



Therapeutic effect of Xanthohumol against highly pathogenic porcine reproductive and respiratory syndrome viruses

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ABSTRACT

The infection by porcine reproductive and respiratory syndrome virus (PRRSV) has a severe impact on the world swine industry. However, commercially available vaccines provide only incomplete protection against this disease. Thus, novel approaches to control PRRSV infection are essential for the robust and sustainable swine industry. In our previous study, Xanthohumol (Xn), a prenylated flavonoid extracted from hops (*Humulus lupulus* L), was screened from 386 natural products to inhibit PRRSV proliferation and alleviate oxidative stress induced by PRRSV via the Nrf2-HMOX1 axis in Marc-145 cells. In this study, we further found that Xn could inhibit PRRSV different sub-genotype strains infection with a low IC₅₀ value in porcine primary alveolar macrophages (PAMs). In addition, it caused decreased expression of interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor-α in PAMs infected with PRRSV or treated with lipopolysaccharide. Animal challenge experiments showed that Xn effectively alleviated clinical signs, lung pathology, and inflammatory responses in lung tissues of pigs induced by highly pathogenic PRRSV infection. The results demonstrate that Xn is a promising therapeutic agent to combat PRRSV infections.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an endemic infectious disease of pigs and one of the most economically significant contagious diseases affecting the swine industry worldwide. A “mystery swine disease” was first observed simultaneously in North America and Europe in the late 1980s (Albina, 1997; Elazhary et al., 1991; Russell et al., 1980). The causative agent of this disease was identified as the PRRS Virus, a single-stranded, positive-sense RNA virus. Although infected pigs of different ages may present various symptoms, PRRSV primarily causes late-term abortions and stillbirths in sows and respiratory disease in piglets (Pejsak et al., 1997). PRRSV is divided into two genotypes: the European genotype (type I) and the North American genotype (type II). Although the overall disease phenotype and gross clinical symptoms are similar, the nucleotide identities of these two genotype virus share is only about 60% (Collins et al., 1992; Mardassi et al., 1994). This degree of genetic heterogeneity suggests there was a protracted period of independent evolution

between the two continents (Nelsen et al., 1999). PRRSV has the intrinsic ability to adapt and evolve, due to its mutation rate of 3.29×10^{-3} substitutions per nucleotide site per year (Kappes and Faaberg, 2015; Tian et al., 2007; Zhang et al., 2017). Thus, the high variability and rapid recombination of the virus increases the complexity and difficulty of its control. A representative example is the epidemic of the highly pathogenic PRRSV (HP-PRRSV) in China in 2006, which presented as high fever, high morbidity, and high mortality among pig farms, resulting in substantial economic losses. Recently, NADC30-like PRRSV has also emerged in China (Zhang et al., 2016).

At present, vaccination remains the most common strategy for the control of PRRSV infections. However, commercially inactivated vaccines provide only incomplete protection. The modified live virus vaccines protect only against homologous strains and have the potential risk of reverting to a more virulent form (Murtaugh and Genzow, 2011; Renukaradhya et al., 2015). Therefore, more effective and safe methods to control PRRSV are urgently needed.

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Many host antiviral factors have been reported, including interferon-stimulated genes, such as viperin (Fang et al., 2016), myxovirus resistance 2 (Wang et al., 2016), 2',5'-oligoadenylate synthetase 1 (Zhao et al., 2016), interferon-induced protein with tetratricopeptide repeat 3 (Zhang et al., 2013), and cholesterol-25-hydroxylase (Song et al., 2019, 2017), and some microRNA, small interfering RNAs, and small hairpin RNAs (Li et al., 2014; Xie et al., 2014). Each of these factors has been shown to inhibit PRRSV replication, at least to some extent. However, there is no effective, commercially available antiviral factor. Natural compounds and compositions provide valuable sources of promising antiviral drugs, many of which have been exhibited anti-PRRSV activities *in vitro*, such as sodium tanshinone IIA sulfonate (Sun et al., 2012), proanthocyanidin A2 (Zhang et al., 2018), and griffithsin (Li et al., 2018).

Xanthohumol (Xn), a constituent of beer, is the major dietary source of prenylated flavonoids and a natural product with multiple biofunctions that is purified from the hop plant *Humulus lupulus L* (Pinto et al., 2012). Xn has been reported to convey anti-inflammatory effects against lipopolysaccharide (LPS)-induced acute lung injury and ischemia reperfusion-induced liver injury in mice (Ge et al., 2017; Lv et al., 2017), as well as anti-proliferative activities towards breast, colon, and ovarian cancer cell lines (Miranda et al., 1999). Xn also reportedly conveys antiviral activities against human immunodeficiency virus, bovine viral diarrhoea virus, and herpes simplex viruses 1 and 2 (Buckwold et al., 2004; Cos et al., 2008). In our previous study, Xn was screened from 386 natural products to inhibit PRRSV proliferation and alleviate oxidative stress induced by PRRSV via the Nrf2-HMOX1 axis in Marc-145 cells (Liu et al., 2019). In this study, we furtherly found that Xn could inhibit PRRSV different sub-genotype strains infection with low IC₅₀ value, and significantly decreased the inflammatory responses in porcine primary alveolar macrophages (PAMs). And it could significantly inhibit PRRSV replication and alleviate lung injury in pigs already infected with HP-PRRSV.

2. Materials and methods

2.1. Cells, viruses, and reagents

Marc-145 cells (an African green monkey embryonic kidney epithelial cell line; American Type Culture Collection, Manassas, VA, USA) and PAMs were cultured in Roswell Park Memorial Institute-1640 supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) at 37 °C. The HP-PRRSV strain BB0907 (GenBank accession number: HQ315835), which is maintained in our laboratory, was used for all experiments and is represented as "PRRSV" in this article. PRRSV strains S1 and FJ1402 were also used, but are specifically mentioned by name (S1, a classical strain; FJ1402, a NADC30-like strain). All of the strains used in these experiments were belong to type II PRRSV.

Xn, purity > 99%, was used for the *in vitro* and *in vivo* experiments (Selleck Chemicals, Houston TX, USA and Chengdu Herbpurify Co., Ltd., Chengdu, China, respectively).

2.2. The 50% cytotoxic concentration (CC₅₀) assay

Xn with different concentration were added into the cultures of PAMs and incubated for 24 h at 37°C. Cell viability was tested using the Cell Counting Kit-8 (Beyotime Institute of Biotechnology, Haimen, China) following the manufacturer's instructions. The CC₅₀ was calculated using GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Dimethyl sulfoxide (DMSO) was used as a negative control.

2.3. The 50% inhibition concentration (IC₅₀) assay

Different concentrations of Xn were added to the PAM culture medium (1–30 μM). DMSO was used as a negative control. HP-PRRSV,

Table 1

Primer sequences probes used for qRT-PCR analysis.

Primer	Sequence (5'→3')
mGAPDH-Fwd	5' CCTTCCGTGTCCCTACTGCCAA 3'
mGAPDH-Rev	5' GACGCCTGCTTACCACCTTCT 3'
mIL-1β-Fwd	5' TCCCACGAGCATAACAACA 3'
mIL-1β-Rev	5' CTTAGCTTCTCCATGGCTACA 3'
mIL-6-Fwd	5' GCTGCAGGCACAGAACA 3'
mIL-6-Rev	5' AAAGCTGCGCAGGATGAG 3'
mIL-8-Fwd	5' CTGGCGGTGGCTCTCTTG 3'
mIL-8-Rev	5' CCTTGGCAAACTGCACC 3'
mTNF-α-Fwd	5' TCCTCAGCCTCTTCTCTTCC 3'
mTNF-α-Rev	5' ACTCCTAAAGTGCAGCAGACA 3'
pIL-1β-Fwd	5' GACCCCAAAAGATACCCAAA 3'
pIL-1β-Rev	5' TCTGCTTGTAGAGGTGCTGATG 3'
pIL-6-Fwd	5' ATGAGAAGTGTGAAAACAG 3'
pIL-6-Rev	5' CATTGTGGTGGGTTAGGG 3'
pIL-8-Fwd	5' GGGTCTCTGCTGGAGGACT 3'
pIL-8-Rev	5' CCCCATCACTCTCTGCCTTC 3'
pTNF-α-Fwd	5' AGAGCATGATCCGAGACGTG 3'
pTNF-α-Rev	5' CAGTAGGCAGAGAGCGTGG 3'
pβ-actin-Fwd	5' CTCATCATGAAGTGGACGT 3'
pβ-actin-Rev	5' GTGATCTCTTCTGCATCTCTGT 3'
PRRSV-ORF7-Fwd	5' AAACAGTCCAGAGGCAAG 3'
PRRSV-ORF7-Rev	5' TCAGTCGAAGAGGAAAT 3'

p means Pigs and m means Monkey.

as 0.01 multiplicity of infection (MOI) was then added to the cell cultures, which were then incubated for 30 h at 37°C. Afterward, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.1% Triton X-100. After washing three times with PBS, the cells were incubated with a monoclonal antibody against the PRRSV N protein (made in our laboratory) for 1 h at 37°C (Chen et al., 2018). Then, the cells were washed three times with PBS and incubated with Alexa Fluor 594-conjugated goat anti-mouse immunoglobulin G (H-L) (1:200; Proteintech, Wuhan, China) for 1 h at 37°C in the dark. Nuclei were stained with 4',6-diamidino-2-phenylindole (Invitrogen Corporation, Carlsbad, CA, USA) for 10 min at room temperature. Immunofluorescence was observed using a Nikon A1 confocal microscope (Nikon Corporation, Tokyo, Japan) and three images of each well were recorded. The degree of fluorescence was determined using ImageJ software (<https://imagej.nih.gov/ij/>), and the IC₅₀ of Xn in PAMs was estimated by GraphPad Prism 7.0 software.

2.4. Western blot assay

Cells were lysed on ice for 15 min in lysis buffer (Beyotime Institute of Biotechnology), and the lysates were collected and then centrifuged at 12,000 × g. The protein pellet was collected and then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane, which was blocked with 5% low-fat milk for 2 h at room temperature, then probed with antibodies against the PRRSV N protein (dilution, 1:1000) and β-actin (1:1000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 h at room temperature. Afterward, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (H-L) (1:1000; Beyotime Institute of Biotechnology). Bound proteins were visualized with the Tanon 5200 chemiluminescence imaging system (Tanon Science & Technology Co., Ltd., Shanghai, China).

2.5. RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using the E.Z.N.A.® Total RNA Kit (Omega Bio-Tek, Inc., Norcross, GA, USA) and then reverse-transcribed using the HiScript II 1st Strand cDNA Synthesis Kit (Vazyme Biotech Co., Ltd., Nanjing, China) in accordance with the manufacturers' instructions. qRT-PCR was performed using AceQ® qPCR

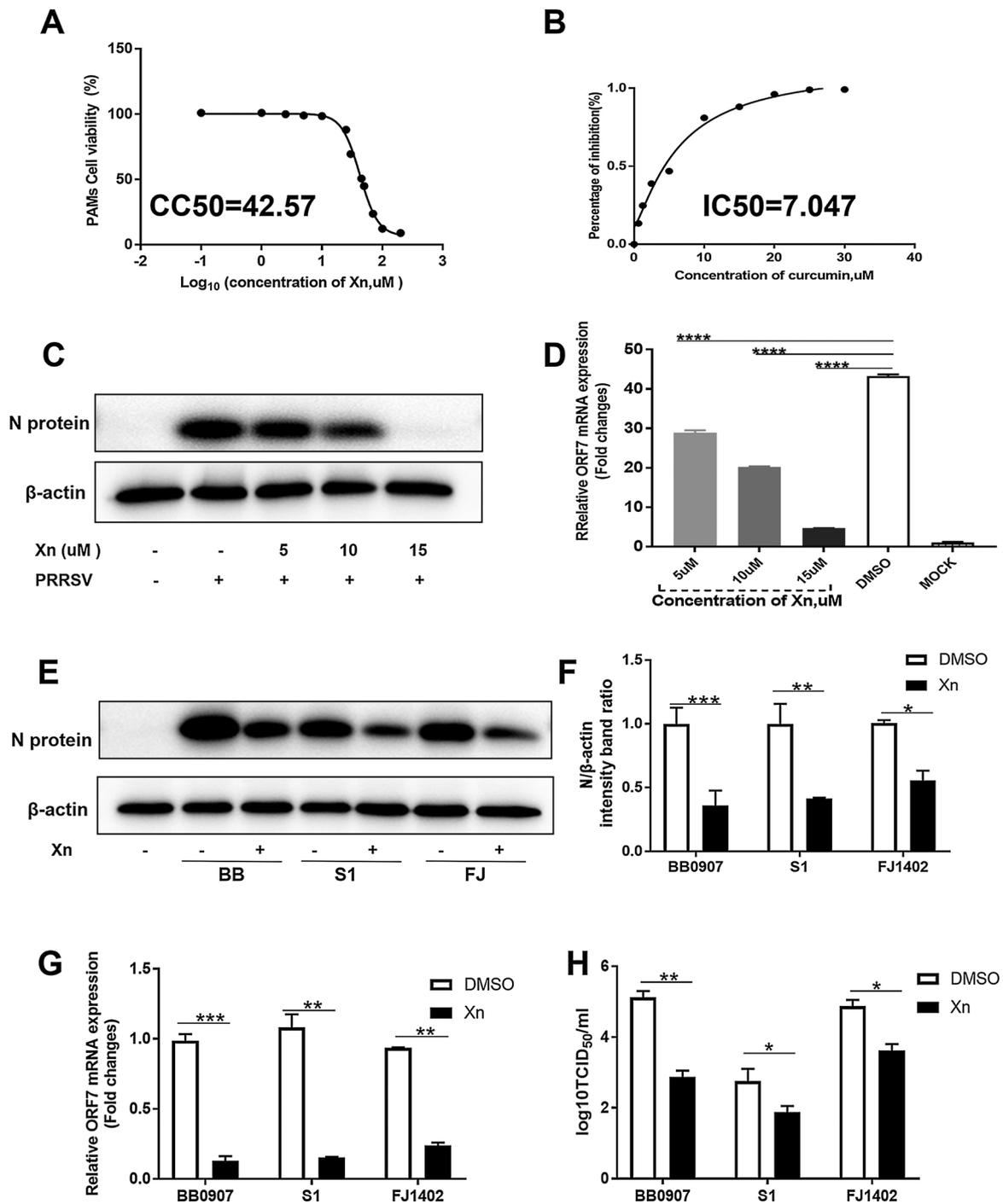


Fig. 1. Effect of Xn on PRRSV infection in PAMs. (A) Determination of the CC₅₀ of Xn in PAMs. PAMs were incubated with DMSO or various concentrations of Xn for 24 h prior to the CCK8 assay. The viability curve and CC₅₀ values were generated using GraphPad Prism 7.0 software. (B) The IC₅₀ of Xn in PAMs for PRRSV strain BB0907. PAMs were pre-treated with different concentrations of Xn for 1 h, then infected with PRRSV (0.1 MOI) for 1 h at 37°C. The cells were washed with PBS and then incubated in fresh medium containing different concentrations of Xn. At 30 hpi, an indirect immunofluorescence assay was performed and the IC₅₀ of Xn in PAMs was determined with the use of ImageJ software and GraphPad Prism 7.0 software. (C–D) PAMs were pre-treated with DMSO or the indicated concentrations of Xn for 1 h, then infected with PRRSV (0.1 MOI) for 1 h at 37°C. Afterward, the cells were washed and incubated in fresh medium containing DMSO or Xn. At 24 hpi, the cells were harvested for western blot (C) and qRT-PCR (D) analyses. (E–H) PAMs were pre-treated with DMSO or Xn (15 μM) for 1 h, then infected with 0.1 MOI of PRRSV of different genotypes for 1 h at 37°C. Afterward, the cells were washed and incubated in fresh medium containing DMSO or Xn. The cells and supernatants were collected for western blot, qRT-PCR, and TCID₅₀ analysis at 24 hpi. The results were confirmed by three independent experiments. Error bars represent the SDs of triplicate experiments. ****p* < 0.001; ***p* < 0.01; **p* < 0.05.

SYBR® Green Master Mix (Vazyme Biotech Co., Ltd.) according to the manufacturer’s instructions. Each reaction was performed in triplicate, and the data are presented as the mean ± standard deviation (SD). The primer sequences for qRT-PCR are listed in Table 1.

For RNA extraction from tissues, approximately 1 g of lung tissue

was homogenized in 3 mL of PBS. After three freeze-thaw cycles, the homogenized tissue samples were centrifuged, and the supernatants were used for RNA extraction. A 150-μL aliquot of the supernatant of each tissue sample and serum sample without dilution was used for RNA extraction. Then, 8 μL of RNA was used to determine the copy

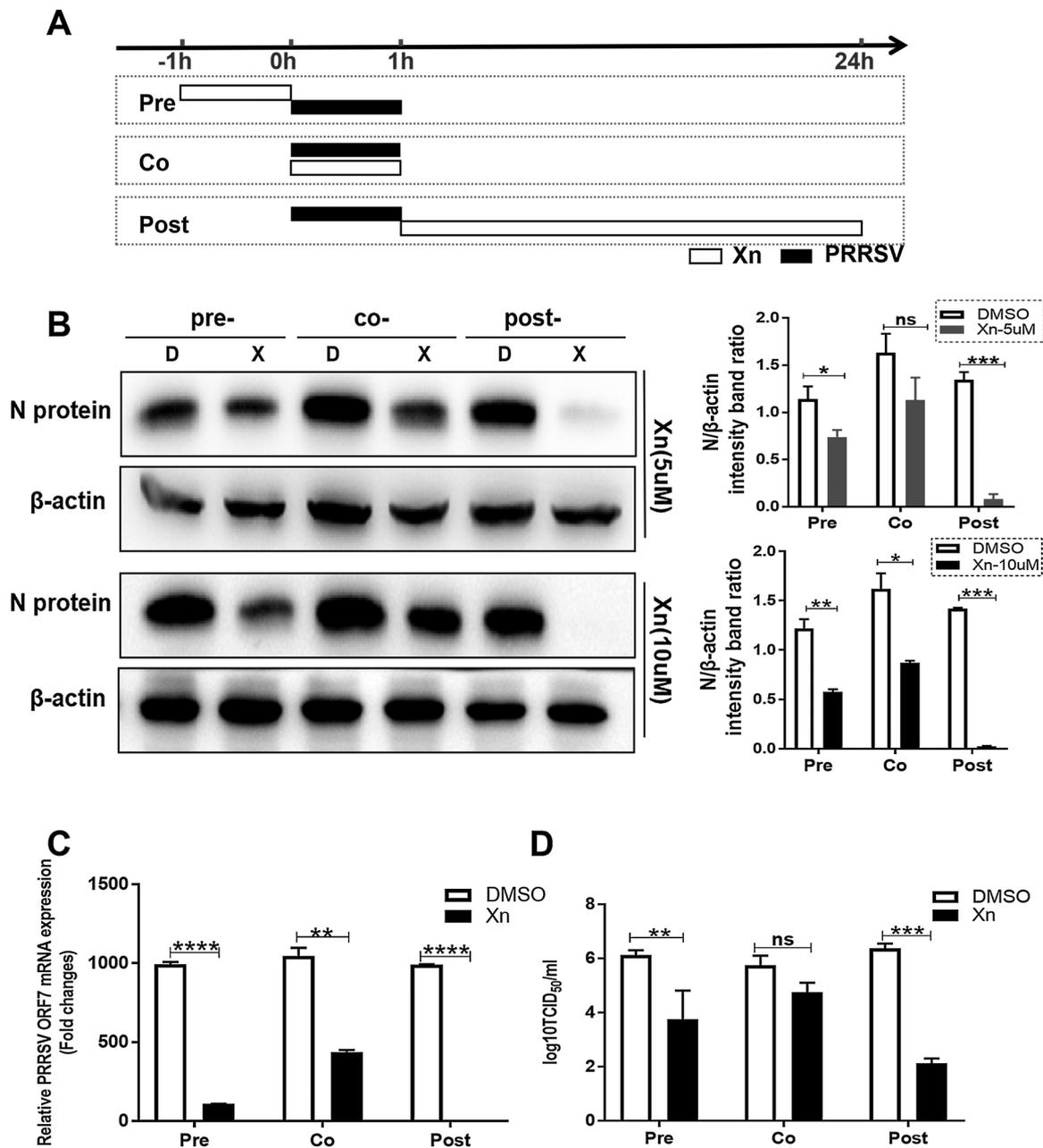


Fig. 2. Anti-PRRSV activity of Xn after PRRSV infection in PAMs. (A) Time-of-addition schematic. PAMs were infected with PRRSV (0.01 MOI) for 1 h (-1 to 0 h), and then treated with Xn at different times of infection, designated as pre-treatment (pre), co-treatment (co), or post-treatment (post). (B) The effect of different concentrations of Xn was detected by western blot analysis and the intensity band ratio of the PRRSV N protein/ β -actin was determined using ImageJ software. (C and D) The effect of 10 μ M Xn was determined by qRT-PCR and $TCID_{50}$ analyses. The results were confirmed by three independent experiments. Error bars represent the SDs of triplicate experiments. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

number of PRRSV genomic cDNAs by qRT-PCR. A recombinant plasmid containing the PRRSV ORF7 gene was used to construct a standard curve (Zhang et al., 2016).

2.6. Virus titration

Marc-145 cells, grown in the wells of 96-well plates, were infected with 10-fold serial dilutions of PRRSV. After 1 h inoculation at 37 $^{\circ}$ C, removing the supernatant and washing cells with PBS for 3 times, then adding fresh DMEM supplemented with 2% FBS. The plates were incubated for an additional 120 h. Cytopathic effect were observed at an inverted microscope and the virus titers were calculated through Reed-Muench methods.

2.7. Time-of-addition experiment

To identify the stage of the PRRSV life cycle that is most affected by Xn, a time-of-addition experiment was performed as shown in the timeline schematic presented in Fig. 2A. PAMs, seeded in the wells of 24-well plates, were either pre-treated, co-treated, or post-treated with Xn relative to PRRSV inoculation. The experiment began when cells completely settled at the bottom of the cell plate (noted as -1 h). At 0 h, the culture supernatants of the pre-treated cells were replaced with DMEM/2% FBS and then the culture was inoculated with PRRSV (0.01 MOI), the co-treated group was treated with 10 μ M Xn and PRRSV, and the post-treated group was inoculated with PRRSV. At +1 h, the culture supernatants in the co- and post-treated groups were replaced with DMEM/2% FBS, and the post-treated group was treated with 10 μ M Xn.

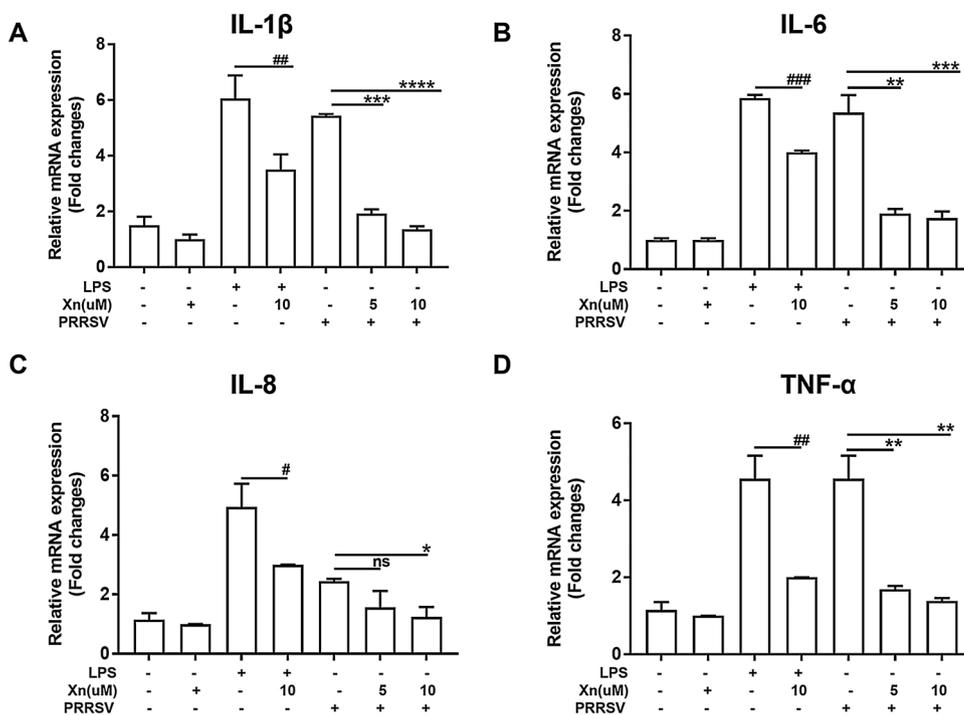


Fig. 3. Effect of Xn on PRRSV-induced inflammatory responses in PAMs. PAMs were treated with 10 μ g/ml LPS or DMEM for 1 h, and then washed three times. Afterward, the culture medium was replaced with fresh DMEM/2% FBS containing 10 μ M Xn or DMSO and the incubation was continued for 6 h. In parallel, PAMs were incubated with PRRSV (0.01 MOI) for 1 h at 37 $^{\circ}$ C and then cultured in medium containing 5 or 10 μ M Xn, or DMSO. Total RNA was extracted from cell lysates at 24 hpi and the expression levels of IL-1 β (A), IL-6 (B), IL-8(C), and TNF- α (D) in PAMs were determined by qRT-PCR analysis. All results are presented as the mean \pm SD of from three independent experiments performed in triplicate. #### p < 0.0001; ### p < 0.001; ## p < 0.01; # p < 0.05 vs. the LPS-treated group. **** p < 0.0001; *** p < 0.001; ** p < 0.01; * p < 0.05 vs. the PRRSV-infected group.

Incubation of all groups was then continued for an additional 24 h.

2.8. Animal challenge

Twenty-five 5-weeks-old piglets (free of PRRSV, porcine circovirus type 2, classical swine fever virus, pseudorabies virus, swine influenza virus, and *Mycoplasma hyopneumoniae*) were randomly allocated to one of five groups (5 piglets per group): i) PRRSV-infected and vehicle (saline + 0.5% DMSO)-treated group; ii) PRRSV-infected and Xn (10 mg/kg)-treated group; iii) PRRSV-infected and Xn (20 mg/kg)-treated group; iv) PRRSV-infected and Xn (25 mg/kg)-treated group; or v) an uninfected and untreated mock group. As shown in Fig. 4A, piglets were challenged intranasally (1 mL) and intramuscularly (1 mL, in the right neck) with PRRSV strain BB0907 (3×10^5 TCID₅₀). Then, the piglets were intramuscularly injected in the left neck with 10, 20, or 25 mg/kg of body weight of Xn or 0.5% DMSO in saline at 24 h after infection. Treatments were administered once every 3 days up to 14 days. After infection, the piglets were monitored daily for general health status and rectal temperature. Serum samples and nasal swabs were collected at 1, 4, 7, 10, and 14 dpi. All piglets were sacrificed on day 14 pi. Serum and lung tissues were collected for determination of the viral RNA load and histopathological analysis.

2.9. Pathological examination

The lung tissues of all piglets were collected at 14 dpi. Macroscopic lung lesions were estimated and scored based on the percentage of infected tissue (Baoqing et al., 2013). Meanwhile, the fixed lung tissues were dehydrated, cleared in xylene, embedded in paraffin wax, then sliced and mounted on slides, which were stained with hematoxylin and eosin, as previously reported (Zhang et al., 2016). The microscopic lung lesions were scored as follows: 0 = no lesions; 1 = mild, focal to multifocal interstitial pneumonia (< 50% affected tissue); 2 = moderate, multifocal to coalescing (50%–75% affected tissue); 3 = severe, patchy to coalescing and extensive (75%–90% affected tissue); and 4 = severe and diffuse (> 90% affected tissue) (Zhang et al., 2017).

2.10. Clinical evaluations

The clinical conditions of the pigs were scored daily after challenge, as previously described (Yue et al., 2008). Briefly, behavior, respiration, and cough were each scored on a 4-point scale from 1 to 4, with 1 being clinically normal and 3 indicating maximal illness. A dead pig was given a score of 4 for each condition. The daily total clinical score was the sum of the scores given for each condition.

2.11. Ethics statement

All animal experiments conformed to the rules of National Guidelines for Housing and Care of Laboratory Animals (China) and were performed after obtaining approval from the Institutional Animal Care and Ethics Committee of Nanjing Agricultural University (permit no. IACECNAU20160102). All piglets were housed in the animal facility of Nanjing Agricultural University (Nanjing, Jiangsu, China).

2.12. Statistical analyses

All statistical analyses were performed using GraphPad Prism 7.0 software. The results were expressed as the mean \pm SD. The significance of differences among groups was determined by one-way or two-way analysis of variance. Differences with p -values < 0.05 were considered significant and designated with an asterisk (*) or pound sign (#) in the figures.

3. Results

3.1. Xn inhibited the replication of different strains of PRRSV in PAMs

The CC50 of Xn was measured using a CCK-8 Kit and calculated to be 42.57 μ M (Fig. 1A). To assess the inhibitory effect of Xn on PRRSV, the IC50 of Xn was detected. As shown in Fig. 1B, the IC50 of Xn is 7.047 μ M. In addition, Western blot and qRT-PCR analyses showed that Xn reduced PRRSV N-protein levels and relative viral mRNA levels in a dose-dependent manner (Fig. 1C–D).

We also investigated whether Xn could inhibit the replication of different sub-genotype strains of PRRSV in PAMs. Western blot, qRT-

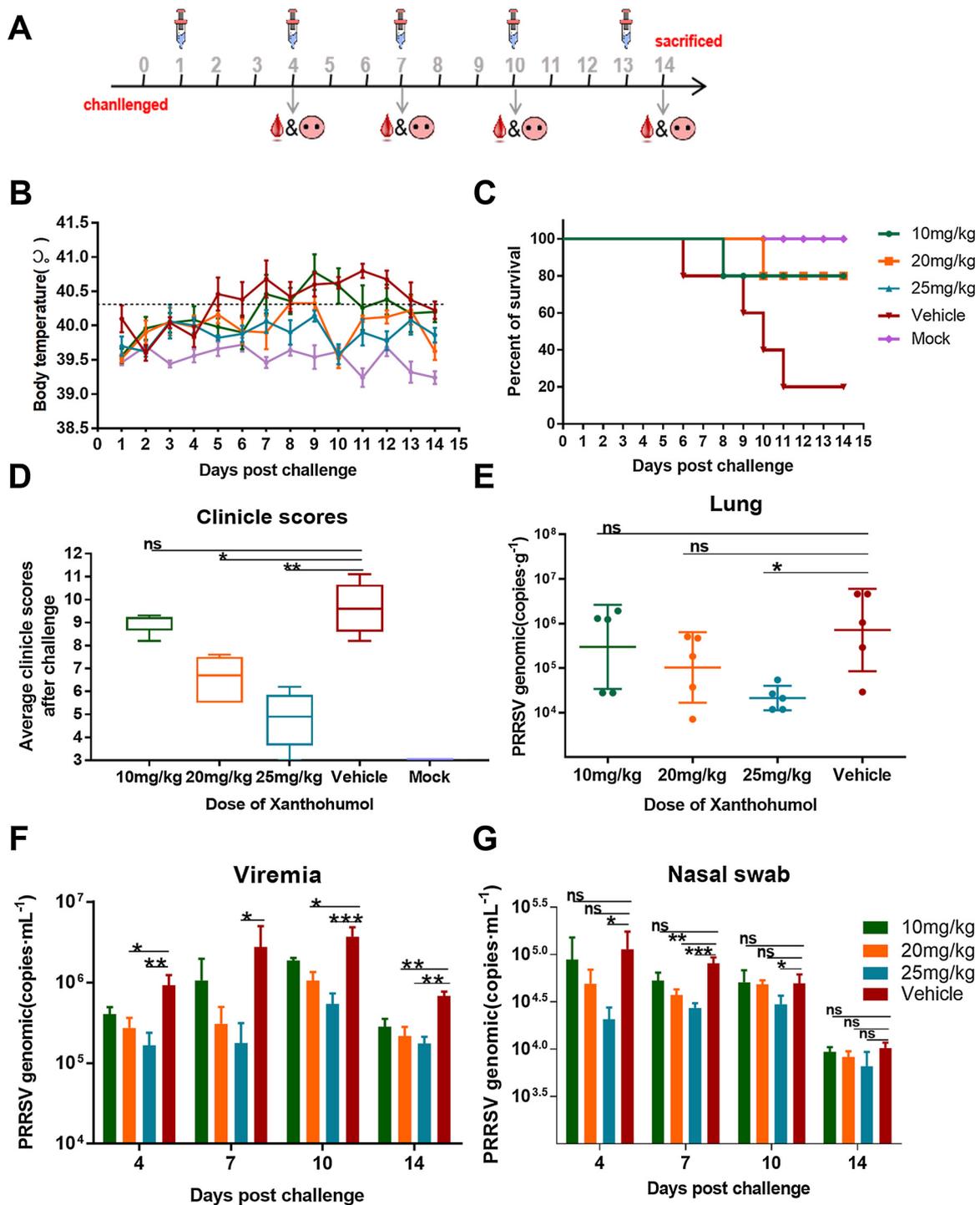


Fig. 4. Therapeutic effect of Xn in PRRSV-infected piglets. (A) A pattern diagram of the animal experiments. (B) Rectal temperatures of piglets from each group presented at the mean \pm SD (error bars). A temperature ≥ 40.5 °C was defined as a clinical fever. (C) The survival rate of each group was monitored until 14 dpi. (D) Average clinical score for each group. Piglets were observed and scored daily after challenge. Error bars represent SDs from pigs on different days. $**p < 0.01$; $*p < 0.05$ vs. the vehicle (saline + 0.5% DMSO)-treated pigs. (E) Viral RNA load in the lungs of pigs (\log copies \cdot g $^{-1}$) at 14 dpi. (F) Viral load in sera of pigs (\log copies \cdot ml $^{-1}$) and (G) nasal swabs (\log copies \cdot ml $^{-1}$) were measured by qRT-PCR. The results are presented as the mean \pm SD (error bars).

PCR, and virus titer analyses demonstrated that Xn effectively reduced replication of C-PRRSV strains S1 and HP-PRRSV strains BB0907. What's more, Xn reduced replication of the NADC30-like strain FJ1402 to some extent (Fig. 1 E–H).

3.2. Xn had therapeutic potential against PRRSV infection of PAMs

To identify the stage of the PRRSV life cycle that is most affected by

Xn, PAMs were treated with Xn before, during, and after PRRSV infection (Fig. 2A). At 24 h post-infection (hpi), the cells were harvested for western blot and qRT-PCR analyses, and the supernatants were harvested for virus titer analysis. The results showed that PRRSV inhibition was greatest in cells treated with Xn for 24 hpi (Fig. 3B–D), suggesting that Xn has therapeutic potential against PRRSV infection of PAMs.

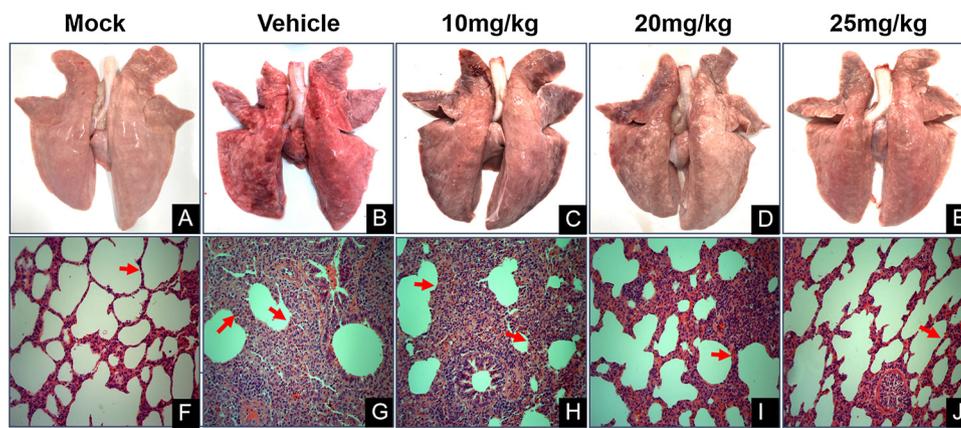
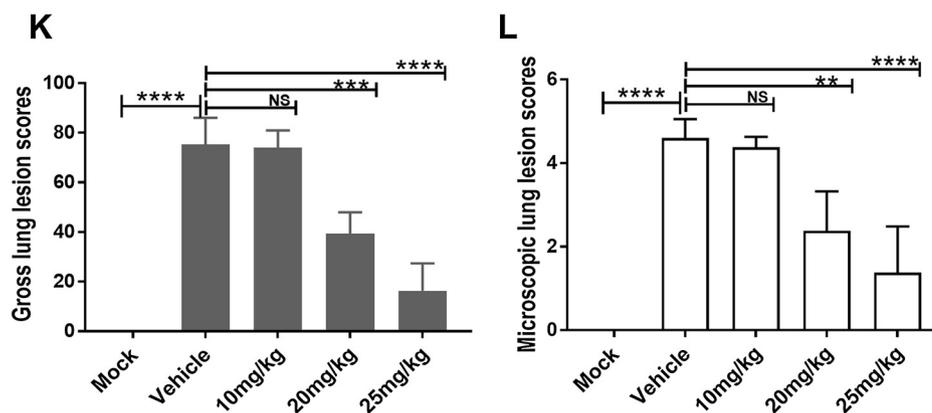


Fig. 5. Gross and microscopic observations of lungs collected from piglets. (A) Lungs from an uninfected and untreated pig. (B) Lungs from an infected and vehicle (saline + 0.5% DMSO)-treated control pig. (C–E) Lungs from pigs infected with PRRSV and treated with the indicated dose of Xn. Microscopic lesions in the lungs of pigs uninfected and untreated (F), infected and vehicle treated (G), and infected and treated with the indicated dose of Xn. (H–J) Original magnification, 200 ×. Gross lung lesion scores (K) and microscopic lung lesion scores (L).



3.3. Xn inhibited the PRRSV-induced inflammatory responses of PAMs

PRRSV infection induces production of cytokines, in return, cytokines interfere with the viral infection. In this study, we first determined the effect of Xn on cellular inflammation in LPS treatment, the dosages of 10 µg/mL LPS was chosen for exposure to PAMs for 1 h and then subjected with Xn (10 µg/mL) for another 6 h. As shown in Fig. 3A–D, Xn had no significant effect on the inflammatory responses and LPS significantly increased the mRNA levels of interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α, whereas Xn (10 µM) considerably inhibited LPS-induced the IL-1β, IL-6, IL-8, and TNF-α. These results were consistent with previous reports which proving Xn having anti-inflammatory activity in many pulmonary inflammatory diseases (Lee et al., 2011; Lv et al., 2017). In order to characterize the expression of these pro-inflammatory cytokines genes in Xn treated PRRSV-infected-cells, the transcript levels of IL-1β, IL-6, IL-8, and TNF-α in PAMs treated with Xn post-infection were investigated. RT-qPCR results displayed that Xn treatment significantly reduced these four cytokines mRNA levels, all of which were up-regulated by PRRSV infection. The transcripts of IL-1β, IL-6, IL-8, and TNF-α in PRRSV-infected cells were increased to 5.4, 5.3, 2.4 and 4.5-fold, respectively. Xn (10 µM) treatment of the virus-infected cells reduced the transcripts of IL-1β, IL-6, IL-8, and TNF-α to 1.3, 1.6, 1.1 and 1.3-fold, respectively (Fig. 3A–D). This demonstrates that Xn treatment effectively reduced inflammatory responses which PRRSV induced.

3.4. Xn had a therapeutic effect on PRRSV in piglets

3.4.1. Clinical signs and changes in body temperature

Following challenge with virulent HP-PRRSV, all piglets in the vehicle control group had a high fever (≥ 40.5 °C) and exhibited a range

of clinical signs, including appetite, lethargy, rough hair coat, dyspnea, periocular edema, and light diarrhea. Four of five pigs in this group died between 5 and 14 dpi. Infected pigs treated with 25 mg/kg of Xn and mock-infected pigs had no clinical fever during the experiment, but experienced a decrease in body temperature that was 0.3–0.8 °C lower than pigs in the vehicle control group. All pigs in these groups had survived. Infected pigs treated with 20 mg/kg of Xn exhibited moderate fluctuations in rectal temperature during the 14 dpi, while one pig died at 10 dpi (Fig. 4 B and C). There was no significant difference in clinical scores between the pigs treated with 10 mg/kg of Xn and those with the vehicle ($p > 0.05$). In contrast, the clinical scores of pigs treated with 20 and 25 mg/kg of Xn were significantly lower than those of the mock-treated pigs ($p < 0.05$) (Fig. 4D).

3.4.2. Viremia and viral load in nasal swabs and lung samples

The viral loads in the lungs of infected pigs treated with 25 mg/kg of Xn were significantly lower than in the vehicle control pigs ($p < 0.05$) (Fig. 4E). Blood and nasal swab samples of the pigs were collected at 1, 4, 7, 10, and 14 dpi. The copy number of PRRSV genomic cDNA was determined by qRT-PCR analysis. Levels of circulating viruses in the serum of infected pigs treated with 20 and 25 mg/kg of Xn were significantly lower than in the vehicle control group at 4–14 dpi (Fig. 4F) ($p < 0.05$). Similarly, the copy number of PRRSV genomic DNA in the nasal swabs of the infected pigs treated with 25 mg/kg of Xn were significantly lower than in the vehicle control group at 4–10 dpi (Fig. 4G) ($p < 0.05$).

3.4.3. Lung pathology

Necropsy examinations revealed that lung tissues of the pigs which infected and treated with the vehicle had significant lesions characterized by pulmonary consolidation (Fig. 5B). In contrast, no

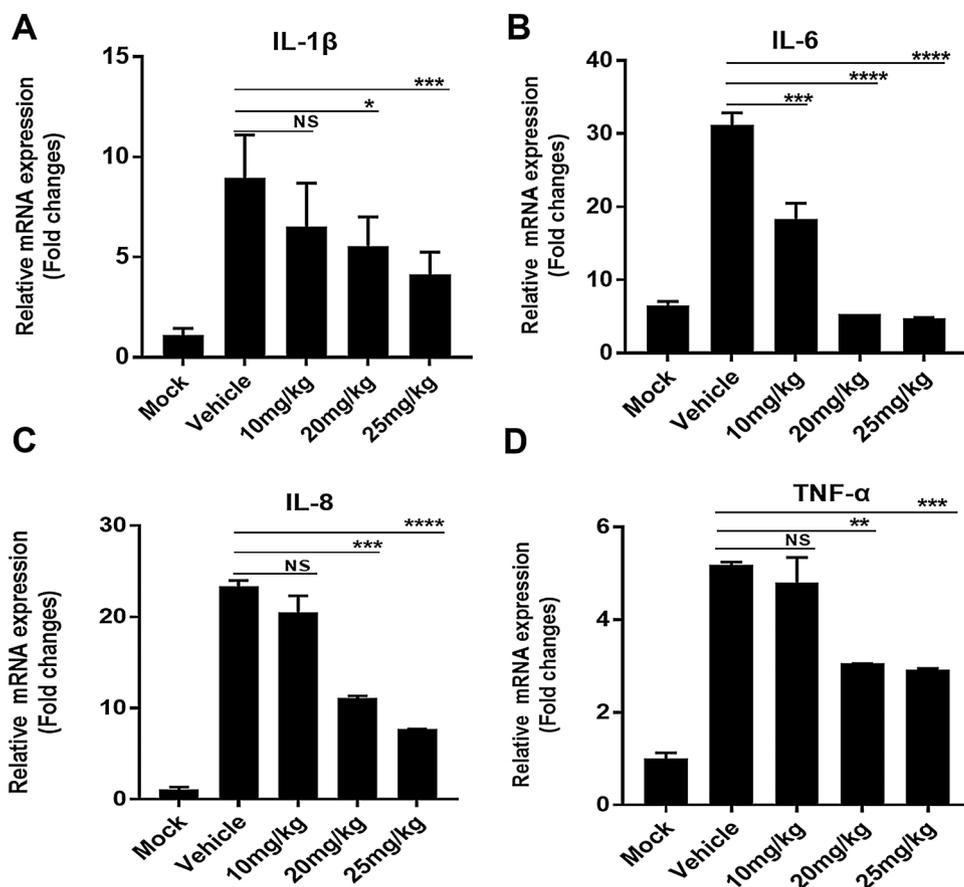


Fig. 6. Effect of Xn treatment on PRRSV-stimulated inflammatory responses in lungs. (A–D) mRNA expression of IL-1 β , IL-6, IL-8, and TNF- α in the lung tissues of each group was measured using qRT-PCR with GAPDH mRNA as an internal loading control. Values are presented as the fold change relative to mock-infected pigs. All assays were repeated at least three times, with each experiment performed in triplicate. Bars represent the mean \pm SD of three independent experiments. **** p < 0.0001; *** p < 0.001; ** p < 0.01; * p < 0.05 as compared with vehicle (saline + 0.5% DMSO)-treated pigs.

pathological lesions were identified in the control pigs (Fig. 5A). The lung lesions of pigs which infected and treated with Xn were significantly smaller than those of the vehicle control pigs, and the effect was dose-dependent (Fig. 5C–E). There was no statistical difference in gross lung lesion scores between the vehicle and the Xn 10 mg/kg group, but the scores of the Xn 20 and 25 mg/kg groups were significantly lower than that of the vehicle group (Fig. 5K). The microscopic lung lesions of vehicle group pigs characterized by thickened alveolar septa, scattered hemorrhaging of alveolar septa, and increased numbers of inflammatory cells. Inflammatory exudation was also found in the trachea of these pigs (Fig. 5F–J). The microscopic lung lesions of the pigs treated with 25 mg/kg of Xn were significantly less severe than those of the vehicle control pigs. Moderate pathological lesions were apparent in pigs treated with 20 mg/kg of Xn (Fig. 5L).

3.4.4. Inflammatory responses in lung tissues

Levels of inflammatory cytokines in lung tissues were detected by qRT-PCR at 14 dpi. PRRSV-infected piglets exhibited increased expression levels of IL-1 β , IL-6, IL-8, and TNF- α , as compared with the mock-infected animals. The levels of IL-1 β , IL-6, IL-8, and TNF- α expression levels in the lung tissues of the infected piglets treated with Xn were attenuated in a dose-dependent manner (Fig. 6A–D).

4. Discussion

Porcine reproductive and respiratory syndrome is an important disease that results in huge economic damage to the global swine industry. Current vaccination strategies cannot control this infectious disease, thus the development of effective anti-PRRSV drugs is necessary to provide additional preventive and therapeutic benefits. Natural products, such as plant-derived flavonoids, have attracted considerable attention in many antiviral studies because of the various biological

activities. Some studies have reported that flavonoids, such as proanthocyanidin A2, have therapeutic effects against PRRSV infection (Zhang et al., 2018). In our previous study, we screened a library of 386 natural products and found that Xn, a prenylated flavonoid found in hops, exhibited significant anti-PRRSV activities via stimulation of the Nrf2-HMOX1 pathway *in vitro*. In this study, we found that Xn conveyed significant anti-PRRSV activities in piglets.

The PRRSV life cycle can be divided into four basic stages: attachment, entry, replication, and release (Kappes and Faaberg, 2015; Wang et al., 2018). Our results showed Xn had the best antiviral effect when it was added after PRRSV infection of PAMs, indicating that Xn conveys a therapeutic effect to infective pigs. Consistent with our hypothesis, results of animal experiment showed that the piglets in the 20 mg/kg of Xn treatment group exhibited only moderate clinical signs, mild injury, and low viral loads, as compared to those in the PRRSV challenge control groups. Moreover, the pigs treated with 25 mg/kg of had minimal clinical symptoms, which were almost identical to those of the mock-infected group.

The replication of PRRSV begins in macrophages, which play a major role in the inflammatory and immune responses (Qiao et al., 2011). Pro-inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6, accumulated in the PAMs during PRRSV infection. Increased levels of these cytokines in the circulation are responsible for the clinical symptoms of PRRSV infection, which include fever, depression, and anorexia. Accumulating evidence has shown that Xn has anti-inflammatory effects and protective effect against LPS-induced acute lung injury (Lv et al., 2017) and immunomodulatory activities in macrophage cell lines (Young-Chang et al., 2008). Long-term inflammatory mediators, including TNF- α , IL-6, IL-1 β , and IL-8, are strongly related to the development of acute and chronic inflammation diseases. Xn has been reported to effectively reduced cellular secretion of TNF- α , IL-6, and IL-1 β (Fairweather et al., 2005). Our results showed that LPS

treatment and PRRSV infection significantly induced the mRNA levels of IL-1 β , IL-6, IL-8, and TNF- α . Xn not only effectively attenuated the PRRSV-induced mRNA levels of these cytokines but also inhibited increases caused by LPS in PAMs. Notably, the decreased expression of inflammatory cytokines is partly attributed to the inhibitory effect of Xn against PRRSV, which indirectly reduces the expression of pro-inflammatory cytokines. These results suggest that Xn could directly intervene in the cellular immune response, at least to some extent, and subsequently inhibit the expression of inflammatory cytokines induced by PRRSV. Xn significantly decreased the mRNA levels of IL-1 β , IL-6, IL-8, and TNF- α in the lung tissues of infected pigs and alleviated lung lesions due to interstitial pneumonia.

It has been reported that Xn and its metabolites were excreted mainly in feces within 24 h of administration in rats (Nookandeh et al., 2004). The lifelong treatment at a daily dose of 100 mg/kg body weight in a two-generation study did not affect the development and fertility of SD rats (Gerhäuser, 2005; Hussong et al., 2010). Furthermore, the mean half-life period of Xn in human was 20 h for the 60 mg dose and 18 h for the 180 mg dose (Legette et al., 2014). It suggests that Xn treatment has a little residual and high safety in animals. But the half-life period of Xn in pigs and the bioavailability of Xn after oral administration should be studied in the future.

In conclusion, Xn, as a well-sourced natural product, had highly effective against highly pathogenic PRRSV infection in both PAMs and swine. It has great potential as a promising anti-PRRSV agent.

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References

Albina, E., 1997. Epidemiology of porcine reproductive and respiratory syndrome (PRRS): an overview. *Vet. Microbiol.* 55, 309–316.

Baoqing, G., Lager, K.M., Henningson, J.N., Miller, L.C., Schlink, S.N., Kappes, M.A., Kehrl, M.E., Brockmeier, S.L., Nicholson, T.L., Han-Chun, Y.J.V., 2013. Experimental infection of United States swine with a Chinese highly pathogenic strain of porcine reproductive and respiratory syndrome virus. *Virology* 435, 372–384.

Buckwold, V.E., Wilson, R.J.H., Aysegul, N., Beer, B.B., Voss, T.G., Turpin, J.A., Buckheit, R.W., Jiayi, W., Michelle, W.M., Walton, E.M., Antiviral Research, J., 2004. Antiviral activity of hop constituents against a series of DNA and RNA viruses. *Antiviral Res.* 61, 57–62.

Chen, X., Bai, J., Liu, X., Song, Z., Zhang, Q., Wang, X., Jiang, P.J.J.O.V., 2018. Nsp1 α of PRRSV strain BB0907 impairs the function of monocyte-derived dendritic cells via the release of soluble CD83. *JVI* 92 00366-00318.

Collins, J.E., Benfield, D.A., Christianson, W.T., Harris, L., Hennings, J.C., Shaw, D.P., Goyal, S.M., McCullough, S., Morrison, R.B., Joo, H.S., et al., 1992. Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. *J. Vet. Diagn. Invest.* 4, 117–126.

Cos, P., Maes, L., Vlietinck, A., Pieters, L., 2008. Plant-derived leading compounds for chemotherapy of human immunodeficiency virus (HIV) infection - an update (1998–2007). *Planta Med.* 74, 1323–1337.

Elazhary, Y., Weber, J., Bikour, H., Morin, M., Girard, C., 1991. 'Mystery swine disease' in Canada. *Vet. Rec.* 129, 495–496.

Fairweather, D., Rose, N.R., Lupus, J., 2005. Inflammatory heart disease: a role for cytokines. *Lupus* 14, 646.

Fang, J., Wang, H., Bai, J., Zhang, Q., Li, Y., Liu, F., Jiang, P., 2016. Monkey Viperin Restricts Porcine Reproductive and Respiratory Syndrome Virus Replication. *PLoS One* 11, e0156513.

Ge, M., Yao, W., Yuan, D., Zhou, S., Chen, X., Zhang, Y., Li, H., Xia, Z., Hei, Z.J.C.D., 2017. Brg1-mediated Nrf2/HO-1 pathway activation alleviates hepatic ischemia-reperfusion injury. *Disease* 8, e2841.

Gerhäuser, C., 2005. Beer constituents as potential cancer chemopreventive agents. *Eur. J. Cancer* 41, 1941–1954.

Hussong, R., Frank, N., Knauff, J., Ittrich, C., Owen, R., Becker, H., Gerhäuser, C.J.M.N., Research, F., 2010. A safety study of oral xanthohumol administration and its influence on fertility in Sprague Dawley rats. *Mol. Nutr. Food Res.* 49, 861–867.

Kappes, M.A., Faaberg, K.S.J.V., 2015. PRRSV structure, replication and recombination: Origin of phenotype and genotype diversity. *Virology* 479–480, 475–486.

Lee, I.S., Lim, J., Gal, J., Kang, J.C., Kim, H.J., Kang, B.Y., Choi, H.J., 2011. Anti-inflammatory activity of xanthohumol involves heme oxygenase-1 induction via NRF2-ARE signaling in microglial BV2 cells. *Neurochem. Int.* 58, 153–160.

Legette, L., Kampracha, C., Reed, R.L., Choi, J., Bobe, G., Christensen, J.M., Rodriguez-Proteau, R., Purnell, J.Q., Stevens, J.F., 2014. Human pharmacokinetics of xanthohumol, an antihyperglycemic flavonoid from hops. *Mol. Nutr. Food Res.* 58, 248–255.

Li, L., Li, Q., Bao, Y., Li, J., Chen, Z., Yu, X., Zhao, Y., Tian, K., Li, N., 2014. RNAi-based inhibition of porcine reproductive and respiratory syndrome virus replication in transgenic pigs. *J. Biotechnol.* 171, 17–24.

Li, L., Tian, X., Chen, J., Li, P., Zheng, Q., Hou, J., 2018. Griffithsin inhibits porcine reproductive and respiratory syndrome virus infection in vitro. *Arch. Virol.* 163, 3317–3325.

Liu, X., Song, Z., Bai, J., Nauwynck, H., Zhao, Y., Jiang, P., 2019. Xanthohumol inhibits PRRSV proliferation and alleviates oxidative stress induced by PRRSV via the Nrf2-HMOX1 axis. *Vet. Res.* 50, 61.

Lv, H., Liu, Q., Wen, Z., Feng, H., Deng, X., Ci, X.J.R.B., 2017. Xanthohumol ameliorates Lipopolysaccharide (LPS)-induced acute lung injury via induction of AMPK/GSK3 β -Nrf2 signal axis. *Redox Biol.* 12, 311–324.

Mardassi, H., Mounir, S., Dea, S., 1994. Identification of major differences in the nucleocapsid protein genes of a Quebec strain and European strains of porcine reproductive and respiratory syndrome virus. *J. Gen. Virol.* 75 (Pt 3), 681–685.

Miranda, C.L., Stevens, J.F., Helmrich, A., Henderson, M.C., Rodriguez, R.J., Yang, Y.H., Deinzer, M.L., Barnes, D.W., Buhler, D.R., J. Food, Toxicology, C., 1999. Antiproliferative and cytotoxic effects of prenylated flavonoids from hops (*Humulus lupulus*) in human cancer cell lines. *Food Chem. Toxicol.* 37, 271–285.

Murtaugh, M.P., Genzow, M., 2011. Immunological solutions for treatment and prevention of porcine reproductive and respiratory syndrome (PRRS). *Vaccine* 29, 8192–8204.

Nelsen, C.J., Murtaugh, M.P., Faaberg, K.S., 1999. Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. *J. Virol.* 73, 270.

Nookandeh, A., Frank, N., Steiner, F., Ellinger, R., Schneider, B., Gerhäuser, C., Becker, H., 2004. Xanthohumol metabolites in faeces of rats. *Phytochemistry* 65, 561–570.

Pejsak, Z., Stadejek, T., Markowska-Daniel, I., 1997. Clinical signs and economic losses caused by porcine reproductive and respiratory syndrome virus in a large breeding farm. *Vet. Microbiol.* 55, 317–322.

Pinto, C., Duque, A.L., Rodriguez-Galdon, B., Cestero, J.J., Macias, P., 2012. Xanthohumol prevents carbon tetrachloride-induced acute liver injury in rats. *Food Chem. Toxicol.* 50, 3405–3412.

Qiao, S., Feng, L., Bao, D., Guo, J., Wan, B., Xiao, Z., Yang, S., Zhang, G.J.V.M., 2011. Porcine reproductive and respiratory syndrome virus and bacterial endotoxin act in synergy to amplify the inflammatory response of infected macrophages. *Vet. Microbiol.* 149, 213–220.

Renukaradhya, G.J., Meng, X.J., Calvert, J.G., Roof, M., Lager, K.M., 2015. Inactivated and subunit vaccines against porcine reproductive and respiratory syndrome: current status and future direction. *Vaccine* 33, 3065–3072.

Russell, P., Atkinson, K., Krishnan, L., 1980. Recurrent reproductive failure due to severe placental villitis of unknown etiology. *J. Reprod. Med.* 24, 93–98.

Song, Z., Bai, J., Nauwynck, H., Lin, L., Liu, X., Yu, J., Jiang, P., 2019. 25-Hydroxycholesterol provides antiviral protection against highly pathogenic porcine reproductive and respiratory syndrome virus in swine. *Vet. Microbiol.* 231, 63–70.

Song, Z., Zhang, Q., Liu, X., Bai, J., Zhao, Y., Wang, X., Jiang, P.J.V.M., 2017. Cholesterol 25-hydroxylase is an interferon-inducible factor that protects against porcine reproductive and respiratory syndrome virus infection. *Vet. Microbiol.* 210, 153.

Sun, N., Zhao, X., Bai, X.Y., Niu, L., Song, M.Q., Sun, Y.G., Jiang, J.B., Li, H.Q., 2012. Anti-PRRSV effect and mechanism of sodium tanshinone IIA sulfonate in vitro. *J. Asian Nat. Prod. Res.* 14, 721–728.

Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., Hu, Y., Chen, X., Hu, D., Tian, X., Liu, D., Zhang, S., Deng, X., Ding, Y., Yang, L., Zhang, Y., Xiao, H., Qiao, M., Wang, B., Hou, L., Wang, X., Yang, X., Kang, L., Sun, M., Jin, P., Wang, S., Kitamura, Y., Yan, J., Gao, G.F., 2007. Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. *PLoS One* 2, e526.

Wang, H., Bai, J., Fan, B., Li, Y., Zhang, Q., Jiang, P., 2016. The interferon-induced Mx2 inhibits porcine reproductive and respiratory syndrome virus replication. *J. Interferon Cytokine Res.* 36, 129–139.

Wang, H.M., Liu, T.X., Wang, T.Y., Wang, G., Liu, Y.G., Liu, S.G., Tang, Y.D., Cai, X.H., 2018. Isobavachalcone inhibits post-entry stages of the porcine reproductive and respiratory syndrome virus life cycle. *Arch. Virol.* 163, 1263.

Xie, J., Zhou, H., Cui, J., Chen, Y., Zhang, M., Deng, S., Zhou, P., Su, S., Zhang, G., 2014. Inhibition of porcine reproductive and respiratory syndrome virus by specific siRNA targeting Nsp9 gene. *Infect. Genet. Evol.* 28, 64–70.

Young-Chang, C., Hyun Jung, K., Young-Jun, K., Kwang Youl, L., Hyun Jin, C., Ik-Soo, L., Bok Yun, K.J.I.L., 2008. Differential anti-inflammatory pathway by xanthohumol in IFN-gamma and LPS-activated macrophages. *Int. Immunopharmacol.* 8, 567–573.

Yue, W., Yajie, L., Jun, H., Burkhart, K.M., Vaughn, E.M., Roof, M.B., Faaberg, K.S., Virology, J., 2008. Attenuation of porcine reproductive and respiratory syndrome virus strain MN184 using chimeric construction with vaccine sequence. *Virology* 371, 418–429.

Zhang, L., Liu, J., Bai, J., Du, Y., Wang, X., Liu, X., Jiang, P., 2013. Poly(I:C) inhibits porcine reproductive and respiratory syndrome virus replication in MARC-145 cells via activation of IFIT3. *Antiviral Res.* 99, 197–206.

Zhang, M., Wu, Q., Chen, Y., Duan, M., Tian, G., Deng, X., Sun, Y., Zhou, T., Zhang, G., Chen, W., Chen, J., 2018. Inhibition of proanthocyanidin A2 on porcine reproductive and respiratory syndrome virus replication in vitro. *PLoS One* 13, e0193309.

- Zhang, Q., Bai, J., Hou, H., Song, Z., Zhao, Y., Jiang, P., 2017. A novel recombinant porcine reproductive and respiratory syndrome virus with significant variation in cell adaption and pathogenicity. *Vet. Microbiol.* 208, 150–158.
- Zhang, Q., Jiang, P., Song, Z., Lv, L., Li, L., Bai, J., 2016. Pathogenicity and antigenicity of a novel NADC30-like strain of porcine reproductive and respiratory syndrome virus emerged in China. *Vet. Microbiol.* 197, 93–101.
- Zhao, J., Feng, N., Li, Z., Wang, P., Qi, Z., Liang, W., Zhou, X., Xu, X., Liu, B., 2016. 2',5'-Oligoadenylate synthetase 1(OAS1) inhibits PRRSV replication in Marc-145 cells. *Antiviral Res.* 132, 268–273.