapeutic way to treat respiratory viral infections in horses, the present study aimed to assess the in vitro virucidal activity of instilled and nebulized C–AgNP on EHV-1 and murine norovirus (MNV), the latter used as surrogate of ERAV and other highly tenacious small non-enveloped equine respiratory viruses. Noroviruses are notorious for their tenacity in the face of inactivating treatments (Ludwig-Begall et al., 2021a; Wielick et al., 2022; Zonta et al., 2016a); inactivation of MNV can be considered a reliable predictor for inactivation of any virus ranking lower in the hierarchy of pathogen resistance.

2. Materials and methods

2.1. Cells

Rabbit kidney epithelial cells (RK13; ATCC CCL-37) and murine macrophage cells (RAW 264.7; ATCC TIB-71) were maintained at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Sigma) complemented with 10% heat-inactivated foetal calf serum (Greiner), 2% of an association of penicillin (10,000 U/mL) and streptomycin (10 mg/mL) (Sigma), and 1% of a preparation of nonessential amino acids (Sigma) (DMEMc). 1% 1 M HEPES buffer (pH 7.6) (Invitrogen) was added to RAW 264.7 cell suspensions.

2.2. Viruses

EHV-1 strain HVS25 and MNV isolate MNV-1.CW1 were propagated in RK13 and RAW 264.7 cells, respectively. For viral stock production, viruses were grown for 72 (EHV-1) or 48 (MNV) hours until cytopathic effects were observable. Subsequently, MNV-infected cells underwent three freeze/thaw cycles to facilitate viral particle release (−80 °C/37 °C). Cells and supernatant were harvested and clarified for 20 min at 3000 rpm at 4 °C. For both viruses, infectious viral titres were determined via the median tissue culture infective dose ($TCID_{50}$) method as previously described (Di Francesco et al., 2020; Ludwig-Begall et al., 2021b; Wielick et al., 2021). Briefly, cells were seeded in flat-bottomed 96-well plates, were incubated for 24 (RK13) or 2 (RAW 264.7) hours to allow them to attach to the bottom of the wells, and were infected with ten-fold serial dilutions of either virus. Following a 3-day incubation at 37 °C with 5% CO_2, allowing EHV-1 to propagate in RK13 and MNV to propagate in RAW 264.7 cells, the plates were stained with 0.2% crystal violet. Titres for EHV-1 and MNV, expressed as $TCID_{50}$/mL, were calculated according to the Reed and Müench transformation (Reed and Müench, 1938). Stocks of EHV-1 and MNV with a titre of, respectively, 4.16×10^6 and 3.56×10^7 $TCID_{50}$/mL were subsequently used.

2.3. Compounds

In the currently commercialized SilvaPlex™ solution, spherical C–AgNP (formula: $[Ag_3(C_6H_5O_7)_{n+1}]^{3n-}$) with a diameter of 71.8 nm are concentrated at 100 parts per million (ppm) in distilled water. In our study, four C–AgNP concentrations (manufactured and provided by Wire2Wire Vet Products) were used: 100, 500, 1000 and 2000 ppm. Hydrogen peroxide 6% (H_2O_2) and phosphate-buffered saline (PBS) were used as positive and negative controls, respectively. The pH ranged from 2.4 to 2.5 for the C–AgNP solutions, was 3.45 for H_2O_2 and 7.27 for PBS.

2.4. Cytotoxicity assays

A quantitative evaluation of cell viability and metabolism after exposure to the compounds was performed. For this, four wells of a 96-well plate containing either RK13 or RAW 264.7 cells were exposed to 50 μL of a solution consisting of 10% DMEM and 90% treatment solution or two-fold dilutions thereof. Following a 2-hour incubation, 100 μL of DMEMc were then added to each well and the plates were incubated for 72 hours at 37 °C with 5% CO_2. The plates were finally stained with 0.2% crystal violet. The maximum non-cytotoxic concentration was determined as the lowest dilution without cytotoxic effect.

2.5. Virucidal effect after instillation

Three different solutions were prepared for each compound: (A) 10%

viruses and 90% treatment solution (C–AgNP at 100, 500, 1000 or 2000 ppm, PBS, H_2O_2), (B) 10% DMEM and 90% treatment solution (cytotoxicity control), and (C) 10% viruses and 90% DMEM (untreated). The three solutions were placed for 2 hours at room temperature and were vortexed every 15 minutes. The treatment solutions and controls were filtered through Microspin S-400 HR filters (Sigma Aldrich) at 2700 rpm during 2 minutes, in order to remove the cytotoxic treatment solutions (without eliminating the viruses). Ten-fold serial dilutions of the filtrates in DMEM were then utilized in a $TCID_{50}$ assay (as described above). Plates containing RK13 cells were first incubated for 2 hours at 37 °C with 5% CO_2 and were then covered with 100 µL of DMEMc and incubated for 72 hours at 37 °C with 5% CO_2. Plates containing RAW 264.7 cells were incubated at 37 °C with 5% CO_2 for 72 hours directly following application of ten-fold filtrate dilutions. Following staining of the plates with 0.2% crystal violet, infectious virus titres of the (A) solutions, expressed as $TCID_{50}$/mL, were calculated. The same approach was used on the (B) solutions to calculate the cytotoxic effect of the solutions, and thus the overall assay limit of detection (LOD). Untreated (C) solutions were used as a control of viral titres and accompanied all assays. The timeline of the protocol involving instillation is presented in Fig. 1.

2.6. Virucidal effect after nebulization

To investigate the virucidal activity of nebulized C–AgNP, 400 µL pure DMEMc (cytotoxicity controls) or DMEMc-virus suspension (at a concentration of $8.29 \times 10^6 \pm 1.71 \times 10^6$ $TCID_{50}$/ml for EHV-1, and $4.88 \times 10^7 \pm 2.49 \times 10^7$ $TCID_{50}$/ml for MNV) were loaded in one well of a flat-bottomed 6-well plate (35 mm well diameter). As sets of two (one plate with DMEMc and one with DMEMc-virus), the open-topped plates were placed within a nebulization chamber (Flexineb C2 Aerosol Box, Nortev Ltd, Galway, Ireland) at 15 cm distance to the overhead nebulization source (Flexineb E2, Nortev Ltd, Galway, Ireland). Then, 2.5 mL of the treatment solutions were nebulized above each plate within the chamber (Fig. 2). A single nebulization chamber contained two medication cups, allowing the (A) DMEMc-virus and (B) DMEMc-only plates to be treated simultaneously with the same treatment solution. Medication cups were changed between treatments. One set of plates (C) remained untreated outside the nebulization chamber and served as a control of viral titres during the whole procedure. Following nebulization, plates were placed for 2 hours at room temperature (virus-aerosol contact). Ten-fold serial dilutions of each unfiltered solution (column

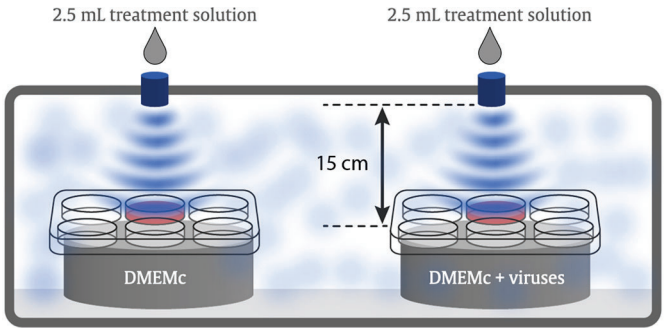

Fig. 2. Schematic representation of the nebulization chamber and the experimental setup therein. Six-well plates, of which one well contained either DMEMc or DMEMc-viruses, were placed within a nebulization chamber at a distance of 15 cm to the overhead nebulization source. Subsequently, 2.5 mL of the treatment solutions were nebulized above each plate within the nebulization chamber.

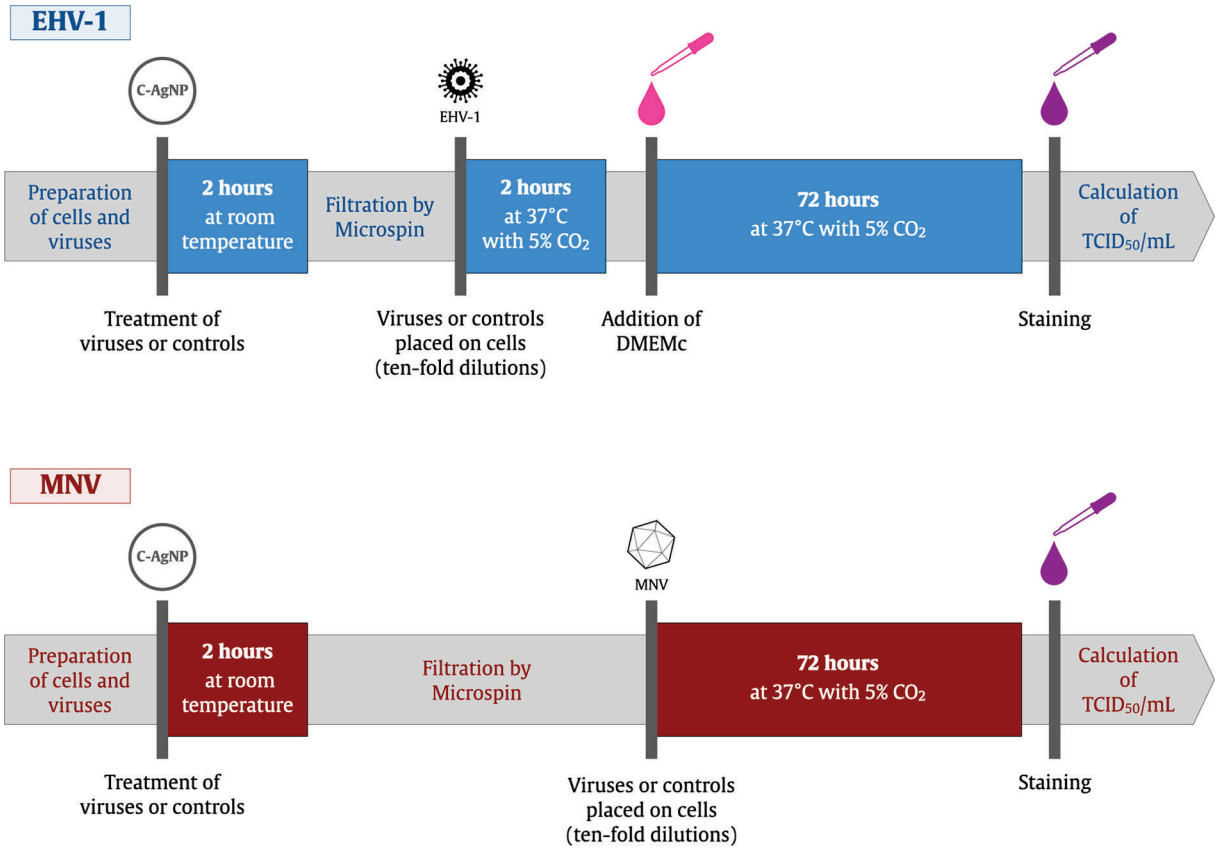

Fig. 1. Timeline of the protocol for the assessment of the virucidal effect of citrate-complexed silver nanoparticles (C–AgNP) when instilled on equine herpesvirus-1 (EHV-1) or murine norovirus (MNV). The same protocol was followed for nebulization without the filtration step. $TCID_{50}$/mL: viral titre expressed as 50% cell culture infectious dose per mL.

filtration not applied due to practical considerations and deemed unnecessary following nebulization pre-tests) were then utilized in a $TCID_{50}$ assay (as described above) and infectious viral titres of the (A) solutions, expressed as $TCID_{50}$/mL, were calculated. The same approach was used on the (B) solutions to calculate the cytotoxic effect of the solutions, and thus the overall assay LOD. Since the absolute volume within the different 6-well plate wells may have been subject to variation depending on the impaction of the nebulized treatment solution in each well, the $TCID_{50}$/mL were corrected to the final volumes. Untreated (C) solutions were used as a control of viral titres and accompanied all assays. The timeline of the protocol involving nebulization is presented in Fig. 1.

2.7. Statistical analysis

All experiments were performed in triplicate. When applicable, data is presented as mean ± standard deviation. Statistical analysis of differences in infectious viral titres was performed using Prism 9 (GraphPad Software) and included *T*-tests for independent means. All analyses considered a significance level of 0.05.

3. Results

3.1. Cytotoxicity

All C–AgNP solutions showed a cytotoxic effect on both RK13 and RAW 264.7 cells until at least the 1:32 dilution. Higher concentrations of C–AgNP induced cytotoxicity at higher dilutions (Table 1). The positive control, H_2O_2, showed the highest cytotoxic effect on both cell lines. The negative control, PBS, did not show any cytotoxic effect on either cell line.

3.2. Virucidal effect after instillation

3.2.1. On equine herpesvirus-1 (EHV-1)

Instillation of C–AgNP at all tested concentrations showed a significant virucidal effect on EHV-1, superior to that of the positive control (H_2O_2), lowering viral titres by at least three orders of magnitude (3 log_{10}) from $4.48 \times 10^5 \pm 1.59 \times 10^5$ $TCID_{50}$/mL to the assay LOD ($6.31 \times 10^1 \pm 8.53 \times 10^{-2}$ $TCID_{50}$/mL) (Fig. 3A).

3.2.2. On murine norovirus (MNV)

Murine norovirus titres were not significantly changed after instillation of C–AgNP 100 ppm. However, instillation of C–AgNP at concentrations of 500, 1000 and 2000 ppm reduced significantly viral titres. The viral reduction with C–AgNP 500 and 1000 ppm was less than one order of magnitude (1 log_{10}) from $3.04 \times 10^5 \pm 9.01 \times 10^4$ to $1.85 \times 10^5 \pm 5.08 \times 10^4$ $TCID_{50}$/mL and $2.95 \times 10^5 \pm 4.41 \times 10^4$ $TCID_{50}$/mL, respectively. Instilled C–AgNP 2000 ppm reduced the viral titres by at least three order of magnitude (3 log_{10}) to $3.56 \times 10^5 \pm 7.90 \times 10^1$ $TCID_{50}$/mL (Fig. 3B).

3.3. Virucidal effect after nebulization

3.3.1. On EHV-1

Nebulization of C–AgNP at all tested concentrations showed a significant virucidal effect, inducing reduction of the viral titres of at least three orders of magnitude (3 log_{10}) from $8.29 \times 10^6 \pm 1.71 \times 10^6$ $TCID_{50}$/mL to the assay LOD ($4.15 \times 10^3 \pm 2.23 \times 10^2$ $TCID_{50}$/mL for C–AgNP 100, 500 and 1000 ppm, and $7.90 \times 10^3 \pm 3.20 \times 10^2$ $TCID_{50}$/mL for C–AgNP 2000 ppm) (Fig. 4A).

3.3.2. On MNV

Nebulization of C–AgNP at all tested concentrations did not significantly reduce infectious MNV titres with all values for C–AgNP-treated virus clustering close to those of the negative control (PBS) and the untreated virus control ($4.47 \times 10^7 \pm 1.60 \times 10^6$ $TCID_{50}$/mL) (Fig. 4B).

4. Discussion

The first objective of the present study was to assess the in vitro virucidal activity of instilled C–AgNP on EHV-1 and MNV. All tested concentrations of C–AgNP (100 to 2000 ppm) significantly reduced EHV-1 titres by at least three orders of magnitude. This observation is in line with previously observed effects of AgNP (chelated to various compounds) on enveloped human viruses such as H1N1 influenza A virus, adenovirus type 3, herpes simplex virus type 1, and SARS-CoV-2 (Baram-Pinto et al., 2009; Chen et al., 2013; Jeremiah et al., 2020; Park et al., 2018), and may be expected to be similarly efficacious against enveloped equine viruses. In contrast, only the highest 2000 ppm concentration showed a significant viral reduction of infectious MNV titres by at least three orders of magnitude when instilled, while lower concentrations (500 and 1000 ppm) showed significant reductions of MNV titres by less than one order of magnitude. This indicates a concentration-response relationship as previously observed against MNV (Castro-Mayorga et al., 2017) and against enveloped human viruses (Baram-Pinto et al., 2009; Chen et al., 2013; Park et al., 2018). The findings in the present study suggest the importance of reaching sufficiently high concentrations for inactivation of hardier small non-enveloped viruses. While lipid-enveloped viruses are susceptible to inactivating treatments, non-enveloped viruses are known to be significantly more resistant. Amongst them, the small, non-enveloped

Table 1
Results of the cytotoxicity assay testing different concentrations of citrate-complexed silver nanoparticles (C–AgNP) as well as negative (phosphate-buffered saline) and positive (hydrogen peroxide 6%) controls on rabbit kidney epithelial cells (RK13) and murine macrophage cells (RAW 264.7). +: complete cytotoxicity (in all triplicates), +/−: partial cytotoxicity (in one or two of the triplicates), −: no cytotoxicity.

		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
RK13											
Negative control		−	−	−	−	−	−	−	−	−	−
C–AgNP	100 ppm	+	+	+	+	+	−	−	−	−	−
	500 ppm	+	+	+	+	+	+	−	−	−	−
	1000 ppm	+	+	+	+	+	+	−	−	−	−
	2000 ppm	+	+	+	+	+	+	+	−	−	−
Positive control		+	+	+	+	+	+	+	+	−	−
RAW 264.7											
Negative control		−	−	−	−	−	−	−	−	−	−
C–AgNP	100 ppm	+	+	+	+	+	+	−	−	−	−
	500 ppm	+	+	+	+	+	+	+	−	−	−
	1000 ppm	+	+	+	+	+	+	+	−	−	−
	2000 ppm	+	+	+	+	+	+	+	+/−	−	−
Positive control		+	+	+	+	+	+	+	+	+/−	−

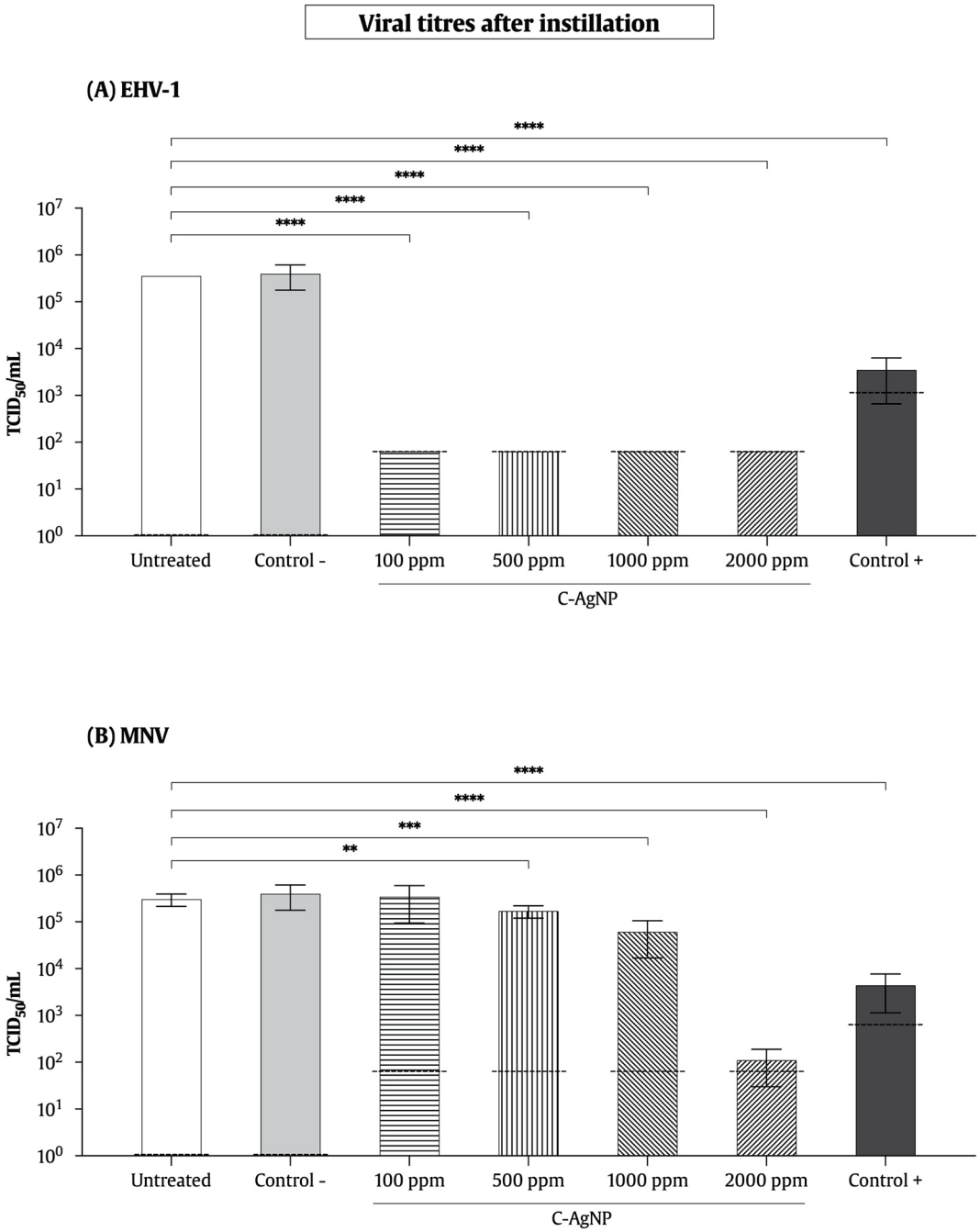

Fig. 3. Virucidal effect of citrate-complexed silver nanoparticles (C–AgNP) at different concentrations (expressed as parts per million [ppm]) on (A) equine herpesvirus-1 (EHV-1) and (B) murine norovirus (MNV) after instillation. Negative and positive controls were phosphate-buffered saline and hydrogen peroxide, respectively. Untreated samples constituted a DMEMc-virus suspension exposed to none of the compounds. The dashed lines represent the varying limits of detection for each specific assay (as determined by differing cytotoxicities of the treatment solutions). Asterisks represent a significant difference in 50% cell culture infectious dose per mL ($TCID_{50}$/mL) between a treated and the untreated virus solution, where ****$p < 0.0001$, ***$p < 0.001$ and **$p < 0.01$.

noroviruses are notorious for their tenacity (Ludwig-Begall et al., 2021a; Zonta et al., 2016b). While the precise origin for observed differences in C–AgNP virucidal/antiviral activity remains unclear (possible interaction at structural [envelope *versus* no envelope] and/or genomic [DNA vs RNA] level), a contributing factor may be a high degree of repulsion between the negatively charged C–AgNP and MNV particles, forming an electrostatic barrier that limits virus-particle interaction, thereby leading to lower inactivation, similar to effects previously observed in non-enveloped MS2 bacteriophages (Sinclair et al., 2021). Whilst the findings of the present study are encouraging, they result from in vitro assays conducted on a single EHV-1 strain and one strain of MNV (both viruses were cultivated in non-equine cells), and cannot be directly extrapolated to the live horses; further investigations are needed to assess the potential in vivo use of C–AgNP.

In horses, respiratory viruses can be present in the mucosa at different levels of the airways: nasopharynx, trachea and/or bronchi. Increasingly, aerosol therapy with equine-adapted devices has garnered interest in veterinary practice and has been used for the direct delivery of several compounds to the equine airways. Nebulization of C–AgNP could represent an innovative integrative treatment in infected horses since viral infections are commonly associated with secondary bacterial infections; nebulized C–AgNP have already been shown to exhibit in

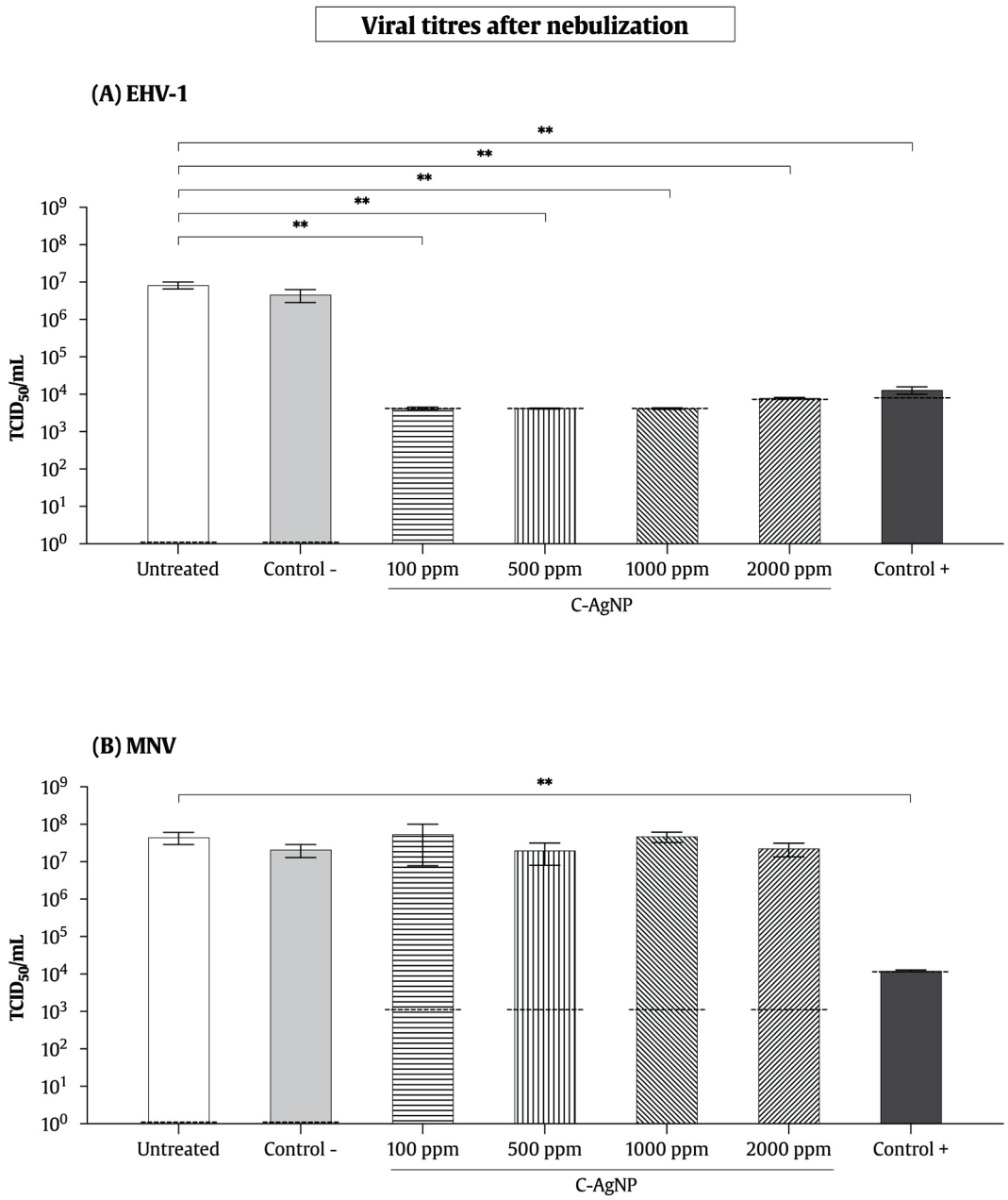

Fig. 4. Virucidal effect of citrate-complexed silver nanoparticles (C–AgNP) at different concentrations (expressed as parts per million [ppm]) on (A) equine herpesvirus-1 (EHV-1) and (B) murine norovirus (MNV) after nebulization. Negative and positive controls were phosphate-buffered saline and hydrogen peroxide, respectively. Untreated samples constituted a DMEMc-virus suspension exposed to none of the compounds. The dashed lines represent the varying limits of detection for each specific assay (as determined by differing cytotoxicities of the treatment solutions). Asterisks represent a significant difference in 50% cell culture infectious dose per mL ($TCID_{50}$/mL) between a treated and the untreated virus solution, where $^{**}p < 0.01$.

vitro bactericidal activity towards common equine respiratory bacteria (Frippiat et al., 2021). Here, we demonstrate preserved virucidal activity of nebulized C–AgNP on EHV-1, with all tested compound concentrations inducing viral titre losses of at least three orders of magnitude to below the assay LOD. For MNV, viral titre reductions were less pronounced following nebulization as opposed to instillation; while 2000 ppm instilled C–AgNP reduced MNV titres by over three orders of magnitude, the same solution achieved no significant MNV infectivity loss following nebulization. Differences are probably attributable to a dilution effect and loss of C–AgNP in the environment upon product nebulization. Please note that differences between nebulization and instillation efficacies were not observable on the less resistant, enveloped EHV-1; while viral EHV-1 titres may have indeed been reduced further via C–AgNP instillation than nebulization, larger reductions were not measurable once titres fell below the assay LOD. In the context of a putative concentration-response relationship of C–AgNP against small, non-enveloped viruses, this highlights the importance of carefully calibrating and adapting nebulized doses.

This in vitro study serves as a proof of concept for the potential use of C–AgNP (marketed for veterinary use) in the treatment of equine respiratory viral infections. We show virucidal activity against both enveloped and non-enveloped viruses following instillation, and demonstrate inactivation of EHV-1 in a nebulization protocol. In vivo virucidal activity can, however, not be directly extrapolated based on the present results and must be carefully evaluated in follow-up studies. These should include a careful calibration of virucidal efficacy *versus* cytotoxicity in different cells and/or explants. In the present in vitro models, C–AgNP showed increasing cytotoxicity with increasing

concentrations of C–AgNP. Nonetheless, it should be noted that both in vitro models relied on cells that are not representative of the ciliated epithelial cells covering the equine airways; viruses were cultivated in rabbit kidney (EHV-1) or murine macrophage (MNV) cells. The cell models used here are thus only of limited in vivo relevance to the equine. A long-term exposure of human lung cells to low-dose AgNP has previously been shown to be pro-fibrotic and induce cell transformation in vitro (Gliga et al., 2018), while a 28-day inhalation of AgNP did not induce significant body changes in rats (Ji et al., 2007).

Further investigations should focus on in vivo assays to pinpoint the effects of breathing and physiological/pathological mucus on the dissemination of C–AgNP within the large equine airways (effective concentrations at the viral and cellular levels must be determined).

5. Conclusion

Nebulized C–AgNP show promises for an integrative approach to the treatment of equine respiratory infections. Here, we investigated their in vitro virucidal potential and demonstrated inhibitory activity against both enveloped and non-enveloped viruses following instillation, and demonstrate inactivation of EHV-1 in a nebulization protocol. While only a cautious extrapolation of these in vitro findings to an in vivo situation is indicated, the results of the present study are encouraging for further research.

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CRediT authorship contribution statement

Thibault Frippiat: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Lorène Dams:** Methodology, Investigation. **Constance Wielick:** Investigation. **Catherine Delguste:** Conceptualization, Writing – review & editing, Supervision. **Louisa F. Ludwig-Begall:** Writing – review & editing, Visualization. **Tatiana Art:** Conceptualization, Writing – review & editing, Supervision. **Etienne Thiry:** Conceptualization, Methodology, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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