Lianhuaqingwen exerts anti-viral and anti-inflammatory activity against novel coronavirus (SARS-CoV-2)

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ABSTRACT

Purpose: Lianhuaqingwen (LH) as traditional Chinese medicine (TCM) formula has been used to treat influenza and exerted broad-spectrum antiviral effects on a series of influenza viruses and immune regulatory effects Ding et al. (2017). The goal of this study is to demonstrate the antiviral activity of LH against the novel SARS-CoV-2 virus and its potential effect in regulating host immune response.
Methods: The antiviral activity of LH against SARS-CoV-2 was assessed in Vero E6 cells using CPE and plaque reduction assay. The effect of LH on virion morphology was visualized under transmission electron microscope. Pro-inflammatory cytokine expression levels upon SARS-CoV-2 infection in Huh-7 cells were measured by real-time quantitative PCR assays.
Results: LH significantly inhibited SARS-CoV-2 replication in Vero E6 cells and markedly reduced pro-inflammatory cytokines (TNF-α, IL-6, CCL-2/MCP-1 and CXCL-10/IP-10) production at the mRNA levels. Furthermore, LH treatment resulted in abnormal particle morphology of virion in cells.
Conclusions: LH significantly inhibits the SARS-COV-2 replication, affects virus morphology and exerts anti-inflammatory activity *in vitro*. These findings indicate that LH protects against the virus attack, making its use a novel strategy for controlling the COVID-19 disease.

1. Introduction

Coronaviruses are a group of enveloped viruses named for their coronary appearance with positive single-stranded RNA genomes [2]. In addition to six known strains of coronaviruses that are infectious to humans, a novel coronavirus (SARS-CoV-2) was detected recently in Wuhan, China [3,4]. Like the other two highly pathogenic coronaviruses SARS-CoV and MERS-CoV, SARS-CoV-2 also caused severe respiratory illness and even death. Moreover, the population's susceptibility to these highly pathogenic coronaviruses has contributed to large outbreaks and evolved into the public health events, highlighting the necessity to prepare for future reemergence or the novel emerging viruses [5].

Similar to SARS-CoV and MERS-CoV, SARS-CoV-2 is initiated by zoonotic transmission likely from bats and spreads rapidly among humans [6]. The basic reproduction number (R0) of person-to-person spread is about at 2.6, which means that the SARS-CoV-2 infected cases grow at an exponential rate. As of February 07, 2020, 57,620 cases of the SARS-CoV-2 have been reported in China, including 26,359 suspected cases, and a sustained increase is predictable. The initial patient cluster with confirmed SARS-CoV-2 infection was reported Wuhan pneumonia with unknown aetiology, which bore some resemblance to SARS-CoV and MERS-CoV infections and was associated with ICU admission and high mortality. Moreover, High concentrations of cytokines

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were recorded in plasma of patients requiring ICU admission, such as GCSF, IP10, MCP1, MIP1A, and TNFα, suggesting that the cytokine storm was associated with disease severity [7]. A retrospective clinical study indicated the risk of fatality among hospitalized cases at 4.3 % in single-center case series of 138 hospitalized patients [8], and the infection fatality risk could be below 1% or even below 0.1 % in a large number of undetected relatively mild infections [9]. However, It is challenging to judge the severity and predict the consequences with the information available so far. Since no specific antiviral treatment for COVID-19 is currently available, supportive cares, including symptomatic controls and prevention of complications remain the most critical therapeutic regimens, especially in preventing acute respiratory distress syndrome [10]. Although the control of SARS-CoV-2 still presents multiple challenges in the short term, more potent antiviral drugs are urgent to be developed [4].

At present, some drugs are effective in eliminating SARS-CoV-2 and improving symptoms. The most promising antiviral drug for SARS-CoV-2 is remdesivir that is currently under clinical development for the treatment of Ebola virus infection [11]. However, the efficacy and safety of remdesivir for SARS-CoV-2 pneumonia patients need to be assessed by further clinical trials. In addition, in the prevention and treatment of COVID-19, Tranditonal Chinese medicines have received broad adoption, especially in treating cases of mild symptoms [12]. Lianhuaqingwen (LH), a Chinese patent medicine composed of 13 herbs, has played a positive role in the treatment of SARS-CoV-2. A retrospective analysis of clinical records was conducted in the SARS-CoV-2 infected patients at Wuhan Ninth Hospital and CR & WISCO General Hospital. LH combination could significantly relieve cardinal symptoms and reduce the course of the COVID-19 [13], making it successively included in the *Guideline for the Diagnosis and Treatment of Novel Coronavirus (2019-nCoV) Pneumonia (On Trials, the Fourth/Fifth/Sixth/Seventh Edition)* issued by National Health Commission of the People's Republic of China and also recommended by 20 provincial health commissions including Hubei, Beijing, and Shanghai as well as National Administration of Traditional Chinese Medicine for the treatment of COVID-19. Moreover, LH exerted broad-spectrum effects on a series of influenza viruses by inhibiting viral propagation and regulating immune function and achieved similar therapeutic effectiveness with Oseltamivir in reducing the course of H1N1 virus infection [1,14,15]. Notably, the anti-influenza activity of LH in infected mice might depend on the regulation of cytokines, particularly in cytokine storm associated cytokines, such as IP-10, MCP-1, MIP1A, and TNF-α [1]. In the present study, we evaluated the antiviral and anti-inflammatory efficiency of LH against a clinical isolate of SARS-CoV-2 from Guangzhou in vitro.

2. Materials and methods

2.1. Cell lines and virus

The African green monkey kidney epithelial (Vero E6) cells and the human hepatocellular carcinoma (Huh-7) cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10 % fetal bovine serum (FBS) at 37 °C. A clinical isolated SARS-CoV-2 virus (Genebank accession no. MT123290.1) was propagated in Vero E6 cells, and viral titer was determined by 50 % tissue culture infective dose ($TCID_{50}$) according to the cytopathic effect by use of Reed-Muench method [17]. All the infection experiments were performed in a biosafety level-3 (BLS-3) laboratory.

2.2. Reagent preparation

LH capsule (Lot No.B2001019) was obtained from Yiling Pharmaceutical Co. Ltd. (Shijiazhuang, China). UPLC fingerprints of LH consist of 32 common peaks. 9 of 32 common peaks are identified. The similarities in 10 batches of LH Capsules samples were all above 0.96

(Supplementary Fig. 1). The black powder of raw material of LH was first dissolved in dimethyl sulfoxide (DMSO) to 240 mg/mL. After shaking for 30 min at room temperature, the LH solution was diluted with serum-free DMEM to 24 mg/mL as a stock solution and stored at −20 °C before using. Remdesivir was kindly provided by Prof. Jiancun Zhang from Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences and was dissolved in DMSO to 100 mM and stored at −20 °C before using. DMEM with 2% FBS was used as the dilution buffer in the follow-up experiments.

2.3. Cytotoxicity assay

The cytotoxic effects of the LH on Vero E6 and Huh-7 cells were evaluated by Methyl Thiazolyl Tetrazolium (MTT) assay. Briefly, monolayers of Vero E6 cells and Huh-7 cells in 96-well plates were rinsed with phosphate-buffered saline (PBS) followed by incubation with indicated concentrations of LH. After 72 h, the cells were stained with MTT solution at 0.5 mg/mL for 4 h. The supernatants were then removed, and the formed formazan crystals were dissolved in 200 μL DMSO. The absorbance was measured at 490 nm using Multiskan Spectrum reader (Thermo Fisher, USA). The 50 % cytotoxic concentration (CC_{50}) was calculated by the GraphPad Prism 7.0 software.

2.4. Cytopathic effect (CPE) inhibition assay

The Vero E6 cell monolayers were grown in 96-well plates and inoculated with 100 $TCID_{50}$ of coronavirus strains at 37°C for 2 h. The inoculum was removed, and the cells were subsequently incubated with indicated concentrations of LH or the positive control remdesivir. Following the 72 h of incubation, the infected cells shown 100 % CPE under the microscope. The percentage of CPE in LH-treated cells were recorded. The 50 % inhibition concentration (IC_{50}) of the virus-induced CPE by LH was calculated by the Reed-Muench method [17].

2.5. Plaque reduction assay

The Vero E6 cell monolayers in 6-well plates were infected with 50 plaque-forming units (PFU) of SARS-CoV-2 for 2 h at 37 °C. After incubation, the cell monolayers were covered with agar overlay (final concentration: 0.6 % agar, 2% FBS, indicated concentrations of LH or remdesivir). The plates were then incubated for 48 h at 37 °C with 5% CO_2. Subsequently, the agar overlays were removed, and the cell monolayer was fixed with 10 % formalin, stained with 1% crystal violet, and then the plaques were counted and photographed.

2.6. RNA isolation and reverse transcriptase-quantitative PCR analysis (RT-qPCR)

The Huh-7 cell monolayers in 12-well plate were rinsed with PBS and then exposed to coronavirus at a multiplicity of infection (MOI) of 1 for 2 h at 37 °C. The inoculum was removed and replaced with the indicated concentrations of LH or mock-treated with DMEM supplemented with 2% FBS for subsequent 48 h incubation at 37 °C with 5% CO_2. The cells were then harvested for RNA isolation and qPCR as described previously [16]. The primer and probe sequences used for analysis are listed in Supplementary Table 1. The relative mRNA expression was calculated using the $2^{-\triangle\triangle Ct}$ method with GAPDH as an internal reference gene.

2.7. Electron microscope

Monolayers of Vero E6 cells in 6-well plates were incubated with SARS-CoV-2 at a MOI of 0.001 for 2 h at 37 °C. The virus inoculum was then removed and replaced with DMEM medium supplemented with 2% FBS containing LH (600 μg/mL) or remdesivir (5 μM). At 48 h p.i., the cells were fixed, dehydrated and embedded as described previously
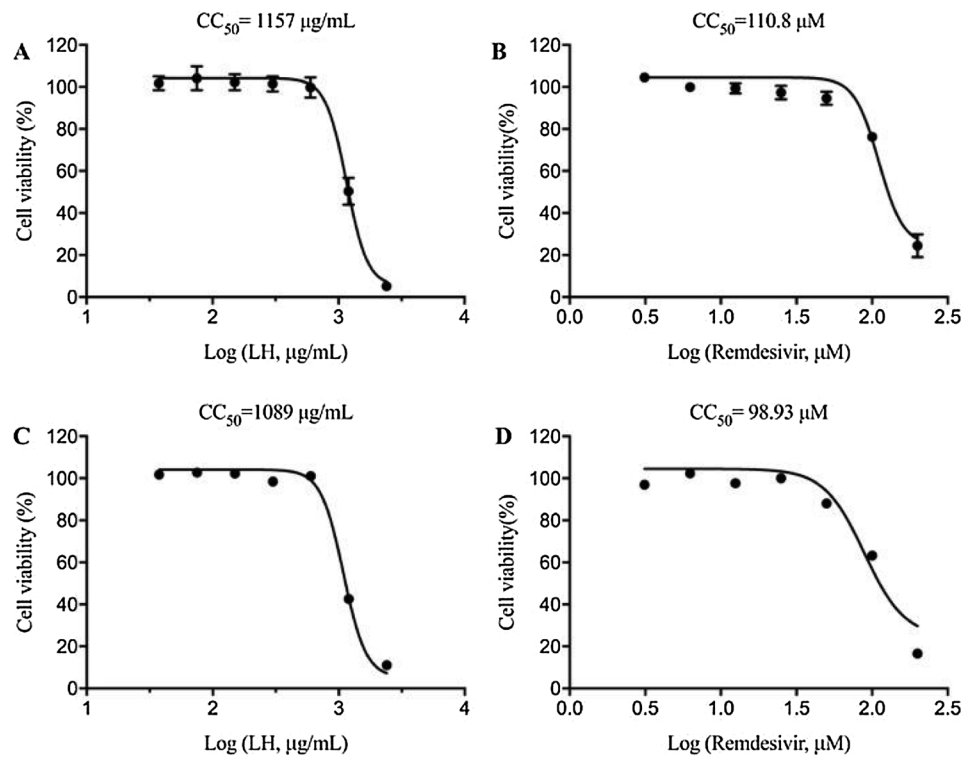
Fig. 1. Cytotoxic effect of the LH and remdesivir on Vero E6 cells (A, B) and Huh-7 cells (C, D). Data are presented as mean ± SD. The experiments were performed in triplicate.

[18]. Ultrathin sections (70 nm) of embedded cells were prepared, deposited onto Formvar-coated copper grids (200 mesh), stained with uranyl acetate and lead citrate, and then observed under JEM-1400 PLUS transmission electron microscopy (Japan Electron Optics Laboratory Co., Ltd., JEM-1400 PLUS).

2.8. Statistical analyses

Statistical analysis was performed using GraphPad Prism 7.0 software. The differences in mRNA expression levels of cytokines were compared using a one-way analysis of variance (ANOVA). Values of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Antiviral activity of LH on SARS-CoV-2 in vitro

The cell viability after LH or remdesivir treatment was determined by MTT assay in both Vero E6 and Huh-7 cells. LH showed unapparent cytotoxicity for both cell lines at concentrations up to 600 μg/mL (Fig. 1A, C). The positive control remdesivir showed no cytotoxicity to cells at a concentration of 50μM (Fig. 1B, D).

To investigate the antiviral effect of LH against SARS-CoV-2 virus, the Vero E6 cells were infected with 100 $TCID_{50}$ of virus and incubated with LH at various concentrations for 72 h. As shown in Fig. 2A, LH inhibited the replication of SARS-CoV-2 virus with an IC_{50} value of 411.2 μg/mL by CPE assay (Fig. 2A). Meanwhile, treatment with LH following infection also had a dose-dependent inhibitory effect on plaque formation of the SARS-CoV-2 virus (Fig. 2C). We selected remdesivir as the positive control in our study and the results showed that remdesivir potently inhibited virus-induced CPE with an IC_{50} of 0.651 μM and a total plaque formation inhibition at 5 μM (Fig. 2B, C).

To further confirm the efficacy of LH in inhibiting SARS-CoV-2 virus replication in cells, we detected the viral particles in ultrathin sections of infected cells under electron microscopy. At 48 h p.i., viral particles

were found in cytoplasm, intracellular vesicles, endoplasmic reticulum, and cell membrane and presented spherical crown-like appearance, which was typical coronavirus morphology (Fig. 3B, G). LH (600 μg/mL) and positive control remdesivir (5 μM) treatment resulted in a reduction of the number of virions compared with mock-treated infected cells (Fig. 3G–J). It was interesting to note that some virions in the surface of LH-treated cells presented spindle sharp which was in contrast to the typical spherical particles in the mock-treated cells (Fig. 3I).

3.2. Inhibition of SARS-CoV-2-induced cytokine and chemokine expression by LH in vitro

To determine the effect of LH on the expression of cytokines and chemokines induced by SAR2-CoV-2, the mRNA expression levels of TNF-α, IL-6, CCL-2/MCP-1, and CXCL-10/IP-10 were detected and compared between the LH-treated and mock-treated Huh-7 cells. The results showed that the elevated expressions of these four cytokines were significantly inhibited by LH treatment in a concentration-dependent manner (Fig. 4).

4. Discussion

Starting from December 2019, a pandemic of respiratory illness caused by a novel coronavirus named SARS-CoV-2 is sweeping the mainland of China. This virus has spread to several foreign countries, threatening to trigger a global outbreak. Several antiviral agents can be envisaged to control or prevent viral infections by antiviral assay in vitro [14,17]. However, the efficacy and safety of novel candidates need validations in vivo, even for those clinically approved medicines, which means that it will take months to years for clinical practices. At present, symptomatic and supportive treatments remain key to clinical practices. Thus, Traditional Chinese Medicines (TCM) carried both the antiviral effect and the symptomatic relief might bring more clinical benefits [12]. As a classical TCM prescription for respiratory diseases,
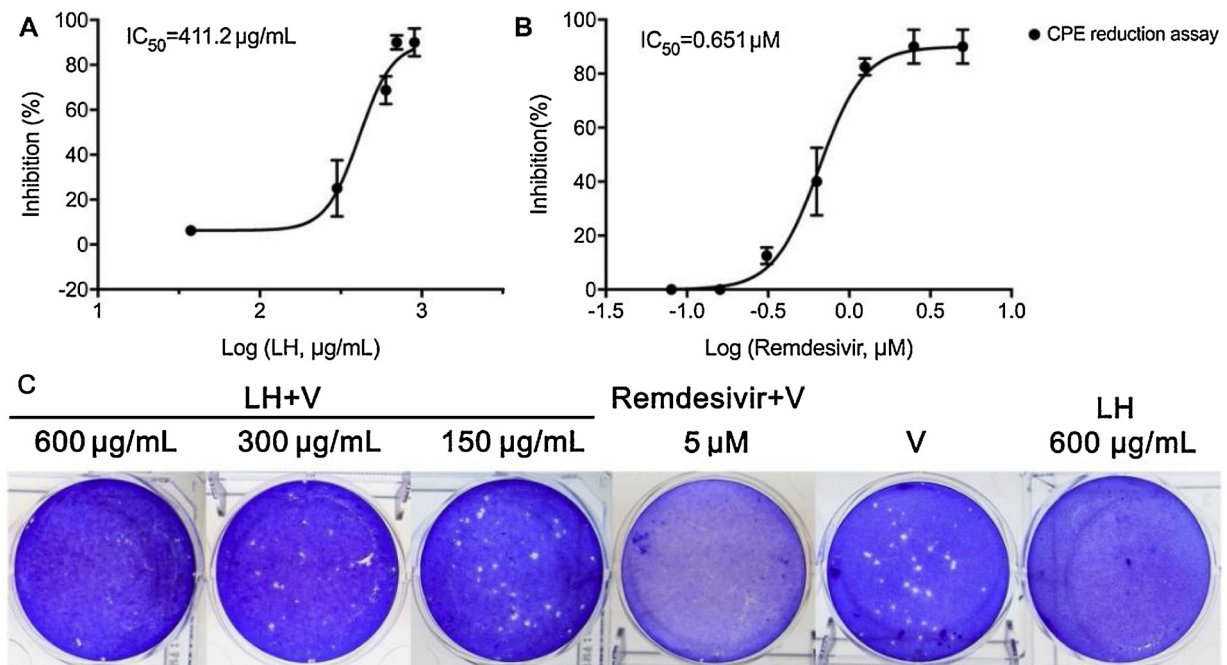
Fig. 2. Antiviral activity of LH and remdesivir against SARS-CoV-2 in Vero E6 cells. (A, B) The inhibitory curve for LH and remdesivir. (C) Plaque reduction assay of LH against the SARS-CoV-2 virus. Data are representative of three independent experiments.
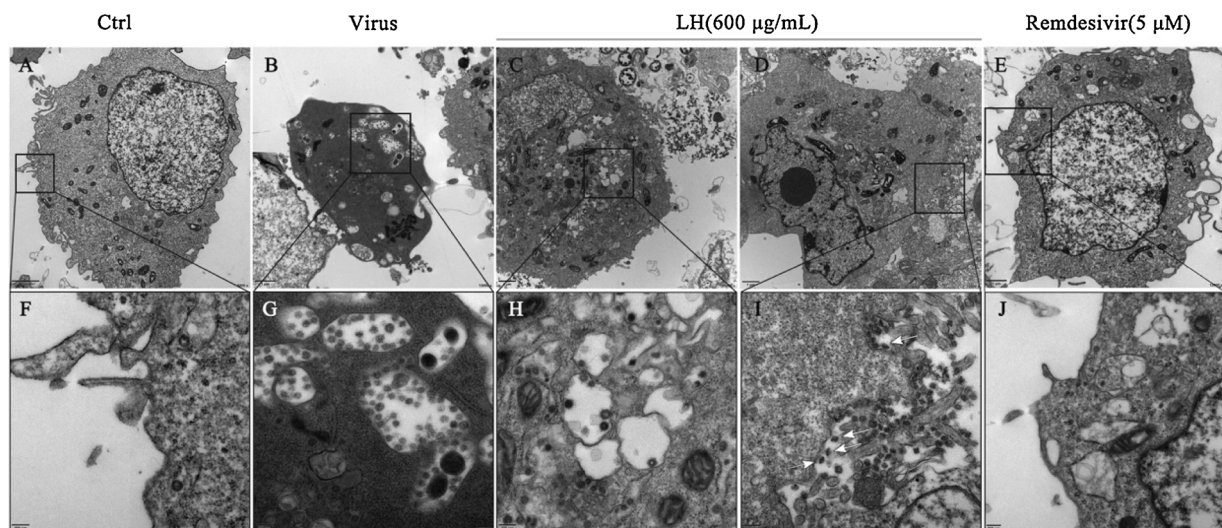

Fig. 3. Virions in the ultra sections of infected Vero E6 cells under electron microscope. (A, F) uninfected cells, (B, G) mock-treated SARS-CoV-2 virus infected cells, (C, D, H, I) infected cells with LH treatment, (E, J) infected cells with remdesivir treatment. White arrows indicated the spindle sharp of viral particles in infected cells with LH treatment.

LH is the only approved medicine in the treatment of SARS and influenza. After the outbreak of SARS-CoV-2, LH as a representative TCM prescription was recommended again in the latest Guideline for the Diagnosis and Treatment of Novel Coronavirus (2019-nCoV) Pneumonia issued by National Health Commission of the People's Republic of China The purpose of this study was to demonstrate whether the therapeutic effects of LH on the COVID-19 targeting virus replication and immunological regulation as it did on the infection caused by influenza viruses.

Our previous study showed that LH exhibited in vitro anti-influenza activity with IC$_{50}$ ranging from 200 to 2000 μg/mL [1]. Here we demonstrated that LH also has a comparable antiviral potency against the SARS-CoV-2 virus with an IC$_{50}$ value of 411.2 μg/mL (Fig. 2). Transmission electron microscopy (TEM) has been a potent tool to observe virus entry, virus particle assembly, viral ultrastructure, and budding

from the plasma membrane [17]. To understand the antiviral details of LH, EM pictures were taken from each group. Abundant virus particles assembled at the surface of membrane, cytoplasm, and plasma vesicles in the SARS-CoV-2 infected cells, decreased in the treatment of LH at 600ug/mL. Notably, slight deformation of virus particles was seen in the LH treatment, which required us to make further studies.

Highly pathogenic coronaviruses such as SARS-CoV and MERS-CoV cause fatal pneumonia, which is mainly associated with rapid virus replication, massive inflammatory cell infiltration and elevated proinflammatory cytokine/chemokine responses. Although the pathophysiology of fatal pneumonia caused by highly pathogenic coronaviruses has not been completely understood, accumulating evidence suggests that the cytokine storm plays a crucial role in causing fatal pneumonia [18]. Excessive amounts of proinflammatory cytokines were reported (e.g., IL-1β, IL-6, IL-12, IFN-γ, IP-10, and MCP-1) in the serum of SARS
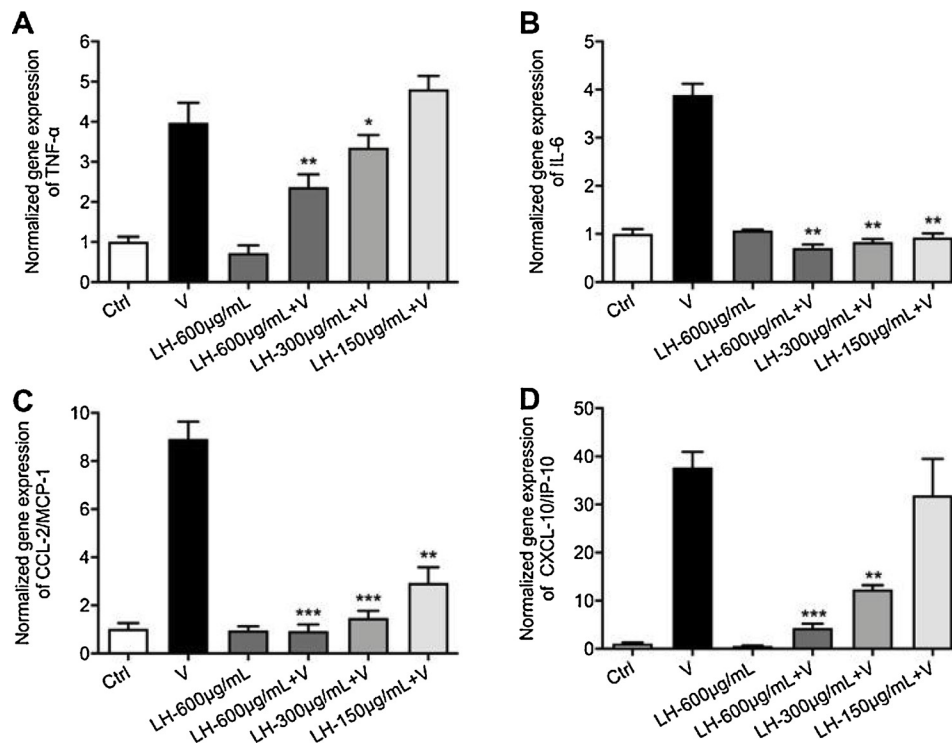
Fig. 4. Effects of LH treatment on the mRNA expression levels of inflammatory mediators in SARS-CoV-2-infected Huh-7 cells. A: TNF-α, B: IL-6, C: CCL-2/MCP-1, D: CXCL-10/IP-10. Data are presented as the mean ± SD obtained from three separate experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$, compared with mock-treated cells.

patients [18], similar in the serum of MERS patients [19]. Chaolin Huang et al. confirmed the occurrence of the cytokine storm in the COVID-19 patients in ICU rather than those in non-ICU patients [7]. Based on the excessive cytokines responses, Suxin Wan et al. claimed that IL-6 and IL-10 levels could be used as one of the bases for predicting the outcome and prognosis of the COVID-2019 [20]. In this study, host cells infected with HCoV-229E and SARS-COV-2 increased the cytokine release such as TNF-α, IL-6, CCL-2/MCP-1, and CXCL-10/IP-10, which was suppressed by LH in a dose-dependent manner. The change of cytokine profiles suggested that LH might have a potential effect on the inhibition of cytokine storm induced by SARS-COV-2, which also needed to be validated *in vivo*.

5. Conclusion

Since the launch of LH, it has been widely used as a broad spectrum of antiviral agent in the clinical practice, especially for various respiratory virus infections. Previous studies have shown that LH a broad spectrum of effects on a series of influenza viruses by interfering with both viral and host reactions. Although LH significantly relieved the clinical symptoms of the COVID-19, the underlying mechanism of antiviral effects on coronavirus, especially in the SARS-COV-2, was still elusive. In this study, we demonstrated that LH exerted its anti-coronavirus activity by inhibiting virus replication and reducing the cytokine release from host cells, which supported the clinical application of LH in combination with existing therapies to treat COVID-2019.

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.

Acknowledgements

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phrs.2020.104761.

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