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25-Hydroxycholesterol provides antiviral protection against highly pathogenic porcine reproductive and respiratory syndrome virus in swine

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) is a severe respiratory disease that leads to huge economic losses in the pig industry throughout the world. Although there are several vaccines available, the protective efficacy is limited. Therefore, new control strategies to prevent PRRS virus (PRRSV) infection are urgently required. We have previously reported that CH25H and 25HC can significantly inhibit the replication of PRRSV by preventing viral entry. In the present study, we found that 25HC with a low IC_{50} value significantly decreased the replication of different PRRSV strains, and increased the production of IL-1β and IL-8 in porcine primary alveolar macrophages and the lung tissue. In pigs challenged with highly pathogenic PRRSV, treatment with 25HC was associated with an obvious reduction in the level of viremia and viral load in lung samples and nasal swabs, as well as decreased lung injury and an increased survival rate. These findings suggest that 25HC could be a promising antiviral drug against PRRSV in the future.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an economically devastating viral respiratory disease that continues to impact the pig industry worldwide (Neumann et al., 2005; Zhang et al., 2016, 2018). The causative agent of PRRS is porcine reproductive and respiratory syndrome virus (PRRSV), which first emerged in America and Europe in the 1980s (Murtaugh and Genzow, 2011). The characteristics caused by this virus is reproductive failure in sows and respiratory disorder in all pigs, especially piglets (Nelsen et al., 1999). The high variability and rapid recombination of the virus increases the complexity and difficulty for its control. Further complicating the situation, highly pathogenic PRRSV (HP-PRRSV) emerged in China in 2006, accompanied by symptoms of a high fever, high morbidity, and high mortality, which led to huge economic losses (Tian et al., 2007). Recently, NADC30-like PRRSV has also emerged and continues to devastate the pig industry (Sui et al., 2018; Wang et al., 2018; Zhang et al., 2016). Although vaccination is a good strategy for controlling PRRSV infection, the safety and efficacy of commercially available vaccines remains questionable. Therefore, the development of antiviral drugs against PRRSV is a novel strategy that can be used to control its infection in the future.

The membrane-associated enzyme cholesterol 25-hydroxylase (CH25 H) plays an important role in cholesterol and lipid metabolism by catalyzing cholesterol to form hydroxycholesterol (25HC) (Li et al., 2017). As a negative regulatory factor, 25HC downregulates liver X receptors (LXRs) and the sterol regulatory element binding protein (SREBP) signaling pathway to decrease cholesterol and lipid synthesis (Janowski et al., 1996; Radhakrishnan et al., 2007). Moreover, lipid biosynthesis is completely indispensable for viral replication, maturation, and secretion, and many virus invasions also require lipid rafts. It has been recently reported that 25HC exhibits broad antiviral activity that can inhibit the replication of several viruses, including enveloped viruses, HIV, HCV, Reovirus, HSV, Zika virus, and MHV68 (Doms et al., 2018a,b; Liu et al., 2013; You et al., 2017), as well as some non-enveloped viruses (e.g., human papillomavirus-16, poliovirus and human rotavirus) by suppressing viral entry (Fessler, 2016). The primary mechanism by which 25HC inhibits HCV replication is through the prevention of membranous web formation and disrupting the function of sterol regulatory element-binding protein 2 (SREBP2) (Anggakusuma et al., 2015; Xiang et al., 2015). In the process of Lassa virus infection, 25HC has been shown to induce aberrant GP1 glycosylation to prevent

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viral propagation (Shrivastava-Ranjan et al., 2016). In addition, 25HC treatment can reduce viremia and provide protection against Zika virus in infection in both mice and rhesus macaques (Li et al., 2017). Furthermore, 25HC also plays an important role in the innate immune response by functioning as an amplifier of inflammation through the recruitment of the AP-1 and regulation of transcriptional responses, resulting in an increased inflammatory response (Gold et al., 2014). Additionally, 25HC can also promote the inflammatory response by increasing IL-6 and IL-8 production through activating NF-κB signaling (Rydberg et al., 2003; Koarai et al., 2012).

In our previous study, we demonstrated that 25HC can significantly inhibit PRRSV infection in PAMs and Marc-145 cells at a relatively low dose (Song et al., 2017). In the present study, we continue to explore the function of 25HC both in vivo and in vitro. It was found that 25HC inhibits the replication of different strains of PRRSV and promotes the production of IL-1β and IL-8 in PAMs. More importantly, 25HC could prevent viral replication and alleviate lung injury in pigs challenged with PRRSV. Thus, the use of 25HC as a natural product of the host may represent a promising antiviral drug against PRRSV.

2. Materials and methods

2.1. Cells and virus

Primary alveolar macrophages (PAMs) and Marc-145 cell which are both highly permissive for PRRSV are maintained in Roswell Park Memorial Institute 1640 medium (RPMI 1640) (Invitrogen, Carlsbad, CA, USA) supplement with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). PRRSV different strains, including S1(Accession: DQ459471.1), a classical PRRSV strain, BB0907 (Accession: HQ315835.1), a HP-PRRSV strain and FJ1402 (Accession: KX169191.1), a NADC30-like strain, were isolated from clinical material and passaged in Marc-145 cells.

2.2. Western blot assay

The virus infected or mock cells were lysis by Radio-immunoprecipitation assay (RIPA) lysis buffer and the lysates were collected for centrifugation at 12,000 × *g* to remove insoluble pellets. The BCA kit was used to detect protein concentration. Then samples were boiled with 5 × LoadingBuffer for 5 min and equal protein samples were used for western blot assay and analyzed the expression of N and β-actin using specific antibodies, respectively.

2.3. $TCID_{50}$ assay

Samples were subjected to 10-fold gradient dilution and inoculated to 96 well Marc-145 cells plates. After 1 h inoculation at 37 ℃, removing the supernatant and wishing cells with PBS for 3 times, then adding 5% DMEM. The plates were incubated for additional 72 h. Cytopathic effect were observed at an inverted microscope and the virus titers were calculated through Reed-Muench methods.

2.4. Animal experiment

Fifteen five-week old piglets which were free of PRRSV, pseudorabies virus (PRV), classical swine fever virus (CSFV) and porcine circovirus type2 (PCV-2) were randomly divided into 3 groups with five piglets in each group. The groups 1 and 2 were challenged intranasally (1 ml) and intramuscularly (1 ml) with PRRSV BB0907 strain ($3 \times 10^5 TCID_{50}$), the group 3 was inoculated with the same dosage of DMEM. At 12 h post challenged, the group 1 was injected intramuscularly with 25HC (1.5 mg/kg/day) and continued administration for 10 days. The group 2 was injected with the same dosage of ethanol (ET), used as challenge control group. And the group 3 was used as negative control (Mock). After challenge, the animals were monitored for 14 days. Rectal temperatures and clinical signs were observed daily. The blood and nasal swabs samples were collected from all animals at 3, 6, 9, 12 and 14-days post challenge (dpc) for the detection of PRRSV. At the end of experiment, all pigs were euthanized for pathological detection.

2.5. Clinical evaluations and gross lesions

The clinical condition of all pigs were evaluated daily after challenging with PRRSV BB0907 strain as previously described (Wang et al., 2008). Briefly, scores ranged from 0 to 4, reflecting the severity of the illness, were determined for three observations including behavior, respiration and cough. The overall score for clinical condition was determined by sum of daily observations. Gross lesions of lungs were evaluated at necropsy. Gross lesions of each lobe were scored and estimated as percentage of lung with grossly visible pneumonia, and then the histological pathology of lungs was determined as described (Halbur et al., 1996).

2.6. Real time PCR

The total RNA of PAMs or tissues were extracted by a Total RNA Kit I (Omega Bio-tek, Shenzhen, China), and used for cDNA synthesis by a HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, China). Real time PCR was performed by an ABI QuantStudio 6 Systems (Applied Biosystems, Foster City, CA, USA) using AceQ® qPCR SYBR® Green Master Mix (Vazyme, China). Each sample was assayed for three times. Gene relative expression levels was calculated through 2-ΔΔCT methods. The primer sequences were referred to previous study (Song et al., 2017).

2.7. Microscopic lesions and immunohistochemistry (IHC)

At 14-days post challenged, all animals were put to death, the lung samples were collected and fixed with 4% polyoxymethylene for microscopcially evaluated by hematoxylin and eosin (H&E) and immunohistochemistry staining as described previously (Zhang et al., 2016) in our lab. The mouse monoclonal against PRRSV N protein was used to detect PRRSV antigen.

2.8. Ethics statement

All experiments about animals were carried out under the Institutional Animal Care and Ethics Committee of Nanjing Agricultural University (NAU) (Nanjing, Jiangsu, China). All animals were raised in the animal facility of NAU. And all operations were in accordance with the International Guiding Principles for Biomedical Research Involving Animals.

2.9. Statistical analysis

Image *J* was used for analyzing the gray density of the pictures and GraphPad Prism 7.0 software (San Diego, CA) was used to analyze data difference by one-way ANOVA followed by the Tukey's t-test, and all data were expressed as means ± SD. Differences were considered statistically significant when the p value < 0.05.

3. Results

3.1. 25HC inhibits the replication of different strains of PRRSV in PAMs

The 50% cytotoxic concentration (CC_{50}) of 25HC was measured using a CCK-8 Kit and calculated to be 69.13 μM (Fig. 1A). To assess the inhibitory effect of this drug on PRRSV, the 50% inhibitory concentration (IC_{50}) of 25HC was detected as previously described (Wang et al., 2017a,b). As shown in Fig. 1B, the IC_{50} of 25HC is 0.634 μM,

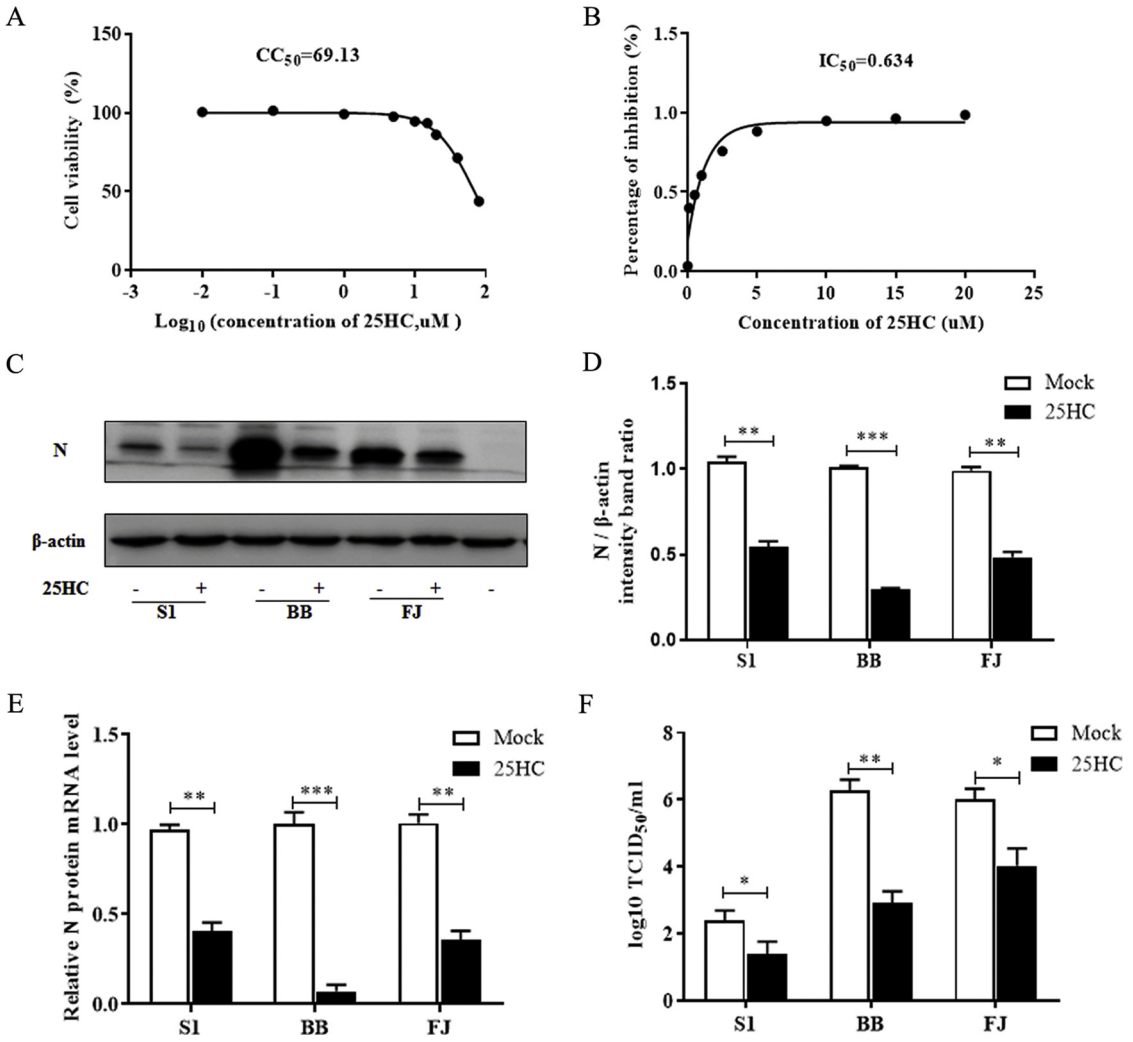

Fig. 1. 25HC inhibits PRRSV replication in PAMs. (A) Cell cytotoxicity assay of 25HC. PAMs were treated with different concentrations of 25HC for 24 h, the cellular cytotoxicity of 25HC was measured using a CCK-8 Kit, and the data was analyzed by GraphPad Prism 7.0 software. The data represent the means of triplicate samples from one independent experiment. (B) The IC_{50} of 25HC. PAMs were pre-treated with the indicated concentrations of 25HC for 1 h following infection with 0.1 MOI of the PRRSV BB0907 strain. At 30 h post-infection (hpi), indirect immunofluorescence assay was performed, and a fluorescence microscope was used to randomly record three photos for each well. Image J software was used to scan the fluorescence value, and the data were analyzed by GraphPad Prism 7.0 software. The data represent the means of triplicate samples from one independent experiment. (C—F) PAMs were pre-treated with 25HC for 1 h following infection with 0.1 MOI of the PRRSV BB0907 strain. The cells samples were collected for Western blot, $TCID_{50}$, and real time PCR assay. The results of the $TCID_{50}$ was confirmed by three independent experiments. The error bars represent the standard deviations of triplicate experiments.

which is quite low, suggesting that 25HC represents an excellent anti-PRRSV drug.

The antiviral effect of 25HC on the different strains of PRRSV was examined using different methods. The Western blot results showed that 25HC can reduce the replication of HP-PRRSV BB0907, C-PRRSV S1, and NADC30-like FJ1402 strains (Fig. 1C and D), which was consistent with the results of the real-time PCR and $TCID_{50}$ assays (Fig. 1E and F). These results indicate that 25HC suppresses the propagation of PRRSV regardless of strain differences.

3.2. 25HC promotes the production of IL-1β and IL-8 in PAMs and PRRSV-infected PAMs

To determine the function of 25HC on inflammation in PAMs, PAMs were treated with 10 μM 25HC for 12 h and 24 h, respectively. The cells were collected and used for RNA extraction. Real-time PCR was performed to detect the level of IL-1β, IL-6, IL-8, TNF-α, and IFN-β mRNA expression. As shown in Fig. 2A and C, 25HC significantly promotes the production of IL-1β and IL-8; however, 25HC did not substantially influence the expression of IL-6 (Fig. 2B), TNF-α (Fig. 2D), and IFN-β (Fig. 2E).

To detect the effect of 25HC on the expression level of inflammatory cytokines, PAMs were pre- or post-treated with different doses of 25HC following infection with 0.1 MOI of the PRRSV BB0907 strain for 24 h, inflammatory cytokines (e.g., IL-1β, IL-6, IL-8, and TNF-α), as well as the PRRSV genome levels were evaluated by real-time PCR. The results showed that 25HC treatment also increased the level of IL-1β (Fig. 3A and F) and IL-8 (Fig. 3C and H) expression compared to the control group, which is consistent with the previous results (Fig. 2); however, the level of IL-6 (Fig. 3B and G) and TNF-α (Fig. 3D and I) significantly decreased with the increase of 25HC. Moreover, the level of PRRSV genome expression significantly decreased following an increase of 25HC, (Fig. 3E and J).

3.3. 25HC treatment inhibits viral replication in piglets

3.3.1. Clinical signs and changes in body temperature

Following challenge with virulent PRRSV, all piglets in the

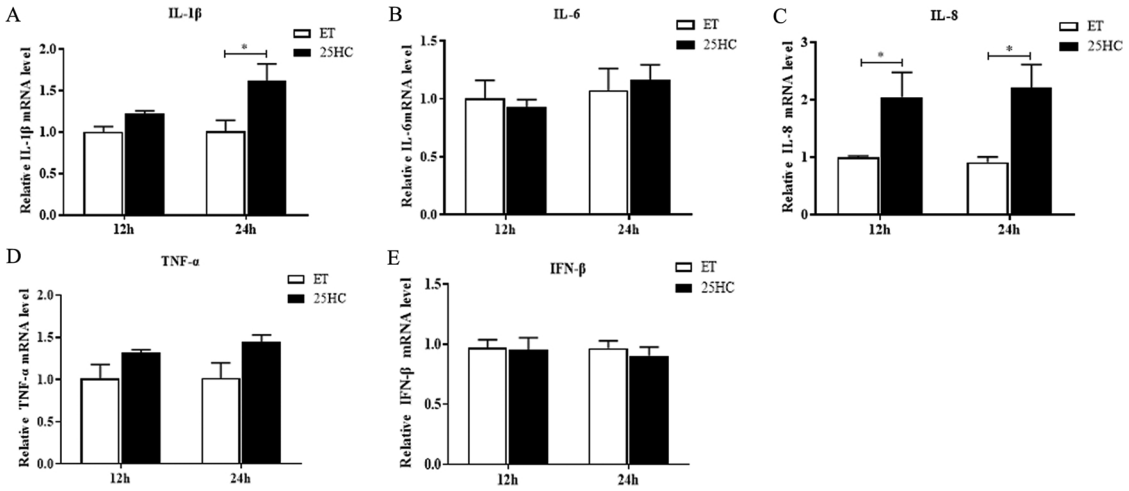

Fig. 2. 25HC promotes the production of IL-1β and IL-8 in PAMs. PAMs were treated with 10 μM 25HC for 12 h and 24 h, then the collected cells were used to measure the production of IL-1β (A), IL-6 (B), IL-8 (C), TNF-α (D), and IFN-β (E) by real-time PCR. The results were confirmed by three independent experiments. Error bars represent the standard deviations of triplicate experiments.

challenge control group had high fever (≥40.5 °C) (Fig. 4B) and displayed a range of clinical signs, including inappetence, lethargy, rough hair coats, dyspnea, periocular edema, eyelid edema, and lightly diarrhea. Additionally, four out of five animals died during the period of 5–12 dpi (Fig. 4C). However, the pigs in the 25HC treatment group only exhibited moderate fluctuation in rectal temperature during the 14-day post-challenge as shown in Fig. 4B. Additionally, only one out of five pigs died during the observation period (Fig. 4C). Moreover, the pigs in the mock group did not develop a clinical fever throughout the entire experiment (Fig. 4B). The scores of the clinical signs in the 25HC treatment group were significantly lower than those in the challenge control group ($P < 0.05$) (Table 1).

3.3.2. Pathological examination and IHC

At 15 dpc, the living pigs were euthanized, and the deceased pigs were collected for pathological examination. The results revealed that all pigs from the challenge control group displayed diffuse tan consolidation of the lungs, and occasional enlargement of the lymph nodes and spleen; however, in the 25HC group, only the deceased pigs displayed diffuse tan consolidation of the lungs. One out of the other four pigs exhibited mild lung lesions. The scores of the lung lesions from the pigs treated with 25HC were significantly lower than those in the challenge group as shown in Table 1 ($P < 0.05$). The histological examination results showed that the lungs in the challenge control group were characterized by thickened increased alveolar walls, intensive macrophage lymphomononuclear cell infiltration, and increased amounts of bronchiole exudates. However, in the 25HC group, all of the piglets, except the deceased, only exhibited moderate interstitial pneumonia, which was mild compared with that of the mock group. IHC straining revealed that a large number of PRRSV-positive epithelial cells and macrophages were observed in the challenge control group, which was substantially greater than that of the 25HC-treated group. Moreover, there were no PRRSV-positive cells observed in the Mock group (Fig. 4D).

3.3.3. Viremia and viral load in the lung samples and nasal swabs

The blood and nasal swab samples of the pigs were collected at 0, 3, 6, 9, 12, and 15 dpc and PRRSV was determined. The results revealed that the pigs inoculated with 25HC were associated with a significantly lower viremia in the blood than those in the challenge control group at 6–12 dpc ($P < 0.05$) (Fig. 4E). The pigs treated with 25HC also displayed a lower viral load in the nasal swabs compared to those in challenge group between 6–12 dpc ($P < 0.05$) (Fig. 4F). Moreover, the viral loads in the lungs of 25HC-treated pigs were significantly lower than those in the challenge control group ($P < 0.05$) (Fig. 4G).

3.3.4. Inflammatory cytokine responses in the lungs

The level of IL-1β, IL-6, IL-8, and TNF-α mRNA expression in the lung was detected using real-time PCR at 14 dpc. The results showed that the mean level of IL-1β and IL-8 mRNA expression in the lungs was higher than that in the challenge control group, while the levels of IL-6 and TNF-α were not (Fig. 5).

4. Discussion

During the past 30 years, PRRSV has been associated with tremendous economic damage, resulting in the persistent devastation of the swine industry. Due to the rapid mutation rate of the virus, several genetically diverse PRRSV strains are emerging, which introduce complexity and present a challenge to the control of PRRSV infection. Although there are several vaccines available, they cannot provide efficient protection against the highly diverse nature of PRRSV strains. Thus, the exploration of novel strategies is required to control this virus infection. In the present study, we found that 25HC can significantly inhibit PRRSV infection in PAMs. Moreover, 25HC can substantially reduce the level of viremia and viral load of HP-PRRSV in lung samples and nasal swabs, as well as increase the survival rate of pigs in challenge experiments.

The screening of small molecule inhibitors against viruses are gaining increased attention. For example, five hit drugs against Japanese encephalitis virus (JEV) were screened through a high-throughput screening assay of an FDA drug library. It was found that these drugs could suppress JEV infection, as well as the infection of another flavivirus, Zika virus, by inhibiting calcium ion channels (Wang et al., 2017a,b). And parthenolide was found to suppress HSV replication by interfering with the efficient infection and production of new viral particles (Benassi-Zanqueta et al., 2018). Recently, multiple drugs have also been identified to have anti-PRRSV effects, including Chinese traditional medicine (Cheng et al., 2013; Gao et al., 2013) and small molecule compounds, such as dozens of hit drugs (Karuppannan et al., 2012), synthetic compounds (Evans et al., 2017). But the antiviral effects of most of these drugs have not be further assessed in vivo and vitro. And the IC_{50} of most anti-PRRSV drugs were in the range of 1–98 μM (Evans et al., 2017). As the oxidation product of cholesterol that is catalyzed by CH25H, 25HC has been identified to have broad antiviral activity against several human viruses (Doms et al., 2018a,b; Liu et al., 2013; You et al., 2017). 25HC can also inhibit animal virus infection, including PRV, PRRSV, and Spring Viremia Carp Virus

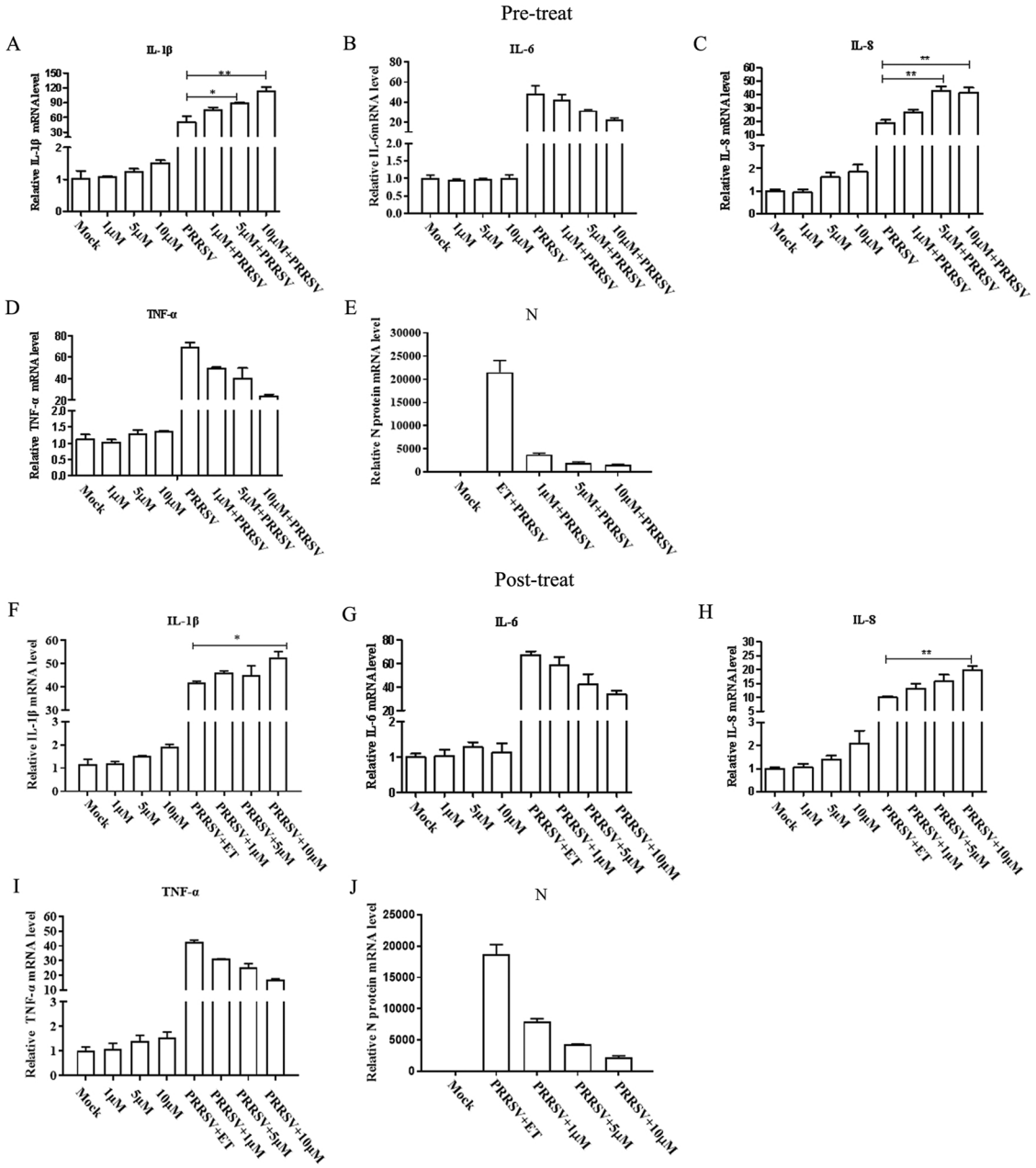

Fig. 3. 25HC inhibits PRRSV infection and promotes the production of IL-1β and IL-8 in PRRSV-infected PAMs. (A—D; F—I) PAMs were pre- or post-treated with indicated 0 μM (ET), 1 μM, 5 μM, and 10 μM of 25HC for 1 h following infection with 0.1 MOI of the PRRSV BB0907 strain. The inflammatory cytokines were measured by real-time PCR, and the PRRSV N gene was also detected (E and J). The experiment was independently performed twice. Error bars represent the standard deviations of triplicate samples.

(SVCV) (Pereiro et al., 2017; Song et al., 2017; Wang et al., 2017a,b). Ke et al., 2017 indicated that CH25H inhibits PRRSV proliferation through two independent mechanisms: 1) 25HC-mediated antiviral activity; and 2) CH25H degrades the PRRSV Nsp1α protein. We have previously demonstrated that CH25H and 25HC can suppress PRRSV infection by blocking viral entry (Song et al., 2017). In the present study, our experimental results demonstrated that 25HC could significantly suppress the different strains of PRRSV infection in PAMs. The IC_{50} and CC_{50} of 25HC against PRRSV in PAMs are only 0.634 μM and 69.13 μM, respectively. Moreover, experimental results in animals showed that the piglets in the 25HC treatment group exhibited only moderate clinical signs, mild injury, and low viral loads compared to those in the challenge control group. These findings suggest that 25HC is a strong PRRSV inhibitor that can significantly inhibit PRRSV replication both in vitro and in vivo.

25HC has several immunological functions, including the suppression of B cell-mediated IgA production, as well as regulating inflammation and monocyte differentiation (Ecker et al., 2010; Bauman et al., 2009). The experimental results of the present study showed that 25HC treatment could significantly increase the level of IL-1β and IL-8 expression in PAMs and PRRSV-infected PAMs, but not IL-6, TNF-α, and IFN-β. And it may be that 25HC induced IL-1β and IL-8 could regulate host immune system to help host defense PRRSV infection. However, it was reported that 25HC promotes IL-6 expression after HSV-1 infection (Cagno et al., 2017), which was different from our results. Thus, more works about the mechanism should to be done to verify these observations in future.

In conclusion, 25HC as a natural product of the host functions as an antiviral agent with low toxicity and a high antiviral effect in both PAM and swine. Therefore, 25HC may represent a promising antiviral agent

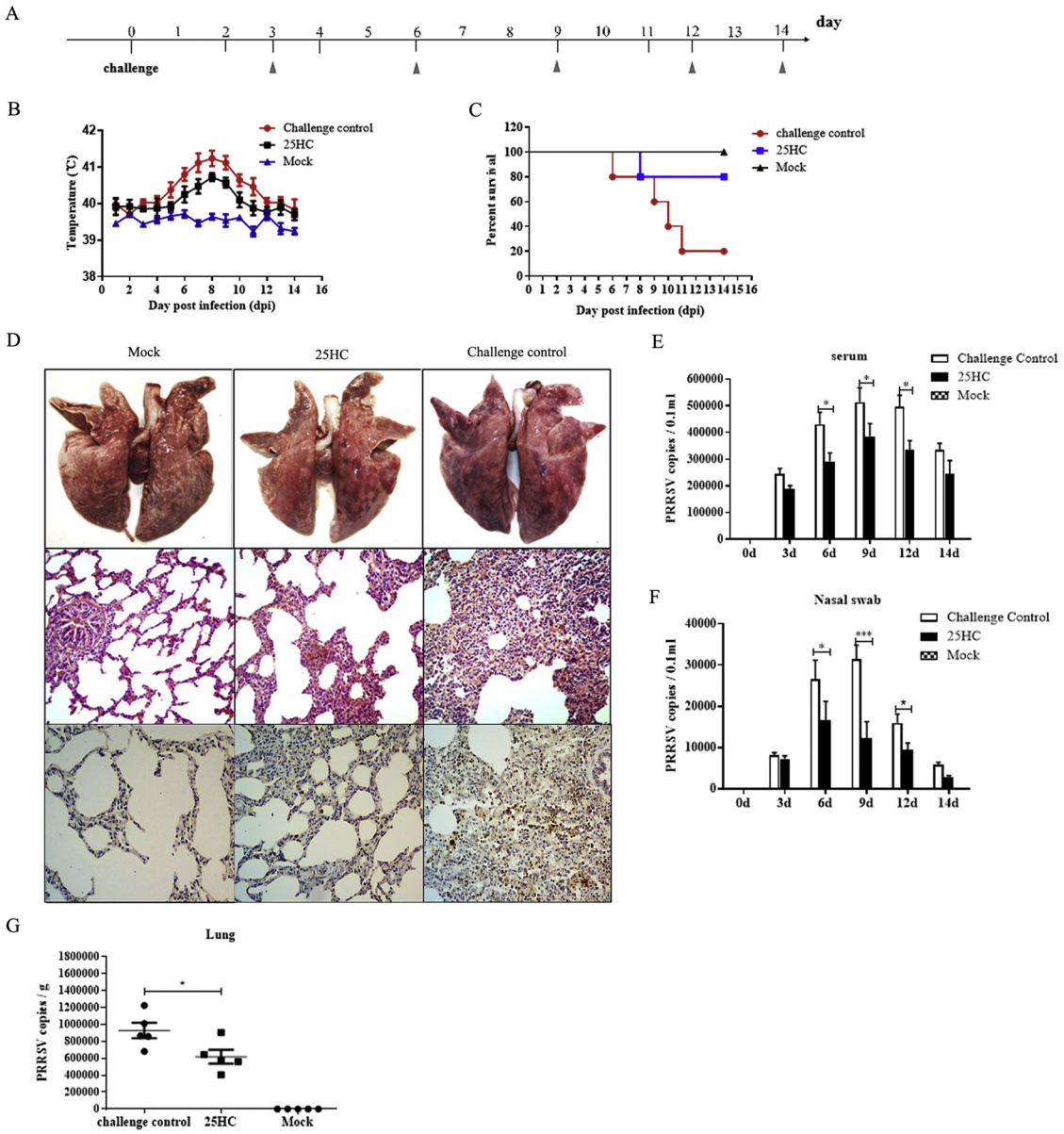

Fig. 4. 25HC treatment inhibits viral replication in piglets and alleviates lung injury. (A) The pattern diagram of animal experiments. (B) The rectal temperature of the animals in the 25HC treatment, challenge control, and mock groups. A fever was defined by rectal temperature of more than 40 ℃. (C) The survival rate of each group was monitored until 14 dpi. (D) Significant development of lung lesions in the challenge control groups characterized by swelling, hemorrhage, consolidation, interstitial lung widening, and an increased number of inflammatory cells; however, in the 25HC treatment group, these symptoms were moderate. In IHC straining (bottom), the amount of positive epithelial cells and macrophages significantly increased in the challenge control group compared to those in the 25HC-treated group (Original magnification: 100×). (E—G) The level of PRRSV mRNA in the serum, nasal swabs, and lungs was measured by real-time PCR. The data are expressed as the means of five samples from one independent experiment. Each sample was measured three times. The error bars represent the standard deviations of five samples.

Table 1
The scores of clinical signs of the pigs after challenging and their lung lesions recorded at 14 dpc[a].

Groups	Clinical signs scores(± S.D.)[b]	Lung lesions scores (± S.D.)[c]
Challenge control group	8.01 ± 3.179^{A}	69.5 ± 6.58^{A}
25HC group	1.67 ± 1.047^{B}	7.8 ± 5.26^{B}
Mock	0^{B}	0^{B}

[a] Within each column, values followed by different letters (A, B) are significantly different ($P < 0.05$).
[b] Scores for clinical signs were determined by sum of daily observations of behavior, respiration and cough according to the severity or the illness.
[c] Evaluation of the percentage of the entire lung affected by pneumonia.

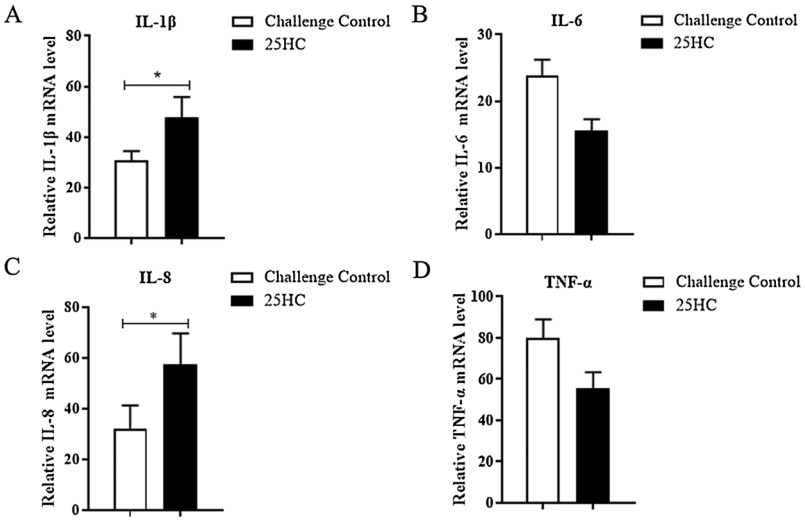

Fig. 5. Treatment with 25HC increases the production of IL-1β and IL-8 in the lungs. (A—D) The inflammatory levels in the lungs were evaluated by IL-1β, IL-6, IL-8, and TNF-α mRNA using real-time PCR. The data are expressed as the means of five samples from one independent experiment. Each sample was measured three times, and the error bars represent the standard deviations of five samples.

for the control of PRRSV infection.

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