Antiviral and virucidal activities of lycorine on duck tembusu virus in vitro by blocking viral internalization and entry

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ABSTRACT Duck tembusu virus (**DTMUV**) was firstly identified in 2010 in China; since then, it has caused enormous economic loss to breeding industry. Great efforts have been made to develop drugs and vaccines against DTMUV. However, current available vaccines or anti-DTMUV drugs are consistently inefficient. Hence, various more broadly effective drugs have become important for the treatment of DTMUV infection; among these, lycorine, one of the important sources of active alkaloids, is a promising example. Nevertheless, it is not known whether lycorine has any antiviral activities against DTMUV. Therefore, the purpose of the present study is to investigate the anti-DTMUV abilities of lycorine. The cytotoxicity of lycorine was evaluated on BHK-21 cells by CCK-8 assay, and its antiviral effect against DTMUV was examined by real-time PCR assays, virus titer determination, Western blot and immunofluorescence (**IFA**) assays, respectively. Furthermore, the underlying mechanisms of the anti-DTMUV effects of lycorine were also investigated. The results indicated that the highest nontoxicity concentration of lycorine on BHK-21 cells was 5 μM. Lycorine possessed the antiviral ability against DTMUV on BHK-21 cells, as demonstrated by the reduction of virus titers and copy numbers in vitro. Western blot and IFA analysis showed the inhibitory effect of lycorine on DTMUV envelope (**E**) protein expression. Moreover, using time-of-addition assays, we found that lycorine displays its antivirus and virucidal activities through blocking viral internalization and entry in vitro. Taken together, our findings firstly demonstrate the antiviral activities of lycorine against DTMUV, suggesting that lycorine can be a potential drug for the treatment of DTMUV infection.

Key words: duck tembusu virus, lycorine, antiviral activity, viral internalization and entry

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INTRODUCTION

Duck tembusu virus (**DTMUV**) is a newly discovered *mosquito-borne Flavivirus* responsible for outbreaks of an infectious viral disease in layer ducks (Cao et al., 2011; Su et al., 2011; Tang et al., 2012). Later, infections and deaths were also reported in chicken (Chen et al., 2014a), geese (Huang et al., 2013), and sparrows (Tang et al., 2013). DTMUV was first described in 2010 in China (Yan et al., 2011). Until now, DTMUV has circulated in China and south eastern Asia, including Malaysia and Thailand, causing huge economic losses (Homonnay et al., 2014; Ninvilai et al., 2018; Thontiravong et al., 2015). Although there are several commercial vaccines in China, the outbreaks of this severe contagious disease still occur in some duck farms. Notably, high levels of antibody against DTMUV were detected in serum samples of duck farm workers (Pulmanausahakul et al., 2021), indicating that DTMUV has potential threats to public health. Hence, developing more effective antiviral drugs against DTMUV is urgent.

Lycorine, one of the important sources of active alkaloids, has been reported to exhibit certain pharmacological properties, including anticancer (Roy et al., 2018), antiviral (Jin et al., 2020), antiparasitic (Nair and van Staden, 2019), antibacterial (Tan et al., 2011), and anti-inflammatory (Zhu et al., 2018) activities. For antiviral research, lycorine possesses inhibitory effects on several viruses, including human enterovirus 71 (**EV71**; Liu et al., 2011), severe acute respiratory syndrome coronavirus 2 (**SARS-CoV-2**; Zhang et al., 2020), herpes simplex virus (**HSV**; Renard-Nozaki et al., 1989), poliovirus (Oluyemisi et al., 2015), and avian influenza virus (**AIV**; He et al., 2013; Yang et al., 2019). Recently, a series of studies have demonstrated that lycorine can

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inhibit many members of the flavivirus family including zika virus (**ZIKV**; Chen et al., 2020), hepatitis C virus (**HCV**; Guo et al., 2016), dengue virus (**DENV**; Wang et al., 2014), and yellow fever virus (**YFV**) replication (Zou et al., 2009). However, the antiviral effect of lycorine against DTMUV has not been investigated, and the underlying mechanisms remain to be evaluated.

In the present study, we investigate the antiviral effect of lycorine against DTMUV in BHK-21 cells. Furthermore, we elucidated the detailed mechanism of lycorine inhibitory action on DTMUV infection. Our findings provide new candidate inhibitors to effectively treat DTMUV infections.

MATERIALS AND METHODS

Cell, Virus, and Drug

BHK-21 (Baby Hamster Syrian Kidney) cells were grown in dulbecco's modified eagle medium (**DMEM**, Gibco) with 10% fetal bovine serum (**FBS**, Gibco, Grand Island, NY), 100 U/mL penicillin, and 100 mg/mL streptomycin. The BHK-21 cells were incubated in a CO_2 incubator at 37°C before use.

DTMUV AQ-19 strain used in this study was isolated in our lab in 2019 (Genbank accession: MT708901). The virus was propagated in BHK-21 cells, and the viral titers determined by Reed and Muench method as the median tissue culture infective dose (**$TCID_{50}$**)/mL.

Lycorine was provided by PureOne Biotechnology in Shanghai, China. Its purity of lycorine was higher than 98%. It can be dissolved in dimethyl sulfoxide (**DMSO**, Sangon, Shanghai, China) and stored at −80°C.

Cytotoxic Effect of Lycorine

In order to eliminate the interference of lycorine concentration on the change of cellular growth, the cytotoxicity of lycorine in BHK-21 cells was assayed by Cell Counting Kit-8 (**CCK-8**, Vazyme, Nanjing, China). Once the BHK-21 cells formed a monolayer, the media was discarded and replenished with fresh medium containing increasing concentrations of lycorine (0μM, 0.1 μM, 0.5μM, 1μM, 5μM, 10μM, 50 μM, 100 μM). After 48 h incubation, 10-μL CCK-8 solution was added to each well. The 96-well plate was incubated at 37°C for 2 h, then the absorbance rates were measured by using a microplate reader at 450 nm.

Lycorine Treatment and DTMUV Infection In Vitro

To investigate the antiviral effect of lycorine on DTMUV, BHK-21 cells were seeded in a 12-well plate. Once the BHK-21 cells formed a monolayer, BHK-21 cells were infected with DTMUV at a multiplicity of infection (**MOI**) of 0.1 in the presence of different concentrations of lycorine (0 μM, 0.1 μM, 0.5 μM, 1 μM, 5 μM, and 10 μM). The cell supernatant was harvested for determining the DTMUV titers, and the cell pellets were collected to quantitate the viral RNA (**vRNA**) load. Meanwhile, the immunofluorescence or Western blot was used to detect the expression of E protein of DTMUV.

Direct Virucidal Assay

To investigate the interaction between DTMUV and lycorine, BHK-21 cells were infected with DTMUV (MOI = 0.1) under 2 different conditions: 1) Lycorine +Virus: the virus was mixed with 5-μM lycorine and then incubated at 37°C for 1 h to assess the virucidal activity. Then, BHK-21 cells were infected with the lycorine-treated viral supernatant for 2 h. The media was replaced by fresh DMEM with 1%FBS and then the cells were incubated at 37°C for 48 h. 2) Lycorine+Cells: BHK-21 cells were incubated with 5 μM lycorine for 1 h at 37°C. And then, the pretreated BHK-21 cells were infected with DTMUV AQ-19 (MOI = 0.1) for 2 h at 37°C. Subsequently, BHK-21 cell media was replaced by fresh DMEM with 1% FBS and incubated at 37°C for 48 h.

Adsorption and Postadsorption Assay

To assess the mechanism of antiviral activity, the time-of-addition effect of lycorine was performed according to a previously described procedure with minor modifications (Visintini Jaime et al., 2013). BHK-21 cells were infected with DTMUV AQ-19 (MOI = 0.1) under 3 conditions: 1) Adsorption: BHK-21 cells were infected with DTMUV AQ-19 in the presence of lycorine and incubated at 4°C for 1 h. Before the BHK-21 cells were incubated at 37°C for 48 h, the media was discarded and replaced with fresh DMEM with 1% FBS. 2) Postadsorption: BHK-21 cells were infected with DTMUV AQ-19 at 4°C for 1 h. Then, BHK-21 cells were washed with PBS and replaced with DMEM in the presence of lycorine at 37°C for 48 h. 3) Simultaneously: BHK-21 cells were infected with DTMUV AQ-19 strain in the presence of lycorine at 37°C for 48 h.

Detection of DTMUV by Quantitative RT-PCR

DTMUV RNA copy number was determined by quantitative RT-PCR as previously described (Zhu et al., 2021). According to the FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme, Nanjing, China) instruction, the total RNA of BHK-21 cells was extracted. Then, first-strand cDNA was synthesized by using HiScript Q RT SuperMix (Vazyme, Nanjing, China). The absolute quantitative RT-PCR was used to measure the vRNA load by using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China).

Indirect Immunofluorescence Assay

To detect the E protein expression levels of DTMUV, the indirect immunofluorescence assay was used. After 2 d postinfection, the cold methanol was used to fix BHK-21 cells at 4°C for 15 min. Then, the cells were washed 3 times with sterile PBS and inoculated with monoclonal antibody against DTMUV E protein which is prepared in our lab. After incubating at 37°C for 1 h, the BHK-21 cells were washed 3 time with sterile PBS and then incubated with FITC-labeled Goat Anti-Mouse IgG at 37°C for 1 h. BHK-21 cells were washed 3 times with sterile PBS, and incubated with diamidine phenylindole (**DAPI**, Solarbio, Beijing, China). The fluorescence microscope (Olympus Inc., Tokyo, Japan) was used to observe the immunofluorescence.

Western Blot

To detect the expression of E protein of DTMUV in BHK-21 cells, the Western blot analysis was used. After 48 h postinfection, the BHK-21 cell pellets were lysed using RIPA buffer (Thermo Fisher Scientific, Inc., Waltham, MA). 15 μg proteins was separated on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (**SDS-PAGE**) and then electrophoretically transferred to PVDF membranes. After that, the PVDF membranes were blocked with 5% skim milk at 37°C for 1 h and then incubated with monoclonal antibody against DTMUV E protein and β-actin used an internal control overnight at 4°C. Then, the membranes were incubated with Goat Anti-Mouse IgG/HRP (Solarbio, Beijing, China) at 37°C for 1 h. The E protein signal was detected using ECL Western blot reagent (Vazyme, Nanjing, China) and the quantification of the bands was performed using Quantity One software (Bio-Rad, Hercules, CA).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA). The results are presented as the means ± SD. Two groups were compared by Student t test. More groups were compared by one-way analysis of variance followed by Tukey's post hoc test. A P value of 0.05 was considered significant.

RESULTS

Dose-Dependent Inhibitory Effect of Lycorine on DTMUV

The molecular structure of lycorine is shown in Figure 1A. First, CCK-8 assays were performed to examine the cytotoxicity of lycorine in vitro. BHK-21 cells were incubated with lycorine at various doses ranging from 0 to 100 μM for 48 h. As shown in Figure 1B, we found that lycorine weakly affected the growth of BHK-21 cells. Specifically, viability of BHK-21 cells was insignificantly affected by lycorine treatment, with the exception of the higher concentrations (10−100 μM), which demonstrate significantly impaired cell viability compared with all other concentrations. The 50% cytotoxic concentrations (**CC50**) of lycorine was 18.99 μM on BHK-21 cells. Finally, 5 μM lycorine was selected for the subsequent experiments.

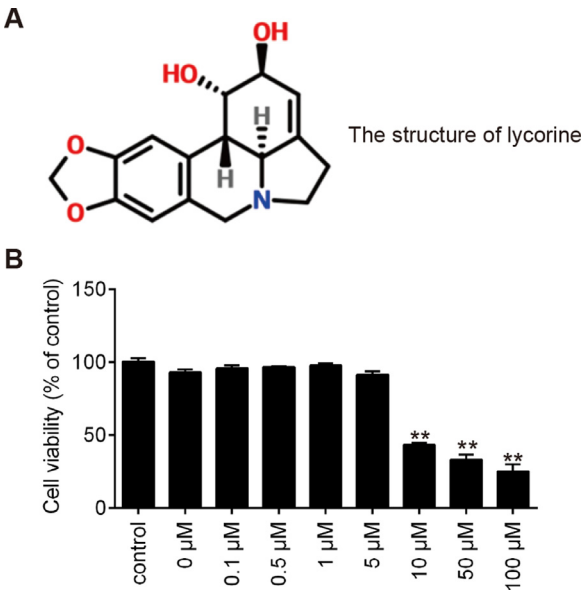

Figure 1. Lycorine cytotoxic effect on BHK-21 cells. (A) The structure of lycorine. (B) BHK-21 cells were incubated with lycorine at various doses ranging from 0− to 100 μM for 48 h. Then, CCK-8 assays were performed to examine the effect of lycorine on the viability of BHK-21 cells. Data are shown as means ± SD of three separate experiments. $^{**}P < 0.01$ vs. control group.

To investigate whether lycorine has anti-viral effect on DTMUV, BHK-21 cells were treated with lycorine (0 μM, 0.1 μM, 0.5 μM, 1 μM, 5 μM, and 10 μM) and subsequently infected with DTMUV at an MOI of 0.1. Then, we analyzed the changes by determining the viral titers in the cell supernatant and the copy numbers in cells. As shown in Figures 2A and 2B, DTMUV titers and copy numbers significantly decreased in a dose-dependent manner upon lycorine treatment in BHK-21 cells. Approximately 80% viral inhibition was observed in the BHK-21 cells treated with 5 μM lycorine. The half-maximal inhibitory concentration ($\mathbf{IC_{50}}$) was estimated to approximately 2 μM (Figure 2C). Moreover, to validate the antiviral effect of lycorine, we applied Western blot and IFA analysis to quantify the E protein levels of DTMUV in BHK-21 cells, which is considered a direct method for estimating the DTMUV load in cells. Levels of E proteins are commonly used to evaluate the level of viral replication. As can be seen in Figures 2D and 2E, viral E protein levels were decreased after lycorine treatment. This inhibitory effect was also a dose-dependent manner. These results indicated that lycorine can inhibit DTMUV replication in BHK-21 cells.

Lycorine Was Directly Virucidal

To investigate the extracellular virucidal ability of lycorine, 2 group treatments were used to confirm

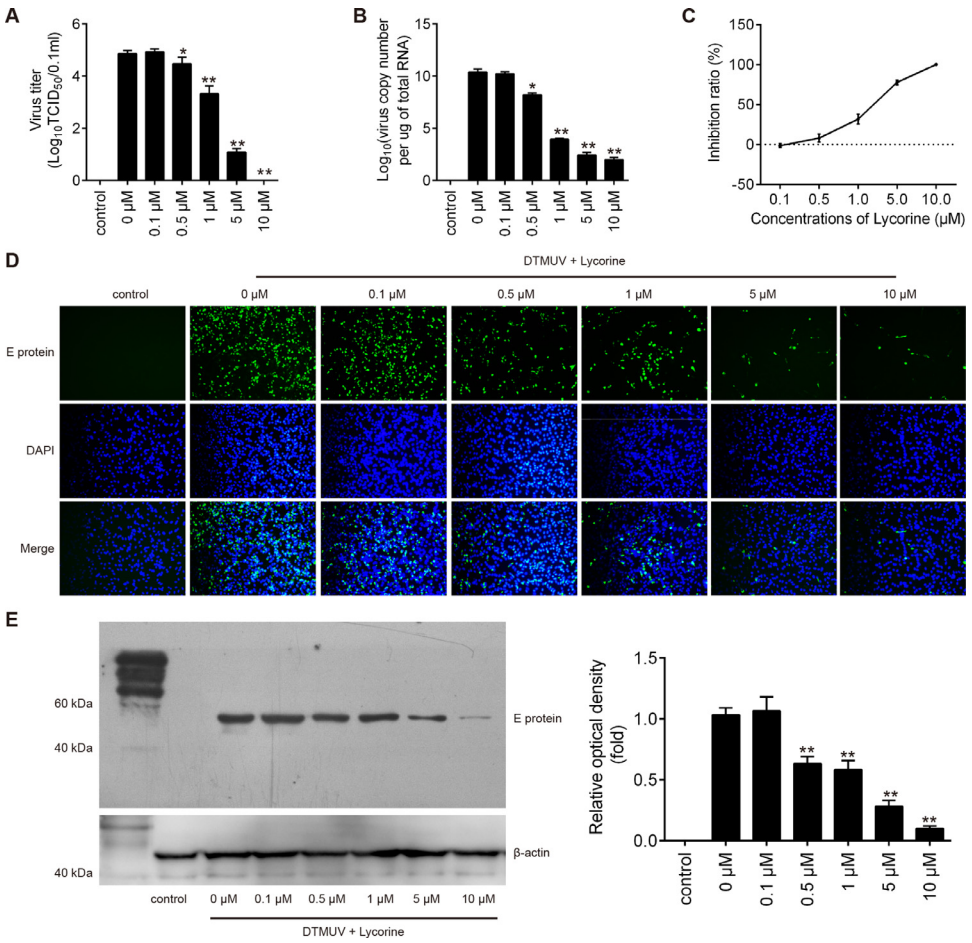

Figure 2. Dose-dependent inhibition of DTMUV infection by lycorine. BHK-21 cells were treated with different concentrations of lycorine (0 μM, 0.1 μM, 0.5 μM, 1 μM, 5 μM, and 10 μM) at 37°C and subsequently infected with DTMUV at an MOI of 0.1 for 48 h. (A) The virus titers were determined by measuring the $TCID_{50}$ assay. (B) The virus copy numbers were quantified by qRT-PCR. (C) The inhibition rate was determined by titrating progeny virus and calculated as follows: [(The virus titers of 0 μM group — virus titers of each compound-treated groups)/(The virus titers of 0 μM group)] × 100. (D and E) DTMUV E protein expression in cells was detected using indirect immunofluorescence staining and Western blot. Data are shown as means ± SD of three separate experiments. * $P < 0.05$, ** $P < 0.01$ vs. DTMUV infection group. Abbreviations: DTMUV, duck tembusu virus; MOI, multiplicity of infection; $TCID_{50}$, tissue culture infective dose.

this ability. In lycorine + virus group, AQ-19 strain DTMUV was incubated with 5-μM lycorine at 37°C for 1 h. Subsequently, the mixture was infected with BHK-21 cells for 48 h. In lycorine + cells group, BHK-21 cells were first incubated with 5-μM lycorine for 1 h then infected with AQ-19 DTMUV at 37°C for 48 h. As shown in Figures 3A and 3B, the virus titers and copy numbers were significantly decreased in lycorine + virus group in comparison with the DTMUV group. However, in lycorine + cells group, no significant influence on the virus titers and copy numbers were observed comparing to the DTMUV group. Approximately 75% viral titer reduction was observed in lycorine + virus group, whereas the antiviral effects in lycorine + cell group was minimal (Figure 3C).

DTMUV E protein was further determined by WB (Figure 3D) and IFA (Figure 3E) in BHK-21 cells. The results showed that, as compared to the DTMUV infection group, the DTMUV E protein expression levels were significantly reduced in lycorine + virus group, while the lycorine + cells group had no significant change. All these data indicated that lycorine can reduce DTMUV infectivity through directly interacting with DTMUV particles.

Lycorine Blocked the Postadsorption Steps of DTMUV in BHK-21 Cells

To determine which stage of the DTMUV life cycle is specifically affected by lycorine, virus adsorption and postadsorption assays were performed in BHK-21 cells. It was shown that both the virus titers and copy numbers were decreased at different degrees in three groups including simultaneously group, adsorption group and postadsorption group, in comparison with the DTMUV infection group (Figures 4A and 4B). Interestingly, compared to the DTMUV group, the simultaneously and postadsorption groups showed great antiviral effect, while a weaker inhibitory effect was observed in adsorption group. As shown in Figure 4C, approximately 100% viral titer reduction was observed in postadsorption group, whereas the inhibition ratio for simultaneously

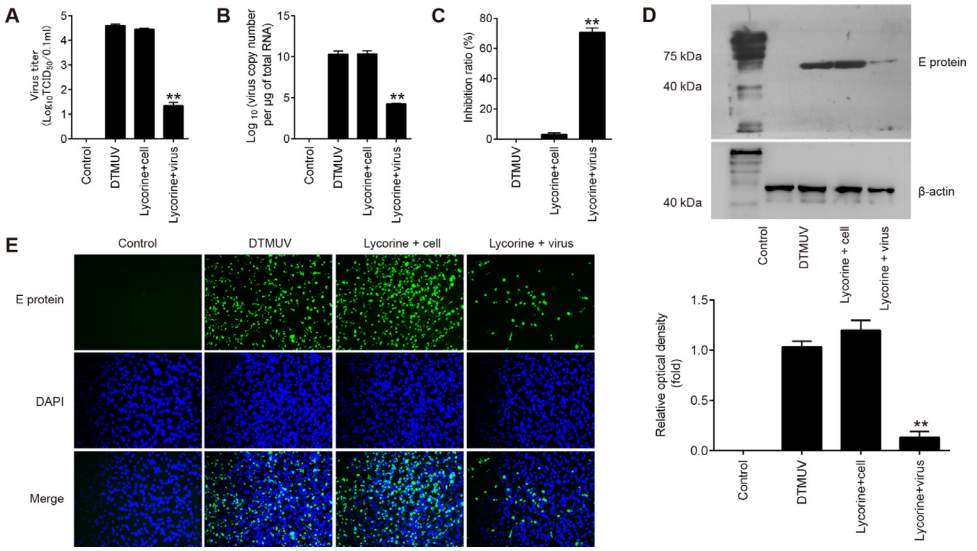

Figure 3. Lycorine inhibited DTMUV infection through direct interaction with DTMUV particles. The timing of lycorine treatment after infection was planned into 2 treatment regimens: lycorine + virus and lycorine + cells. The cells and cell supernatants were collected after 48 h infection for subsequent experiments. (A) The virus titers were determined by $TCID_{50}$ assay. (B) The virus copy numbers were measured by qRT-PCR. (C) The inhibition rate was determined by titrating progeny virus and calculated as follows: [(The virus titers of DTMUV group—virus titers of each compound-treated groups)/(The virus titers of DTMUV group)] × 100. (D and E) DTMUV E protein expression in cells was detected using Western blot and indirect immunofluorescence staining, respectively. Data are shown as means ± SD of three separate experiments. ** $P < 0.01$ vs. DTMUV group. Abbreviations: DTMUV, duck tembusu virus; $TCID_{50}$, tissue culture infective dose.

group and adsorption group was 70 and 25%, respectively (Figure 4C). As expected, we observed similar results for the viral E protein level detection in all 3 groups compared to the DTMUV group (Figures 4D and 4E). In summary, these results showed that lycorine inhibits DTMUV infection mainly by blocking the post-adsorption stage.

DISCUSSION

In the present study, we found that lycorine treatment showed antiviral activity against DTMUV in BHK-21 cells. This antiviral activity of lycorine is mainly achieved by exerting its virucidal activity and inhibiting viral internalization and entry into host cells. Our

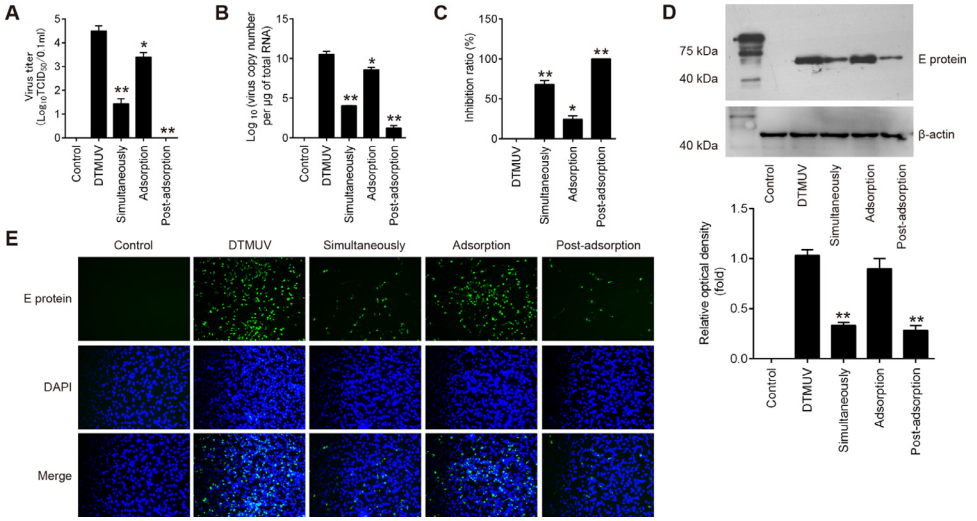

Figure 4. Lycorine inhibited DTMUV infection through blocking the postadsorption of DTMUV in BHK-21 cells. BHK-21 cells underwent 3 types of treatment as follows: 1) Simultaneous treatment: BHK-21 cells were treated with 5 μM lycorine at 37°C and simultaneously infected with DTMUV at an MOI of 0.1. 2) Adsorption: BHK-21 cells in 12-well plates were infected with DTMUV at an MOI of 0.1 for 1 h at 4°C in the presence of 5 μM lycorine. After removal of the pretreatment medium, the cells were incubated with compound-free media for 48 h. 3) Postadsorption: DTMUV at an MOI of 0.1 was preincubated with BHK-21 cells in 12-well plates for 1 h at 4°C. After removal of the pretreatment medium, the cells were incubated with 5 μM lycorine at 37°C for 48 h. (A) The virus titers were determined by $TCID_{50}$ assay. (B) The virus copy numbers were measured by qRT-PCR. (C) The inhibition rate was determined by titrating progeny virus and calculated as follows: [(The virus titers of DTMUV group—virus titers of each compound-treated groups)/(The virus titers of DTMUV group)] × 100. (D and E) DTMUV E protein expression in cells was detected using Western blot and indirect immunofluorescence staining, respectively. Data are shown as means ± SD of three separate experiments. * $P < 0.05$, ** $P < 0.01$ vs. DTMUV group. Abbreviations: DTMUV, Duck tembusu virus; MOI, multiplicity of infection; $TCID_{50}$, tissue culture infective dose.

findings showed that lycorine has promising therapeutic potential against DTMUV infection.

Current findings showed that lycorine has significant antiviral activities against several viruses. For example, Chen et al. (2020) reported that lycorine could effectively inhibit ZIKV infection by restraining RNA-dependent RNA polymerase (**RdRp**) activity in vitro and in vivo. In addition, lycorine was reported to inhibit the expression of hsc70, which is important for HCV replication (Chen et al., 2015). Nevertheless, the antiviral effect of lycorine against DTMUV has not been showed in previous study. In our study, we demonstrated that lycorine can effectively inhibit DTMUV replication, as demonstrated by reduction of virus titers, virus copy numbers, and expression levels of DTMUV E protein. Our findings suggested that lycorine treatment is effective in preventing infection in vitro.

The viral life cycle includes attachment to the target cell, viral internalization, and replication and release from the target cell. Many antiviral drugs work by blocking one or more of these steps (Chen et al., 2014b; Liu and Thorp, 2002). For instance, Tsai showed that tryptanthrin prevents the early and late stages of human coronavirus NL63 (**HCoV-NL63**) replication, by blocking viral RNA genome synthesis and papain-like protease 2 activity (Tsai et al., 2020). Li et al. (2019) found that Griffithsin reduced porcine epidemic diarrhea virus (**PEDV**) infection by preventing viral attachment to host cells and disrupting cell-to-cell transmission. Previous studies have reported that lycorine can inhibit virus infection through a variety of potential mechanisms. For example, Liu et al. (2011) found that lycorine affects the elongation of the viral polyprotein during EV71 replication. It is well-known that time-of-addition assay can provide a preliminary understanding of the infectious phase upon which lycorine acts. Our results demonstrated that the antiviral effect of lycorine on DTMUV can be achieved by interfering with viral internalization and entry.

In summary, we demonstrated that lycorine inhibits DTMUV infection in vitro by blocking viral internalization and entry. These results provided support for lycorine acting as a useful drug candidate for DTMUV prevention, which warrants further research in the application of Lycorine in DTMUV treatment.

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DISCLOSURES

The authors declare no competing interests.

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