

Glycyrrhizin inhibits porcine epidemic diarrhea virus infection and attenuates the proinflammatory responses by inhibition of high mobility group box-1 protein

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Abstract Porcine epidemic diarrhea (PED), caused by porcine epidemic diarrhea virus (PEDV) infection, leads to significant economic losses in the swine industry worldwide. In our studies, we found that glycyrrhizin, the major component of licorice root extracts, could moderately inhibit PEDV infection in Vero cells, when analyzed by western blot, qRT-PCR and a plaque formation assay. We also revealed that glycyrrhizin inhibited the entry and replication of PEDV. In addition, we demonstrated that glycyrrhizin decreased the mRNA levels of proinflammatory cytokines. Since glycyrrhizin is a competitive inhibitor of high mobility group box-1 (HMGB1), we confirmed that TLR4 and RAGE (associated with PEDV pathogenesis during the infection in Vero cells). In summary, our studies provide a molecular basis for developing novel therapeutic methods to control PEDV infection, based on glycyrrhizin and its derivatives.

Introduction

Porcine epidemic diarrhea virus (PEDV), an enveloped, single-stranded and positive-sense RNA virus, is the causative agent of porcine epidemic diarrhea (PED) [1, 2]. PEDV belongs to the *Alphacoronavirus* genus, the family of *Coronaviridae* and the subfamily of *Coronavirinae* [3]. PEDV encodes several structural proteins, including the

spike (S), envelope (E), membrane (M), and nucleoprotein (N) [4–6]. PEDV infection causes 80 to 100% fatality rates in suckling piglets [7]. It was originally reported in Belgium and the United Kingdom [8]. PEDV outbreaks emerged in the United States in 2013 [9–11]. In 2014, PEDV swept through three farms in South-Western Germany [12]. More importantly, PEDV has been proposed to be a potential threat to other species, especially humans [13]. Currently, no antiviral drugs are available to control the infection of PEDV.

Glycyrrhizin (GLY) is the major component of licorice root extracts, which is the most intensively investigated bioactive compound in licorice root (*Glycyrrhiza Radix*) [14]. GLY is a glycosylated saponin, containing one molecule of glycyrrhetic acid and two molecules of glucuronic acid [15, 16]. It has been used as a traditional Chinese medicinal herb for treating hepatitis because of its anti-inflammatory properties [17]. GLY possesses several beneficial activities including expectorant, anti-ulcer, anti-allergy, anti-coagulative, anti-oxidative, antiviral, anti-tumor, and anti-inflammatory activities [18–21]. The mechanism of how GLY exerts these diverse effects remains largely unclear.

The antiviral effect of GLY has been reported previously on various viruses, such as human cytomegalovirus [22], influenza virus [23], severe acute respiratory syndrome [24], herpes simplex type 1 [25], hepatitis A [16] and B virus [26]. However, an antiviral effect of GLY against PEDV has not yet been reported.

GLY is a competitive inhibitor of high mobility group box1 (HMGB1) that can inhibit the cytokine activity of HMGB1 [27]. Extracellular HMGB1 functions as a damage-associated molecular pattern (DAMP) molecule and activates proinflammatory signaling pathways by activating pattern recognition receptors including Toll-like receptors-

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2, -4 [28, 29] and the receptor for advanced glycation end-products (RAGE) [30, 31]. HMGB1 is a unique mediator of innate immune responses and inflammation-associated events [32–35]. In addition, extracellular HMGB1 contributes to the pathogenesis of various chronic inflammatory and autoimmune diseases [36–40].

In our studies, we first explored the antiviral effect of GLY against PEDV infection. Next, we revealed that GLY inhibited entry and replication of PEDV. In addition, we demonstrated that GLY also decreased the mRNA levels of proinflammatory cytokines. We also confirmed that TLR4 and RAGE (receptors for HMGB1) might be associated with PEDV infection-related pathogenesis.

Material and methods

Cell and virus

The African green monkey kidney cell line (Vero) was cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% newborn calf serum (16010-159, GibCO). Porcine epidemic diarrhea virus (strain HLJBY) was propagated in Vero cells in DMEM supplemented with 2% newborn calf serum. PEDV HLJBY strain was isolated from the feces of piglets suffering from severe diarrhea and was propagated in Vero cells [41]. PEDV was adapted for eight passages in Vero cells. PEDV was inactivated by UV light exposure [42]. The loss of infectivity of UV-inactivated virus was confirmed using the plaque formation assay.

Reagents and antibodies

Glycyrrhizin was purchased from Sigma. Small interfering RNAs (siRNAs) were purchased from Biotend (China). The sequence of the siRNA specifically targeting RAGE was 5'-GCCGGAAAUUAUAGAUUCUdTdT-3', and the negative control siRNA was 5'-UUCUCCGAACGUGU CACGUTT-3'. Antibodies against RAGE were obtained from Cell Signaling Technology. The polyclonal antibody for the PEDV-N protein was previously generated in our lab.

Plasmid constructs

In order to construct a HA-tagged HMGB1 protein-expressing plasmid, HMGB1 was first amplified using PCR with specific primers (Table 1) carrying *EcoRI* and *XhoI* restriction sites in the forward and reverse primers. The PCR product was digested with *EcoRI* and *XhoI* and ligated into pCAGGS-HA (PCA), previously digested with the same enzymes for 16 h at 4 °C. The HMGB1 mutants

HMGB1-C45S(C45S), HMGB1h-C106S(C106S), and HMGB1-C45S/C106S (C45S/C106S) were prepared with MutExpress® II Fast Mutagenesis kit (Vazyme, China) using specific primers (Table 1) according to the manufacturer's instructions using pCAGGS-HMGB1 as the template.

RNA interference

Vero cells were grown to 50–60% confluency in 6-well cell culture plates and then transiently transfected with siRNAs targeting RAGE (siRAGE) using Lipofectamine 2000. The silencing efficiency of siRNA was analysed by western blotting and qRT-PCR. The control non-targeted siRNA (NC) was used as the negative control.

Antiviral activity of GLY

To assess the antiviral effect of GLY against PEDV infection, Vero cells were treated with different concentrations of GLY (diluted with DMEM medium supplemented with 2% newborn calf serum) for 2 h. The cells were then washed with PBS for three times before being infected with PEDV (multiplicity of infection (MOI)=0.1, 1 or 10) for 24 h in the presence of different concentrations of GLY. The supernatant was collected for a plaque formation assay, and the cells were collected for western blot or qRT-PCR analysis.

The effect of GLY on PEDV entry

Vero cells were first grown in a 6-well plate to 70–80% confluency and then incubated with GLY (0.1–0.8 mM) at 37 °C for 2 h. Next, the cells were incubated with UV-inactivated PEDV (MOI =1) at 4 °C for 1 h before one-hour incubation at 37 °C. The cells were then washed with the citric acid solution (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0) for 3 times to remove un-internalized virus particles. The cells were next washed with PBS 3 times. Total protein was then prepared from these Vero cells for western blot analysis.

The effect of GLY on PEDV replication

To assess the inhibitory effect of GLY on PEDV replication, Vero cells were seeded a 6-well plate and then infected with PEDV (MOI=0.1) for 1 h at 37 °C. The cells were washed with PBS for three times before being incubated with fresh DMEM supplemented with 2% newborn calf serum in the presence of GLY (0.4 mM). The cells were collected at 4, 8, 12 h post infection (hpi) for western blot or qRT-PCR analysis.

Table 1 Sequences of the primers used for cloning or qRT-PCR

Name	Sequence
PEDV-ORF3	Forward: TTTGCACTGTTTAAAGCGTCT Reverse: AGTAAAAGCAGACTAAACAAAGCCT
GAPDH	Forward: AGGTCGGAGTCAACGGATT Reverse: TAGTTGAGGTCAATGAAGGG
IL-1 β	Forward: GGAAGACAAATTGCATGG Reverse: CCCAACTGGTACATCAGCAC
IL-6	Forward: AGAGGCACTGGCAGAAAAC Reverse: TGCAGGAACTGGATCAGGAC
IL-8	Forward: AGGACAAGAGCCAGGAAGAA Reverse: ACTGCACCTTCACACAGAGC
TNF- α	Forward: TCTGTCTGCTGCACTTTGGAGTGA Reverse: TTGAGGGTTTGCTACAACATGGGC
HMGB1	Forward: GACGTGAATTCATGGGCAAAGGAGATCCTA Reverse: GACGTCTCGAGTTATTTCATCATCATCTTCTTCT

The effect of GLY on PEDV assembly and release

To explore the effect of GLY on virus assembly, Vero cells were infected with PEDV in the presence of GLY at 37 °C for 24 h. The supernatant and cells were collected separately for qRT-PCR analysis. The ratio between ORF3 RNA levels in the supernatant and in the cells was used as the index for virus assembly. To explore the role of GLY on virus release, virus titers in the supernatant and in the cells was determined using a plaque formation assay. The ratio between virus titers in the supernatant and in the cells was used as an index of virus release [42].

The effect of GLY on levels of proinflammatory cytokine mRNAs

Vero cells were seeded in a 24-well plate. The cells were pre-treated with different concentrations of GLY for 2 h before PEDV infection (MOI=0.1, 1 h) in the presence of different concentrations of GLY. The cells were further incubated for 5, 11, or 23 h in the presence of GLY before harvest. The mRNA levels of proinflammatory cytokines were determined by qRT-PCR.

Western blotting analysis, qRT-PCR, and plaque formation assay

Vero cells were washed with 1 ml ice-cold PBS for three times. The cells were lysed with 100 μ l 2 \times SDS loading buffer. Proteins were subjected to SDS-PAGE before being transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 3% BSA in PBST (4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween-20) for 1 h at room temperature, followed by incubation for 2 h with the appropriate

primary antibody (anti-PEDV-N, actin or RAGE). After extensive washing with PBST, the membranes were further incubated for 1 h with the appropriate secondary antibody (HRP-anti-rabbit IgG or HRP-anti-mouse IgG). Immunoreactive bands were detected by an ECL enhanced chemiluminescence system (Biouniquer, China) and analyzed using ImageJ software.

Total RNA was extracted and purified from cells using TRIZOL reagent (Invitrogen). The reverse transcription and qRT-PCR were performed according to the previously described method [43]. The primer sequences for qRT-PCR or cloning (IL-1 β , IL-6, IL-8, TNF- α , GAPDH, ORF3 of PEDV) are listed in Table 1. GAPDH was used as the internal control. The relative expression levels of the detected genes were compared with that of the GAPDH gene by the $2^{-\Delta\Delta C_t}$ method. Each qRT-PCR assay was performed in triplicate.

Virus culture supernatant was 10-fold diluted (from 10² to 10⁵) and added to 6-well plates with a confluent monolayer of cells for 1 h before overlay medium (2.5% low melting point agarose in DMEM medium containing 4% newborn calf serum) was added to each well. The cells were further incubated at 37 °C with 5% CO₂ for 3 days before being stained with 0.5% crystal violet.

Cytotoxicity assay

Approximately 2 \times 10⁴ Vero cells per well were added to a 96-well cell culture plate and cultured for 24 h at 37 °C in the presence of 5% CO₂. The medium was replaced with fresh DMEM (supplemented with 2% newborn calf serum) in the presence of inhibitors (GLY). The plates were incubated for up to 24 h. The cytotoxicity was assayed by measuring lactate dehydrogenase (LDH) released from the cells using Cytotox-One homogenous membrane integrity

kit (Promega, USA), according to the manufacturer's instruction.

Statistical analysis

All data were determined in triplicate and are representative of at least two separate experiments. The results represent the means \pm standard deviations of each triplicate data set. The differences between means were considered to be significant at * $p < 0.05$, and very significant at ** $p < 0.01$. All analyses were performed by one-way ANOVA using the SPSS software package (version 16.0, SPSS Inc., Chicago, IL, USA).

Results

Antiviral effect of glycyrrhizin against PEDV infection

The antiviral activity of glycyrrhizin (GLY) has been reported previously on several viruses. To assess the antiviral effect of GLY against PEDV infection, Vero cells were treated with different concentrations of GLY for 2 h before PEDV infection (MOI=0.1). The infected cells were further incubated for 24 h in the presence of GLY before western blotting analysis, which showed that PEDV-N protein expression was moderately reduced in a dose-dependent manner (Fig. 1A), demonstrating the antiviral activity of GLY against PEDV infection in Vero cells. The antiviral activity of GLY was further confirmed by qRT-PCR assay, which showed that GLY treatment resulted in, approximately, a 70% reduction of viral ORF3 gene expression at a concentration of 0.8 mM (Fig. 1B). A similar dose-dependent inhibition of virus infection was observed in the plaque formation assay (Fig. 1C). The cytotoxicity experiment showed GLY did not cause significant cytotoxic effects in Vero cells (at concentrations up to 0.8 mM for 24 h) (Fig. 1D). In summary, our data established the antiviral activity of GLY against PEDV infection in Vero cells.

In addition, the antiviral activities of GLY were investigated at two MOIs (1 and 10), which indicated that GLY had improved inhibitory effects on PEDV infection when cells were infected at a lower MOI (Fig. 1E).

The effect of GLY on PEDV entry, replication, assembly and release

To investigate the effect of GLY on PEDV entry Vero cells, pre-treated with GLY (37 °C, 2 h), were infected with UV-inactivated PEDV (MOI=1) for 1 h at 4 °C before 1 h

incubation at 37 °C. The un-internalized PEDV was washed away with the citric acid solution. The cells were immediately subjected to western blot analysis of PEDV-N protein. The result revealed that PEDV-N protein levels were decreased in a dose-dependent manner (Fig. 2A). Therefore, our results suggested that virus entry was affected by GLY.

To determine whether GLY has an inhibitory effect on PEDV replication, Vero cells were incubated at 37 °C in the presence of GLY (0.4 mM) after Vero cells had already been infected with PEDV (MOI=0.1, 1 h, 37 °C). PEDV-N protein levels in the infected cells were analyzed at 4, 8 and 12 hpi by western blot (Fig. 2B). The result showed PEDV-N expression was inhibited by GLY. In addition, viral ORF3 RNA levels were also decreased after GLY treatment, as demonstrated by qRT-PCR analysis at 4 hpi (~25%), 8 hpi (~33.3%) and 12 hpi (54.5%) (Fig. 2C). The results suggested that GLY inhibited the replication of PEDV.

To study the effect of GLY on virus assembly, we analyzed RNA levels of the PEDV-ORF3 gene in supernatant and in cells. Vero cells were incubated at 37 °C for 24 h with different concentrations of GLY after Vero cells had been infected with PEDV (1 h). The supernatant and the cells were collected for qRT-PCR analysis. The ratio between ORF3 RNA levels in the supernatant and in the cells was similar between GLY-treated and mock-treated samples (data not shown), indicating that GLY might not affect PEDV assembly.

To determine whether GLY affected virus release, virus titers in the supernatant and in cells were determined using a plaque formation assay. Vero cells were incubated with different concentrations of GLY after Vero cells had already been infected. The cells were freeze-thawed three times after PBS washing. The plaque formation assay revealed the virus titer ratio between supernatant and cells was similar between GLY-treated and mock-treated cells (data not shown), suggesting that GLY might not affect virus release either.

GLY reduces levels of proinflammatory cytokine mRNAs during PEDV infection

Low amounts of proinflammatory cytokines may be protective against viral invasion. However, overproduced cytokines will sabotage the host immune responses [44]. It is reported that host cells initiate immune responses by producing various proinflammatory cytokines during the infection of various viruses, including West Nile virus [45], SARS-CoV [46–48], and hepatitis (A, B, C) viruses [49]. Therefore, we studied whether GLY treatment affected the levels of proinflammatory cytokine mRNAs during PEDV infection. Our data showed that PEDV infection increased

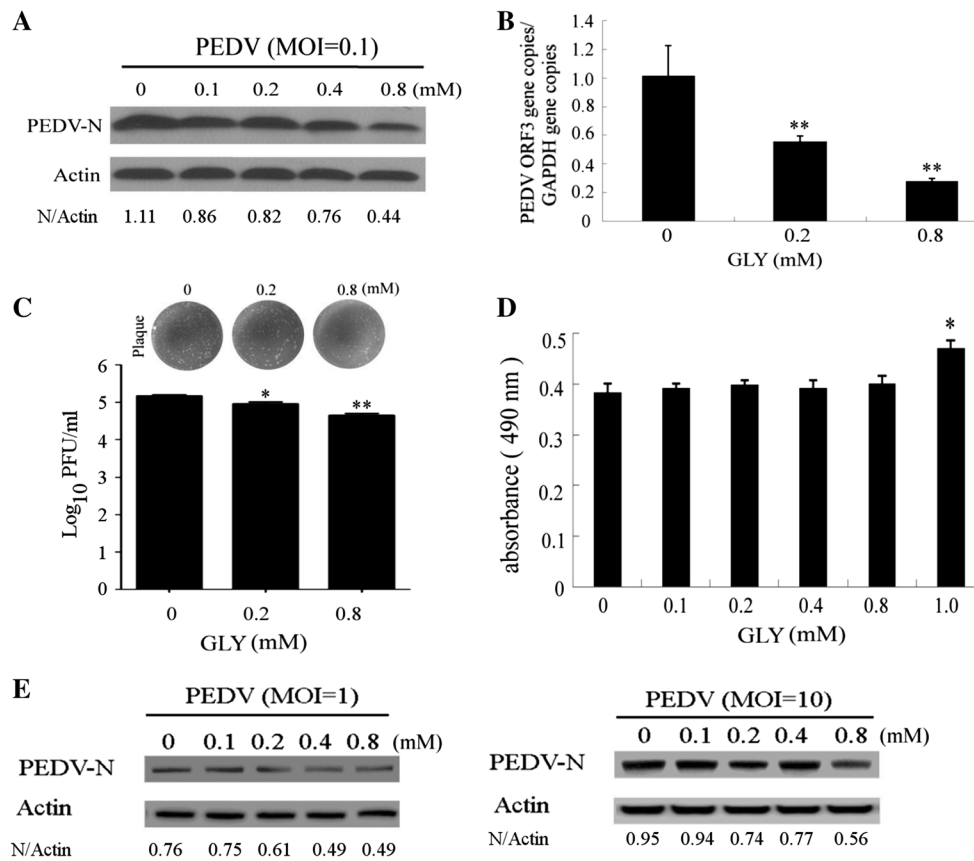


Fig. 1 Antiviral effect of GLY against PEDV infection. (A) Vero cells were pretreated with GLY for 2 h before being infected with PEDV (MOI=0.1) in the presence of GLY. Vero cells were lysed at 24 h post-infection (hpi). The PEDV-N protein expression level was analyzed by western blot. Beta-actin was used as the sample loading control. (B) RNA levels of the ORF3 gene were evaluated by qRT-PCR. (C) Virus titer in the supernatant after GLY treatment was

measured using the plaque formation assay. (D) The cytotoxicity of GLY was measured by Cytotox-One homogenous membrane integrity kit at 24 h. (E) The cells were pretreated with GLY for 2 h before being infected with PEDV (MOI=1 or 10) in the presence of GLY. The expression level of the PEDV-N protein was analyzed by western blot at 24 hpi. The results are representative of at least two different experiments

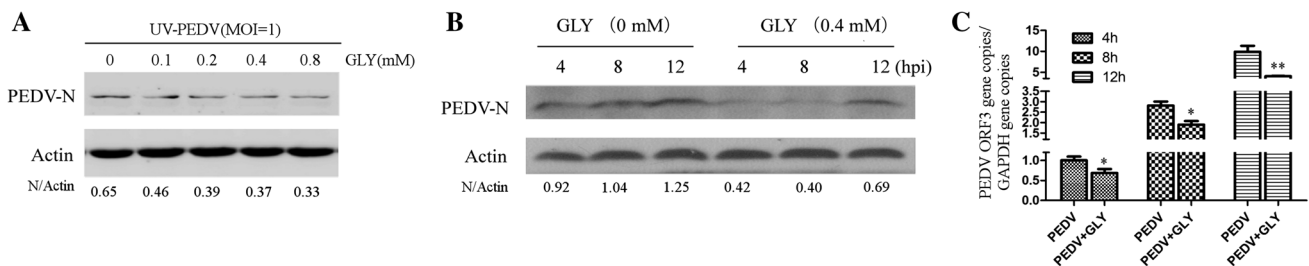


Fig. 2 Effects of GLY on PEDV entry and replication. (A) Vero cells were pretreated with different concentrations of GLY for 2 h at 37 °C. Vero cells were infected with UV-inactivated PEDV (MOI=1) for 1 h at 4 °C before one-hour incubation at 37 °C. The un-internalized PEDV was washed away with citric acid solution. The total proteins were extracted from the Vero cells for western blot. (B) Vero cells

were incubated at 37 °C in the presence of GLY (0.4 mM) after Vero cells were infected with PEDV for 1 h at 37 °C. PEDV-N protein levels were analyzed at 4, 8, 12 hpi by western blot. (C) Vero cells were incubated at 37 °C in the presence of GLY (0.4 mM) after Vero cells were infected with PEDV for 1 h at 37 °C. PEDV ORF3 gene was analyzed at 4, 8, 12 hpi by qRT-PCR

the mRNA levels of the proinflammatory cytokines IL-1 β , IL-6, IL-8, TNF- α , while GLY treatment decreased the mRNA levels of these cytokines at 6, 12 and 24 hpi: IL-1 β (20%, 49%, 75%), IL-6 (39%, 53%, 80%), IL-8 (46%, 47%, 94%), and TNF- α (51%, 56%, 91%) (Fig. 3A, B and C).

Since we had previously found that GLY treatment affected PEDV entry, we performed another experiment to rule out the possibility that the effect of GLY on proinflammatory cytokines might be caused by a decrease in the MOI. The cells were first infected with PEDV for 1 h to

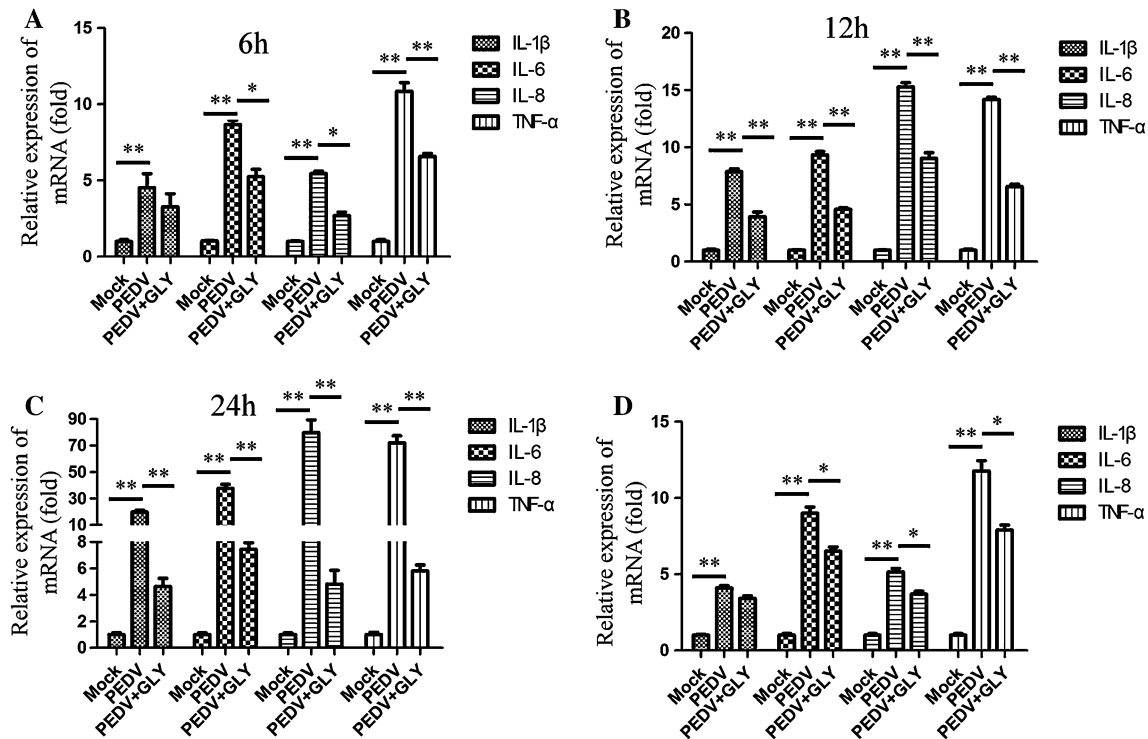


Fig. 3 GLY inhibits the expression of proinflammatory cytokines induced by PEDV infection. Vero cells were pretreated with GLY (0.8mM) for 2 h before being infected with PEDV (MOI=0.1) in the presence of GLY (0.8mM). (A–C) The mRNA levels of the proinflammatory cytokines IL-1 β , IL-6, IL-8 and TNF- α were measured at 6, 12 and 24 hpi. (D) Vero cells were infected with

PEDV for 1 h before being treated with GLY (0.8mM). The mRNA levels of the proinflammatory cytokines IL-1 β , IL-6, IL-8 and TNF- α were analyzed at 4 hpi by qRT-PCR (before the first round of virus release). The results are representative of at least two different experiments

enable virus entry. Unbound viruses were then removed using the citric acid solution. The infected cells were then incubated with GLY before the first round of virus release into the extracellular environment (6 h). We analyzed the levels of proinflammatory cytokine mRNAs at 4 hpi by qRT-PCR, and revealed that GLY indeed decreased the levels of proinflammatory cytokine mRNAs: namely IL-1 β (12%), IL-6 (34%), IL-8 (33%), and TNF- α (41%) (Fig. 3D). All these results suggest that GLY treatment attenuated the proinflammatory responses of the cells during virus infection.

HMGB1 exerts its biological function during PEDV infection through TLR4 and RAGE

GLY is a competitive inhibitor of High Mobility Group Box-1 (HMGB1). Many studies have found that HMGB1 induces proinflammatory cytokine expression through the TLR4 signal pathway. When HMGB1 exerts its effect through TLR4, a disulfide bond forms between Cys23 and Cys45 [50] and a reduced Cys106 in HMGB1 is required [51]. We therefore constructed three HMGB1 mutants to investigate their effects on virus infection. Vero cells were transfected for 12 h with HMGB1 mutant plasmids

HMGB1-C45S, HMGB1-C106S, HMGB1-C45S/C106S or control plasmid pCAGGS-HA (PCA) before PEDV infection (MOI=0.1). The cells were collected at 24 hpi for western blot analysis. The expression of HMGB1-C45S, C106S and C45S/C106S was confirmed (shown in Fig. 4A). PEDV-N protein expression levels decreased about 29%, 20%, and 48% in HMGB1-C45S, C106S, and C45S/C106S over-expressing cells, respectively (Fig. 4A). The RNA levels of the viral ORF3 gene were also decreased by approximately 51%, 20%, and 65% in HMGB1-C45S, C106S, and C45S/C106S over-expressing cells, respectively (Fig. 4B). In addition, the effect of the double mutant on mRNA levels of IL-1 β , IL-8, and TNF- α was more significant when compared to the single mutants (Fig. 4C).

RAGE is one of the main receptors of HMGB1 [52]. We knocked down RAGE expression by siRNA to determine the influence of RAGE on PEDV infection. As expected, a decline in PEDV-N expression and PEDV ORF3 RNA levels (62%) was observed after RAGE knockdown (85% knockdown efficiency) (Fig. 5A, B, C). We determined the effect of RAGE knockdown on infection using the plaque formation assay, which showed that the virus titer in the supernatant was decreased (Fig. 5D). Furthermore,

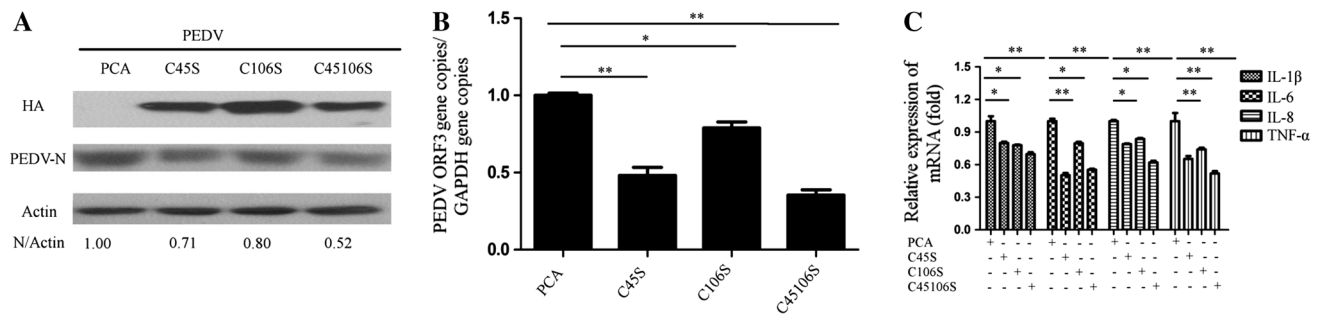


Fig. 4 HMGB1 exerts its biological function during PEDV infection through TLR4. (A–C) Vero cells were transfected with HMGB1 mutant plasmids C45S, C106S, C45S/C106S or the control pCAGGS plasmid (PCA) for 24 h, and then were infected with PEDV (MOI=0.1) for 24 h. (A) The expression of HMGB1 mutant plasmids and PEDV-N expression was analyzed by western blot. (B) The

effects of the HMGB1 mutants on the RNA levels of the ORF3 gene were analyzed by qRT-PCR. (C) The effects of the HMGB1 mutants on the mRNA levels of the proinflammatory cytokines IL-1 β , IL-6, IL-8 and TNF- α were evaluated by qRT-PCR. The results are representative of at least two different experiments

siRAGE treatment significantly reduced the levels of IL-1 β (23%), IL-6 (22%), IL-8 (25%), and TNF- α (52%) mRNA, when compared to NC-treated cells (Fig. 5E). Based on these experiments, we concluded that HMGB1 might exert its biological function through TLR4 and RAGE during PEDV infection in Vero cells.

Discussion

Glycyrrhizin (GLY), the main component of licorice root extracts, inhibits the infection of many viruses. In our studies, we revealed that GLY could moderately inhibit PEDV infection in Vero cells (Fig. 1). It was reported previously that GLY affects porcine reproductive and respiratory syndrome virus (PRRSV) entry [53] and inhibits the replication of SARS-CoV *in vitro* [24]. In our studies, we also demonstrated that GLY inhibited the entry and replication of PEDV, but had no effects on virus assembly and release.

GLY is a competitive inhibitor of high mobility group box1 (HMGB1) that can inhibit the cytokine activity of HMGB1. Our previous studies show that PEDV infection results in the acetylation and release of HMGB1, which would promote the release of proinflammatory cytokines [54]. In this study, we demonstrated that GLY inhibited the increase in proinflammatory cytokines induced by PEDV infection (at the mRNA level) (Fig. 3A, B, C). A similar result was observed in infected cells which were treated with GLY after virus internalization (Fig. 3D).

HMGB1 binding to TLR4 to trigger cytokine release requires the reduced C106, and a disulfide bond between C23 and C45 in HMGB1 [55]. Our studies on HMGB1 mutants (C45S, C106S, and C45S/106S) corroborates that extracellular HMGB1 binding to TLR4 promotes inflammatory responses (Fig. 4), implying that the correct redox

state of HMGB1 is essential for its cytokine activity. We also confirmed the involvement of RAGE in PEDV pathogenesis using a RAGE knockdown experiment (Fig. 5).

Our studies suggest GLY could be used as an immunomodulatory agent against PEDV infection because our *in vitro* experiments showed that PEDV infection results in a significant increase in proinflammatory cytokines, whereas GLY treatment attenuated the production of these cytokines accompanied by a decrease in virus infectivity. An animal experiment shows that suckling pigs and weaned pigs infected by PEDV release a large amount of TNF- α at different time points, while serum IL-8 levels were, remarkably, higher in infected weaned pigs when compared to infected suckling pigs [56]. Although the study did not determine the expression levels of other proinflammatory cytokines, we suspect PEDV infection might cause the un-controlled release of cytokines in pigs. The aberrant release of cytokines has been suggested to play a role in the pathogenesis of diarrhea. Proinflammatory and anti-inflammatory cytokine production locally, or in other organs, induces inflammation and cellular infiltration in to the lamina propria and other layers of the intestinal wall, which subsequently causes diarrhea and finally dehydration [57]. Therefore, we propose that manipulation of the proinflammatory responses by a chemical agent such as GLY will attenuate the severe impact of PEDV infection on animals.

GLY has been shown to protect vital organs against porcine endotoxemia through modulation of systemic inflammatory responses, by reducing the protein and mRNA levels of HMGB1 and other pro-inflammatory cytokines [58]. It is known that administration of large amount of GLY (licorice extract) causes hypokalemia and serious hypertension in both animals and humans [59, 60], but these effects are reversible after GLY withdrawal [61].

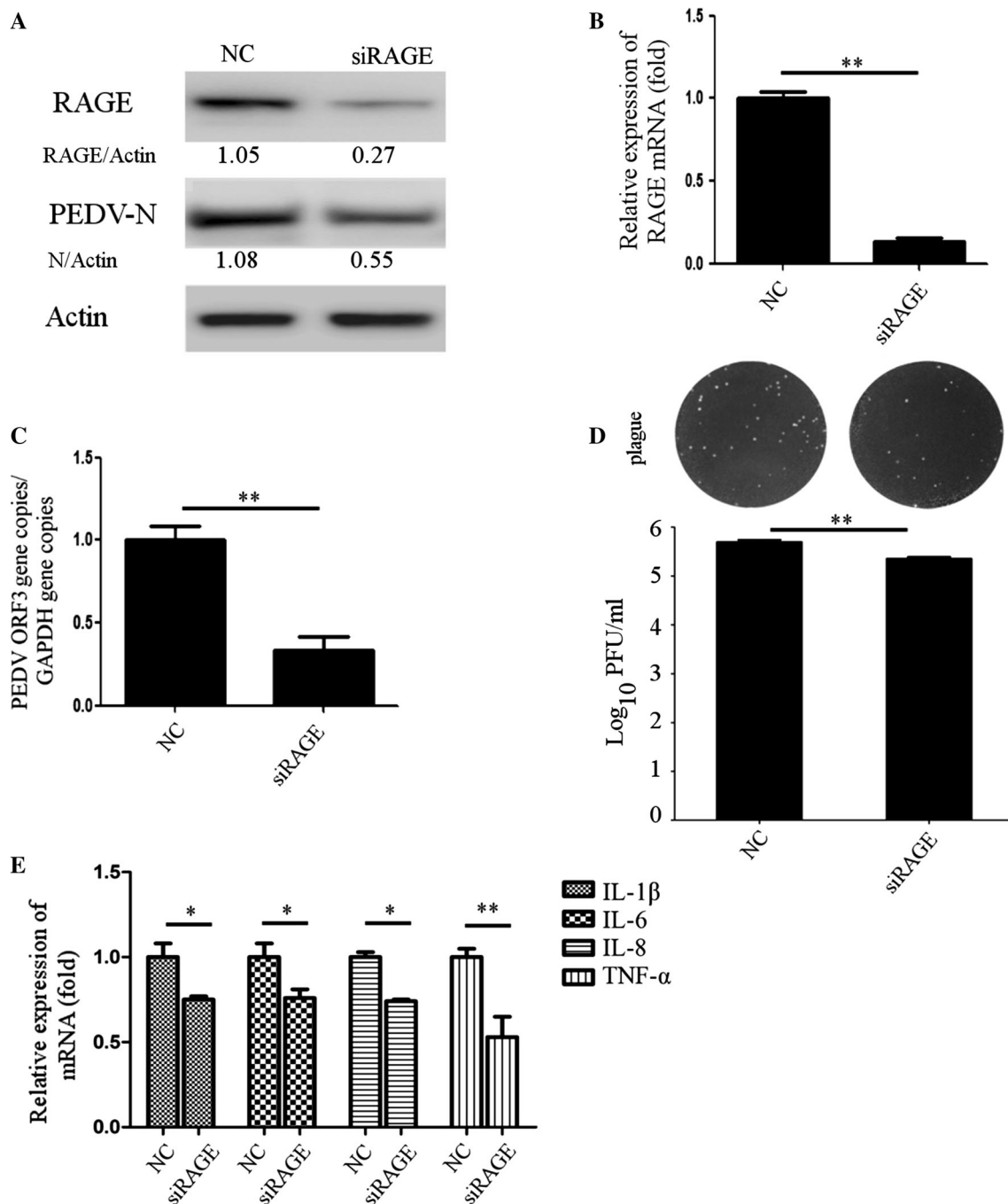


Fig. 5 HMGB1 exerts its biological function during PEDV infection through RAGE. Vero cells were transfected with specific siRNA to knockdown RAGE expression for 24 h. NC was used as the negative control. (A) The RAGE knockdown cells were infected with PEDV for 24 h. The expression of RAGE and PEDV-N were analyzed by western blot. (B, C) The effects of siRAGE on the RNA levels of

RAGE and PEDV ORF3 were measured by qRT-PCR. (D) The virus titer in the supernatant after RAGE knockdown was measured using the plaque formation assay. (E) The effect of siRAGE on the mRNA levels of the proinflammatory cytokines IL-1 β , IL-6, IL-8 and TNF- α was evaluated by qRT-PCR. The results are representative of at least two different experiments

Hence, short-term administration of GLY or its derivatives might not lead to significant harm to animals.

Collectively speaking, our study suggested GLY might be used as an immunomodulatory agent to attenuate the severe clinical symptoms in pigs infected by PEDV.

Compliance with ethical standards

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Conflict of interest All authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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