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Antiviral effects of human placenta hydrolysate (Laennec®) against SARS-CoV-2 *in vitro* and in the ferret model

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The COVID-19 pandemic has caused unprecedented health, social, and economic crises worldwide. However, to date, there is an only a limited effective treatment for this disease. Human placenta hydrolysate (hPH) has previously been shown to be safe and to improve the health condition in patients with hyperferritinemia and COVID-19. In this study, we aimed to determine the antiviral effects of hPH against SARS-CoV-2 *in vitro* and *in vivo* models and compared with Remdesivir, an FDA-approved drug for COVID-19 treatment. To assess whether hPH inhibited SARS-CoV-2 replication, we determined the CC_{50}, EC_{50}, and selective index (SI) in Vero cells by infection with a SARS-CoV-2 at an MOI of 0.01. Further, groups of ferrets infected with $10^{5.8}$ $TCID_{50}$/ml of SARS-CoV-2 and treated with hPH at 2, 4, 6 dpi, and compared their clinical manifestation and virus titers in respiratory tracts with PBS control-treated group. The mRNA expression of immune-related cytokines was determined by qRT-PCR. hPH treatment attenuated virus replication in a dose-dependent manner *in vitro*. In a ferret infection study, treatment with hPH resulted in minimal bodyweight loss and attenuated virus replication in the nasal wash, turbinates, and lungs of infected ferrets. In addition, qRT-PCR results revealed that the hPH treatment remarkably upregulated the gene expression of type I (IFN-α and IFN-β) and II (IFN-γ) IFNs in SARS-CoV-2 infected ferrets. Our data collectively suggest that hPH has antiviral efficacy against SARS-CoV-2 and might be a promising therapeutic agent for the treatment of SARS-CoV-2 infection.

Keywords: SARS-CoV-2, Laennec®, ferret, antiviral, interferon

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Introduction

In early December 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was identified as the cause of severe pneumonia, named coronavirus disease 2019 (COVID-19), in human patients in Wuhan City, China (Huang *et al.*, 2020). Since then, the number of SARS-CoV-2 cases has increased continuously, resulting in the COVID-19 pandemic. As of May 2021, SARS-CoV-2 has spread through 220 countries and caused more than 163 million confirmed cases and 3.3 million deaths worldwide (WHO, 2021). SARS-CoV-2 infection causes clinical symptoms, such as nausea, vomiting, diarrhoea, cytokine storm, lymphopenia, as well as tissue damage to the lungs, liver, kidneys, heart, and other organs, eventually resulting in death in some severe cases (Huang *et al.*, 2020). Notably, convergent evolution of SARS-CoV-2 has resulted in novel variants able to evade host immune responses (CDC, 2021), making the virus difficult to control. Thus, there is an urgent need to develop novel vaccines and antiviral drugs for effective control of SARS-CoV-2 infections.

In October 2020 the FDA approved the use of remdesivir, an antiviral drug for RNA viruses, for use as a therapeutic for COVID-19 (FDA, 2020). Remdesivir was originally developed by Gilead Sciences in 2009 to target hepatitis C and respiratory syncytial viruses (Mackman *et al.*, 2021). A recent study revealed that the Remdesvir inhibits the viral RNA-dependent RNA polymerase to terminate viral RNA synthesis (Eastman *et al.*, 2020). Further, pre-treatment with remdesivir effectively inhibits virus replication *in vitro* (Wang *et al.*, 2020a), and reduces clinical signs in SARS-CoV-2 infected rhesus macaques (Williamson *et al.*, 2020). Clinical trials have shown that remdesivir accelerates clinical improvement in COVID-19 patients (Singh *et al.*, 2020; Wang *et al.*, 2020b). Nevertheless, a systematic review and network meta-analysis showed that remdesivir may have little or no effect on the length of hospital stay (Siemieniuk *et al.*, 2020). Thus, the need for therapeutics to effectively control SARS-CoV-2 and treat COVID-19 still exists.

Human placenta hydrolysate (hPH; Laennec®, GC Wellbeing, Co. Ltd.), a safe therapeutic agent for the improvement of hepatic function, was authorized by the PMDA (Pharmaceuticals and Medical Devices Agency) in Japan in 1974 and by MFDS (Ministry of Food and Drug Safety) in Korea in 1993 for hepatic function improvement. Preclinical studies showed that Laennec® could stimulate liver regeneration, anti-stress mechanisms and hair growth in rats (Liu *et al.*, 1998; Kwon *et al.*, 2015; Park *et al.*, 2018), as well as promote ligament healing in rodents (Shin *et al.*, 2019) and antioxidant effects in mice (Bak *et al.*, 2019). In clinical studies, hPH has been reported to improve the health status of elderly Koreans (Kong and Park,

2012) and to alleviate menopausal symptoms and fatigue in middle-aged Korean women (Kong *et al.*, 2008; Lee *et al.*, 2009). Moreover, it was also approved for use as an i) hepatoprotector, ii) immune-modulating agent for the treatment of atopic dermatitis, psoriasis and acne, and iii) for the treatment of recurring herpes (varicella-zoster virus) infection by the Russian Ministry of Health in 2003. Recently, Maksimov *et al.* (2020) demonstrated that Laennec® administration improved the health of hyperferritinemia patients with COVID-19 by decreasing ferritin levels, reducing lung damage, and increasing blood oxygenation.

Given the various functions of hPH, in this study we determined the CC_{50}, EC_{50}, and selective index (SI) of hPH in Vero cells to investigate the antiviral effects of hPH following infection with SARS-CoV-2 at an MOI of 0.01. Further, we used a ferret infection model, which has high natural susceptibility in the respiratory tract (Park *et al.*, 2020), to evaluate the antiviral effect of this therapeutic and compared the results with those of a remdesivir-treated group.

Materials and Methods

Virus and cells

Vero cells (CCL-81) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) (Gibco). A SARS-CoV-2 virus (NMC-nCoV02) was isolated from a patient with confirmed COVID-19 in South Korea (Kim *et al.*, 2020). The virus was propagated in Vero cells in DMEM supplemented with 1% PS and 0.5 μg/ml L-tosylamino-2-phenylethyl chloromethyl ketone (TPCK)-trypsin (Worthington Biochemical) at 37°C for 72 h. Propagated viruses were harvested and stored at -80°C until further use. The 50% tissue culture infective dose ($TCID_{50}$) of the virus was measured through fixation and crystal violet staining.

Animals

Male and female ferrets, 10–12 months old, and seronegative for influenza A viruses, MERS-CoV and SARS-CoV, were maintained in isolators (Woori IB Corporation, Daejeon, South Korea) in a biosafety level 3 laboratory at Chungbuk National University, South Korea. All ferrets were housed under a 12/12 h light/dark cycle and allowed access to food and water *ad libitum*. Body weight and temperature were measured every two days, before and after virus infection, during all experimental periods. Animal handling and experiments using SARS-CoV- 2 were carried out following protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Chungbuk National University.

Preparation of human placenta hydrolysate (hPH)

Human placenta hydrolysate (Laennec®) was manufactured by GC Wellbeing Co., Ltd. (Seongnam, South Korea), as previously described (Kong and Park, 2012). Briefly, hPH, an aqueous placenta extract, is known for abundant source of various bioactive substances, such as peptides, amino acids, nucleic acids, and minerals. It is prepared by the hydrolysis of human placenta with HCl and pepsin. The final product is aliquoted 2 ml in ampule as liquid. Each ampule of hPH possesses various amino acids including arginine (0.08%), lysine (0.1%), phenylalanine (0.08%), tyrosine (0.03%), leucine (0.12%), methionine (0.03%), valine (0.04%), alanine (0.08%), seine (0.07%), and threonine (0.06%). hPH has been investigated to possess various therapeutic properties ranging from anti-inflammation to immunomodulation.

Antiviral activities of hPH *in vitro*

To evaluate the antiviral efficacy of hPH, Vero cells were cultured overnight in 96-well plates at a density of 2×10^4 cells/well. The cells were infected with SARS-CoV-2 at a multiplicity of infection (MOI) of 0.01. The virus was then removed, and cells were further cultured with fresh medium containing different concentrations of hPH. After 72 h, the cells were fixed with formaldehyde and stained with 0.1% crystal violet. The CellTiter 96® Non-Radioactive Cell Proliferation Assay Kit (Promega) was used to determine the cytotoxic concentration (CC_{50}) and effective concentration (EC_{50}). The values were calculated from the percentage of cells whose viability was inhibited by various concentrations of hPH.

Virus infection and antiviral treatment of ferrets

Ferrets (n = 10 per group) were anesthetized with isoflurane and then infected with 30 μl of $10^{5.8}$ $TCID_{50}$/ml of SARS-CoV-2 through the intranasal route. The infected ferrets were injected with either hPH (4 ml/animal by intravenous injection) on days 0, 2, 4, and 6 post-infection (dpi), or remdesivir (17.6 mg/kg on day 1 as a loading dose and 8.8 mg/kg daily on days 2–4 post infection as maintenance doses). A group of ferrets injected daily with PBS from days 0-4 post infection was used as the PBS-control group. The body weight and temperatures of the ferrets were measured every 2 days until 14 dpi.

Preparation of ferret lung, nasal wash, and nasal turbinate samples

The animals were anesthetized using 150 μl Zoletil/xylazine mixture (Zoletil 50®, 80 mg/kg, Virbac; Rompun®, 20 mg/kg, Bayer HealthCare) through intramuscular injection. Nasal washes were then collected in 1 ml PBS containing antibiotics at 0, 2, 4, 6, 8, and 10 dpi. Lung tissue and nasal turbinates were collected at 3 and 6 dpi and homogenized in a medium containing penicillin G (2×10^6 U/L), polymyxin B (2×10^6 U/L), ofloxacin HCl (60 mg/L), and sulfamethoxazole (0.2 g/L). After centrifugation at 12,000 × *g* for 10 min at 4°C the supernatants were collected. All samples were stored at -80°C until further use.

Virus titrations

To determine infectious SARS-CoV-2 titers, 10-fold serial dilutions of the lung, nasal wash, and nasal turbinate homogenates were incubated with confluent Vero cells in 96-well flat-bottom plates for 1 h at 37°C. The cells were washed with PBS and incubated with DMEM containing 1% PS and 0.5 μg/ml TPCK-trypsin for 4 days at 37°C. The cytopathic effect (CPE) was recorded daily, and the $TCID_{50}$ was calculated

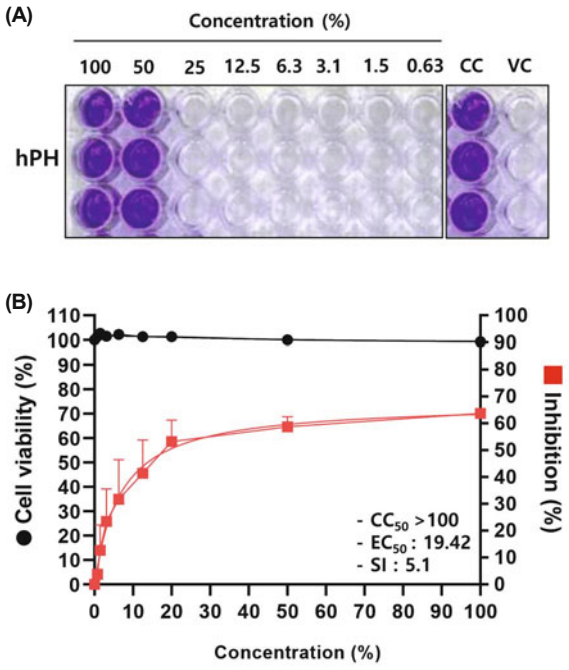

Fig. 1. Antiviral effects of hPH against SARS-CoV-2 *in vitro*. (A) Inhibition of SARS-CoV-2-induced CPE by hPH. In a 96-well plate, Vero cells were infected with SARS-CoV-2 virus (100 $TCID_{50}$/well) and then treated with various concentrations of hPH. At 72 h post-infection (hpi), cells were stained using crystal violet. The cell control (CC) column refers to cells without treatment and virus infection. The virus control (VC) column refers to cells left untreated, but infected with virus. (B) Cell viability and the antiviral effect of hPH were determined in Vero cells. The black line (left Y-axis) indicates cell viability and the red line (right Y-axis) indicates inhibition of SARS-CoV-2 infection. Experiments were done in triplicate. CC_{50}, EC_{50}, and SI are noted on the graph. CC_{50}, 50% cytotoxic concentration; EC_{50}, 50% effective concentration; SI, selective index.

according to the Reed and Muench method (Leed and Muench, 1938) and expressed as $\log_{10}$ $TCID_{50}$/ml.

Quantitative RT-PCR (qRT-PCR) for mRNA cytokine expression

Briefly, total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific), or an RNeasy Kit (Qiagen), and cDNA was generated with an oligo (dT) primer by reverse transcription using the QuantiTect Reverse Transcription Kit (Qiagen). qRT-PCR was performed using the SYBR Green Supermix (Bio-Rad) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) with specific primer sets (IFN-α: Forward: 5′-AACATCATCCCTGCTTC-3′, Reverse: 5′-AGGCCATGCCAGTGAGCT-3′, IFN-β: Forward: 5′-GGTGTATCCTCCAAACTG-3′, Reverse: 5′-ACTCCACACTGCTGCTGC-3′, and IFN-γ: Forward: 5′-CCATCAAGGAAGACATGC-3′, Reverse: 5′-GAAACACACTGTGACT-3′). Finally, the target mRNA was quantified, normalized to GAPDH (endogenous housekeeping gene) and relative to the calibrator, using the $2^{-\Delta\Delta CT}$ method. Thus, all experimental samples are expressed as a fold-change relative to the calibrator.

Statistical analysis

Statistical significance between groups was determined by two-way analysis of variance (ANOVA) and a subsequent Dunnett test using GraphPad Prism version 8.20 (GraphPad Software). Statistical significance was set at $p < 0.05$.

Results

Antiviral activity of hPH *in vitro*

To assess the antiviral efficacy and cell cytotoxicity of hPH, the CC_{50}, EC_{50} and selective index (SI) of this extract were measured in Vero cells. Briefly, SARS-CoV-2 infected cells were treated with different concentrations of hPH, and inhibition of cytopathic effect (CPE) was visually observed using crystal violet staining. The CPE inhibition assay revealed that hPH treatment reduces SARS-CoV-2 induced CPE in Vero cells at both a 50% dilution and its original concentration (Fig. 1A). Additionally, cell viability and antiviral activity were measured using the CellTiter 96® Non-Radioactive Cell Proliferation Assay and hPH did not show any cytotoxicity in Vero cells at up to 100% with the EC_{50} values of hPH (EC_{50} = 19.42%). Therefore, we concluded that the SI (CC_{50}/EC_{50}) value of hPH was 5.1 (Fig. 1B). These results suggest that hPH has antiviral effects against SARS-CoV-2 *in vitro* and with low cytotoxicity.

Antiviral effect of hPH against SARS-CoV-2 in a ferret model

To investigate the antiviral effects of hPH against SARS-CoV-2 *in vivo* groups of ferrets (n = 10/group) infected

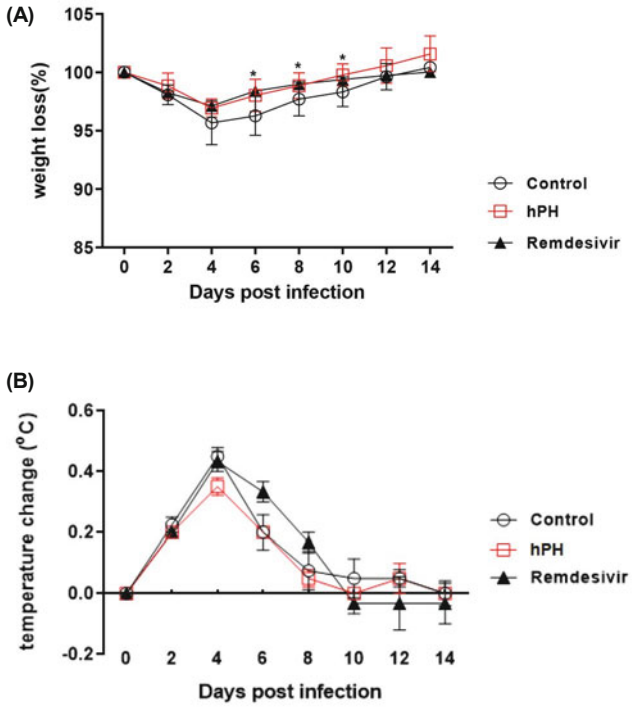

Fig. 2. Clinical features of drug-treated ferret groups following challenge with SARS-CoV-2. Ferrets (n = 10 per group) were inoculated intranasally with $1 \times 10^{5.8}$ $TCID_{50}$ of SARS-CoV-2. One day post-infection, remdesivir (D1, 17.6 mg/kg, and D2-4, 8.8 mg/kg) was dosed to each group. (A) Body-weight loss and (B) temperature change during the experimental period.

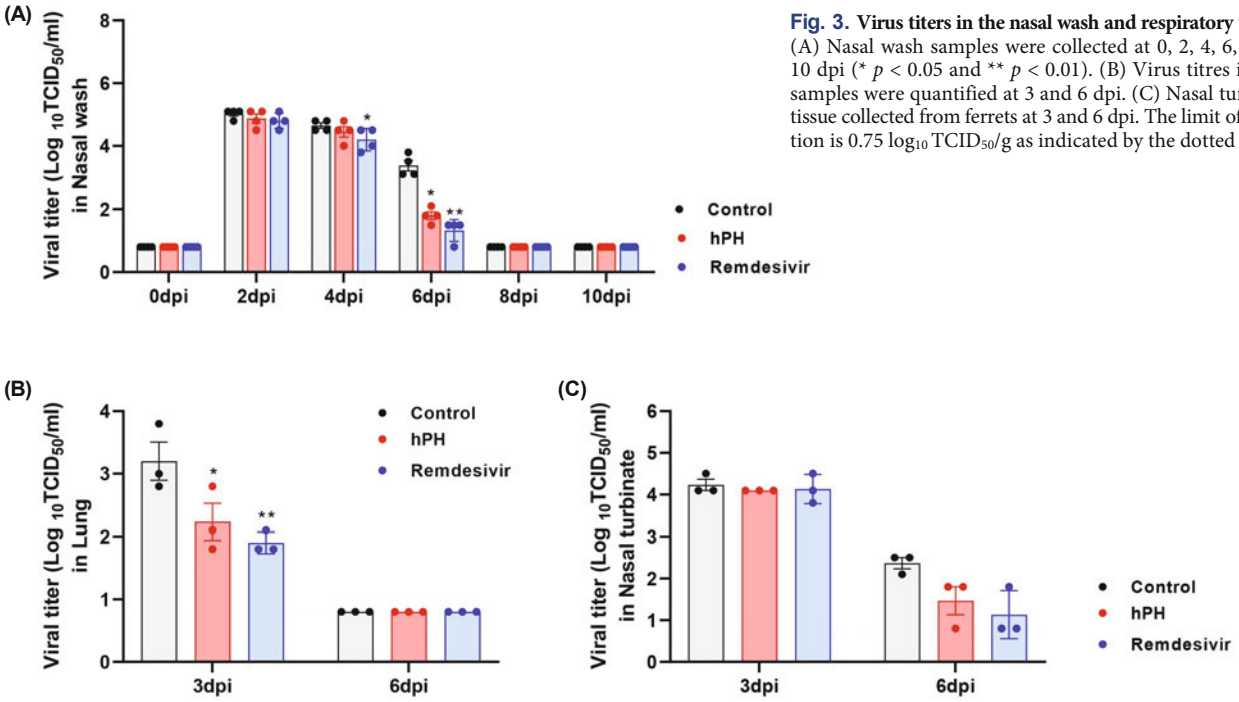

Fig. 3. Virus titers in the nasal wash and respiratory tissues. (A) Nasal wash samples were collected at 0, 2, 4, 6, 8, and 10 dpi (* $p < 0.05$ and ** $p < 0.01$). (B) Virus titres in lung samples were quantified at 3 and 6 dpi. (C) Nasal turbinate tissue collected from ferrets at 3 and 6 dpi. The limit of detection is 0.75 $\log_{10}$ $TCID_{50}$/g as indicated by the dotted line.

with $10^{5.8}$ $TCID_{50}$/ml of SARS-CoV-2 were treated with hPH from 24 h post-infection (hpi) as described in the Methods. All SARS-CoV-2 infected fqerrets showed a gradual decrease in body weight (~6%) until 4 dpi, but recovered to their initial weight by 10–14 dpi. Compared with the PBS-treated control group, the ferrets treated with hPH or remdesivir showed significantly reduced body weight loss and earlier recovery to their initial weight (Remdesivir, $p < 0.05$). There was no difference in weight loss between ferrets treated with hPH or remdesivir (Fig. 2A). However, there were no significant differences between groups in body temperature, as all groups of animals had slightly elevated body temperature at 4 dpi and recovered after 10–14 dpi (Fig. 2B).

To evaluate the antiviral activity of hPH, we measured infectious virus titers in nasal washes and tissues from each ferret group. Infectious SARS-CoV-2 was detected in nasal washes from all groups until 6 dpi. However, the hPH and remdesivir-treated groups showed significantly lower virus titers than

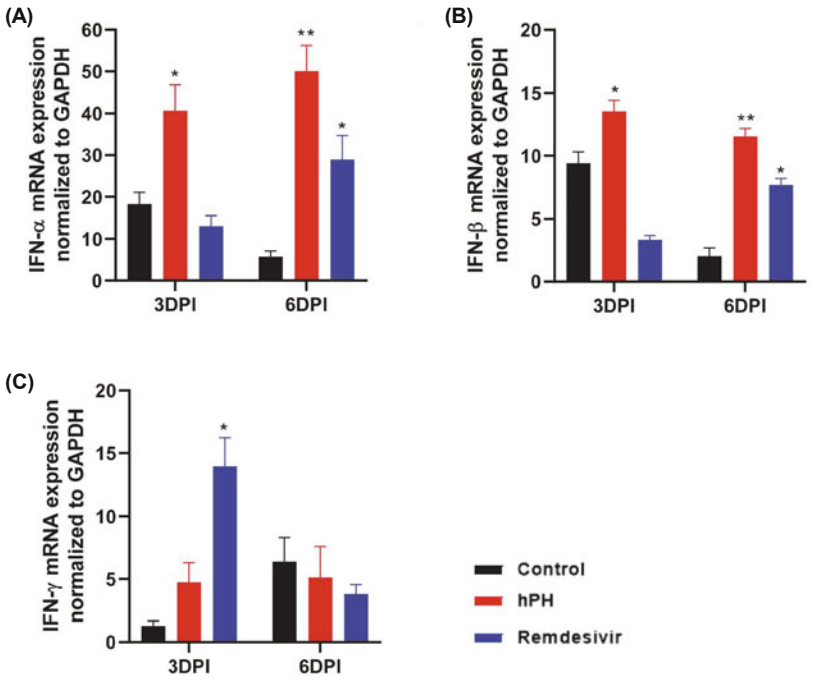

Fig. 4. Comparison of IFN-α, -β, -γ mRNA expression in lung tissue of SARS-CoV-2 infected ferrets. mRNA expression profiling in the lung for IFN-α, IFN-β, and IFN-γ by qRT-PCR. RNA copy numbers are shown as means ± standard error of the mean (SEM) for three animals. Asterisks indicate statistical significance between the control and each treatment group, as determined by two-way ANOVA and subsequent Dunnett's test (* $p < 0.05$ and ** $p < 0.01$).

those of the PBS control group at 6 dpi (hPH, $p < 0.05$; remdesivir, $p < 0.01$) (Fig. 3A). Further, SARS-CoV-2 titers in the lung tissue of hPH and remdesivir-treated groups were significantly lower than that of the control group at 3 dpi (Fig. 3B). In addition, hPH and remdesivir-treated groups showed attenuated viral titers in the nasal turbinates compared to PBS-treated ferrets at 6 dpi (Fig. 3C). Collectively, these data demonstrate that hPH exerts antiviral activity which leads to a reduction of the viral burden in the nasal wash, lungs, and nasal turbinates of SARS-CoV-2 infected ferrets with efficacy comparable with that of remdesivir.

Effect of hPH on immune-related gene expression in lungs of SARS-CoV-2 infected ferrets

To identify potential host factors contributing to the antiviral activity of hPH treatment, lung tissues from each group of ferrets were collected at 3 and 6 dpi, and the mRNA expression levels of immune-related cytokines were determined by qRT-PCR. Interestingly, hPH-treated SARS-CoV-2 infected ferrets showed a significant increase in expression of type I IFN cytokines, including IFN-α and IFN-β at 3 and 6 dpi, compared with that of the PBS-treated control (Fig. 4A and B). Further, although IFN-γ expression was lower in hPH-treated ferrets compared to remdesivir-treated ferrets at 3 dpi, the IFN-γ expression was higher in the hPH-treated group than in the PBS control group. The expression of IFN-γ at 6 dpi was similar in all groups (Fig. 4C). Taken together, our results suggest that administration of hPH reduces the viral burden and clinical symptoms in SARS-CoV-2 infected ferrets by enhancing the expression of type I and II IFNs.

Discussion

Due to the uncontrolled nature of the COVID-19 pandemic, remdesivir was given emergency use approval for the treatment of COVID-19. However, its effect on the disease is not yet fully understood (Liang *et al.*, 2020), and multiple clinical trials are ongoing to evaluate its safety and efficacy as a treatment for COVID-19 (Eastman *et al.*, 2020). Thus, there is still a need to explore other potential antiviral drugs for this disease. Recently, hyperferritinemia contributes to a cytokine storm and is considered an indicator of severe infection in COVID-19 patients (Maksimov *et al.*, 2020). hPH is a safe and approved drug for the improvement of hepatic function (Kong and Park, 2012) and for treatment of hyperferritinemia patients with COVID-19 (Maksimov *et al.*, 2020), making it a potential candidate drug for the treatment of COVID-19. Recently, Maksimov *et al.* (2020) reported that the short peptide fragment in hPH was deduced to improve the serum ferritinemia through inactivation of the pro-inflammatory factor NF-κB and, consequently, to a decrease in the level of chronic inflammation. Further, hPH is already approved by the PMDA in Japan and by the MFDS in Korea for therapeutic treatment in humans, hPH did not show any cytotoxicity *in vitro* while it showed strong antiviral activity against SARS-CoV-2.

Regarding the antiviral activity of remdesivir, many studies have reported that therapeutic treatment results in a reduction in clinical signs in rhesus macaques infected with MERS-CoV (de Wit *et al.*, 2020) and SARS-CoV-2 (Kim *et al.*, 2021) and clinical improvement was observed in 68% of COVID-19 patients treated with remdesivir in a clinical trial (Abdolvahab *et al.*, 2021). Further, a recent study demonstrated that remdesivir-treated ferrets showed reduced virus titers in the nasal wash and lung tissues compared with the control group (Kim *et al.*, 2021).

Thus, for the systemic evaluation of antiviral efficacy of hPH, we adapted the ferret as an *in vivo* evaluation model for SARS-CoV-2 infection and compared antiviral effects of hPH with those of remdesivir, an FDA-approved drug for COVID-19. Our results show that remdesivir treatment following SARS-CoV-2 infection in ferrets resulted in minimal body weight loss and attenuated virus titers in the respiratory tract, which are well within agreement with the previous study. Further, notably, hPH-treated ferrets showed a significantly reduced SARS-CoV-2 viral load in the nasal wash and nasal turbinates at 6 dpi and in the lung at 3 dpi compared to PBS treatment. Importantly, virus titers in hPH-treated ferrets were comparable to those of the remdesivir treatment group indicating that Laennec® has comparable antiviral activity with remdesivir in ferrets.

It is well known that type I IFNs, including IFN-α and IFN-β, are involved in innate antiviral defense. Once IFNs are induced and bind their receptors, STAT (signal transducer and activator of transcription) signaling is activated, which triggers the transcription of numerous IFN-stimulated genes, ultimately eliciting an effective antiviral response (Platanias, 2005; Schoggins *et al.*, 2011). Further, recent studies reported that treatment with IFN-α or IFN-β reduces SARS-CoV-2 virus titers in Vero cells (Mantlo *et al.*, 2020). Moreover, a phase II clinical trial revealed that adminiatration of IFN-β-1b in combination with lopinavir-ritonavir and ribavirin can shorten the duration of virus shedding and reduce clinical symptoms in COVID-19 patients (Mantlo *et al.*, 2020).

In this study, we observed significantly enhanced IFN-α and IFN-β mRNA expression following hPH treatment compared to treatment with remdesivir or PBS. Notably, administration of hPH induced the production of IFN-α and IFN-β at an earlier time point (3 dpi) than was seen following remdesivir treatment. Thus, hPH treatment appears to induce an early and strong type I IFN antiviral response compared to the control group. Unlike virus-induced type I and III IFNs, IFN-γ (known as type II IFN) is mostly produced by immune cells and initiates antiviral activity through expansion of the cytotoxic T lymphocyte population and through activation of monocytes and macrophages to express cytokines. Therefore, IFN-γ is also listed as a potential treatment for COVID-19 (Abdolvahab *et al.*, 2021). Herein, an increase in IFN-γ mRNA expression at an early point (3 dpi) was found in the lungs of SARS-CoV-2 infected ferrets following hPH treatment. Thus, this suggests that the hPH treatment may enhance IFN-γ cytokine expression in lungs and contribute to the inhibition of SARS-CoV-2 replication. Although Vero cells could not secret functional IFN-alpha or beta due to the deletion of the type I interferon gene cluster set, it still have type I IFN receptors and other interferon-inducible genes (ISG) set. Actually, hPH is a human placenta extract which is a cocktail of biologically active compunds including various peptides, growth factors, amino acids, antioxidants, vitamins, minerals etc. Thus such multiple component could exert multiple tar-

get mode of action for antiviral activity as well as induction of type I IFNs. Further, although type I IFN response is important for antiviral activity, there are many other alternative antiviral pathways (Sa Ribero *et al*., 2020; Mdkhana *et al*., 2021) that the other biologically active compounds found in hPH may exert a broad antiviral effects. This study encompass the multiple component, multiple target (MCMT) concept which has been favored to identify therapeutic agents with a broad spectrum antiviral activity using Vero cells. Although a recent study (Maksimov *et al*., 2020) demonstrated that hPH contains numerous compounds that can inhibit virus activation, fusion, replication and budding, however, exact antiviral pathway *in vitro* against SARS-CoV-2 remains to be elucidated. Therefore, to understand the detailed antiviral mechanisms of hPH, further expanded molecular studies are needed.

Collectively, our results demonstrate that hPH has antiviral effects against SARS-CoV-2 and is as effective as remdesivir at reducing the clinical features of virus-infected animals. Moreover, these effects may be associated with enhanced type I and II IFN expression. Although studies of the safety of hPH and elucidation of its detailed antiviral mechanisms are needed, hPH could be a promising candidate for the treatment of COVID-19.

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Conflict of Interest

The author(s) have declared that no conflicts of interest exists.

Ethical Statesments

All animal experiments were approved by the Medical Research Institute, a member of the Laboratory Animal Research Center of Chungbuk National University (approval number CBNUA-1352-20-02), and all animal experiments were conducted in strict accordance and adherence to relevant policies regarding animal handling as mandated under the Guidelines for Animal Use and Care of the Korea Centers for Disease Control. Viruses were handled in an enhanced biosafety level 3 (BSL3) containment laboratory, as approved by the Korean Centers for Disease Control (KCDC-14-3-07). The materials and budget were sponsored by GC Wellbeing Co. Ltd.

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