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Research paper

Chlorine dioxide inhibits the replication of porcine reproductive and respiratory syndrome virus by blocking viral attachment

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) causes a great economic loss to the swine industry globally. Current prevention and treatment measures are not effective to control the outbreak and spread of porcine reproductive and respiratory syndrome (PRRS). In other words, new antiviral strategies are urgently needed. Chlorine dioxide (ClO_2) is regarded as a broad-spectrum disinfectant with strong inhibitory effects on microbes and parasites. The purpose of this study was to evaluate the inhibitory effects and underlying molecular mechanisms of ClO_2 against PRRSV infection in vitro. Here, we identified ClO_2 (the purity is 99%) could inhibit the infection and replication of PRRSV in both Marc-145 cells and porcine alveolar macrophages (PAMs). ClO_2 could block PRRSV binding to cells rather than internalization and release, suggesting that ClO_2 blocks the first stage of the virus life cycle. We also demonstrated that the inhibition exerted by ClO_2 was attributed to the degradation of PRRSV genome and proteins. Moreover, we confirmed that ClO_2 could decrease the expression of inflammatory cytokines induced by PRRSV. In summary, ClO_2 is an efficient agent and potently suppressed PRRSV infection in vitro.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most important pathogens that severely impacts swine industry worldwide (Yun and Lee, 2013). Porcine reproductive and respiratory syndrome (PRRS), caused by PRRSV, is an acute viral infectious disease. PRRS is characterized by reproductive failure in pregnant sows and respiratory diseases in pigs of all ages, which causes huge economic losses every year (Gomez-Laguna et al., 2013; Lunney et al., 2010). PRRSV is a member of the genus *Porartevirus* of the family *Arteriviridae* of the order *Nidovirales*, PRRS-1 (type 1 or European PRRSV) and PRRS-2 (type 2 or American PRRSV) are the two species in this genus (Guo et al., 2018; King et al., 2018). It is an enveloped, single-stranded, positive-sense RNA virus, which has an approximately 15 kb genome encoding at least 11 open reading frames (ORF). ORF1a and ORF1b accounting for about 75% of the full-length genome encode the non-structural proteins (NSP), which mainly act as RNA replicase and RNA polymerase (Fang and Snijder, 2010; Li et al., 2015); ORF2a, ORF2b, ORF3 and ORF4 encode the membrane glycoprotein GP2, GP3 and GP4, which are the secondary structural proteins of virus; ORF5, ORF6 and ORF7 encode glycoprotein GP5, matrix protein M and the nucleocapsid protein N, respectively, which are relevant to viral entry and immunogenicity (Doan and Dokland, 2003; Dokland, 2010; Sarah K. Wootton et al., 1998; Yang et al., 2013; Zhang and Yoo, 2015). PRRSV can cause immunosuppression and induce persistent infection which has resulted in the difficulty of prevention and control of the disease (Kimman et al., 2009). Previous studies have reported that many natural compounds, microRNAs and new vaccinations can control PRRSV infection (Du et al., 2017a; Du et al., 2017b; Liu et al., 2017; Vu et al., 2017). However, there is limited protection against PRRSV infection even though current vaccination strategies were widely used. Therefore, developing a safe and effective antiviral strategy is needed.

Chlorine dioxide (ClO_2) is a strong oxidizing agent with a broad range of biological activity against bacteria, virus, fungi and parasites (Sigstam et al., 2014). ClO_2 has more effective disinfection potential and produces fewer harmful by-products than other chloride (Dodd, 2012). ClO_2 is regarded as a potent and suitable disinfectant widely used in recent years. Many studies have certified that several viruses were inactivated effectively by the chlorination of ClO_2, including human rotavirus (HRV) (Chen and Vaughn, 1990), human norovirus (HNoV) (Lim et al., 2010; Montazeri et al., 2017), feline calicivirus (FCV) (Montazeri et al., 2017), enterovirus71 (EV71) (Jin et al., 2013),

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poliovirus 1 (PV1) (Simonet and Gantzer, 2006) and echovirus 11 (E11) (Zhong et al., 2017). However, the mechanism that the inactivation of PRRSV by ClO_2 has not been reported.

In this study, we explored the antiviral effects of ClO_2 against PRRSV infection in vitro. We found that PRRSV infection and replication was effectively inhibited by ClO_2. ClO_2 restrained PRRSV infection through blocking PRRSV binding to cells. Meanwhile, ClO_2 exerted extracellular antiviral activity against PRRSV as a result of the destruction of viral genome and proteins. Taken together, ClO_2 is of high value in clinical applications, which could be helpful for the antiviral therapies against PRRSV infection.

2. Materials and methods

2.1. Cells and viruses

Marc-145 cells (China Center for Type Culture Collection, China), an immortalized cell line derived from African green monkey kidney cells, were cultured in Dulbecco's modified Eagle's medium (DMEM) (Corning, USA) containing 10% fetal bovine serum (FBS) (PAN, Germany), which were permissive to PRRSV replication and commonly used in laboratories. Porcine alveolar macrophages (PAMs) were isolated from broncho-alveolar lavage fluid of three six-week-old PRRSV-negative pigs. Lungs were removed from thoracic cavity and washed the surface clean with phosphate buffer solution (PBS, Corning, USA). Lungs were lavaged with PBS and were gently massaged for three times. Cells were collected from bronchoalveolar lavage fluid (BALF) by centrifugation for 10 min at 1000 rpm, and then resuspended with RPMI-1640 medium (Gibco, USA) and centrifuged for 5 min at 1000 rpm. Finally, cells were frozen in 40% RPMI-1640 medium, 50% FBS, and 10% DMSO (Sigma, USA). PAMs were maintained in RPMI 1640 medium with 10% FBS at 37 °C in 5% CO_2. No antibiotic was used in the culture during the experiments. All animal experiments were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University.

Three PRRSV strains, CHR6 (Classical North American type PRRSV strain) and Li11 (a highly pathogenic PRRSV isolate), were provided by Dr. Heng Wang from South China Agricultural University, and PRRSV-EGFP, a recombinant virus shows growth replication characteristics similar to those of the wild-type virus in the infected cells, was provided by Dr. Shuqi Xiao from Northwest A&F University (Wang et al., 2013). CHR6 and PRRSV-EGFP were used to infect Marc-145 cells, while PAMs were just infected with CHR6 in our research, because of the low infectivity of PRRSV-EGFP on PAMs. The three virus strains were propagated in Marc-145 cells and titrated as 50% tissue culture infective dose ($TCID_{50}$).

2.2. Cytotoxicity assay

The cytotoxicity of ClO_2 was detected with the alamarBlue® assay (Invitrogen, USA) according to the manufacturer's instructions. Marc-145 cells (1×10^4/well) and PAMs (1×10^5/well) were seeded in 96-well plates respectively. Different concentrations of ClO_2 were added in DMEM and RPMI 1640 medium when cells grew to 60%–70% confluence. After incubation for 48 h in Marc-145 cells and PAMs, 10 μL of alamarBlue® was added to each well, and incubated for another 3 h. At last, the fluorescence value was detected using Multi-Mode Reader (Synergy2, BioTek, USA) at the absorbance of 570 nm.

2.3. Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)

In order to detect the relative expression of PRRSV ORF7 and cytokines, qRT-PCR should be used. Total RNA was extracted from cultured cells using TRIzol reagent (Magen, China). Reverse Transcription System (A3500, Promega, USA) were used for reverse transcription in 20 μL reaction volume following the manufacturer's instructions. The Reverse-transcription primers were Oligo (dT) 15 primer (C110A, Promega, USA) and Random primer (C118A, Promega, USA). Reverse-transcription products were amplified by a LightCycler 480 Real-Time PCR System (LC480, Roche, Switzerland) using 2 × RealStar Green Power Mixture (GenStar, China). The primers used for qRT-PCR are listed in Table 1. qRT-PCR reaction system was run under the following conditions: 95 °C for 10 min, then 95 °C for 15 s, 60 °C for 1 min and 72 °C for 30 s went through 40 cycles, finally 72 °C for 10 min. Data were normalized to GAPDH in each individual sample. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative expression changes. Relative expression (fold changes) was compared to mock infected cells.

Table 1
List of primers for RT-PCR.

Primer[a]	Sequence (5′–3′)[b]
N-F	AAAACCAGTCCAGAGGCAAG
N-R	CGGATCAGACGCACAGTATG
mGAPDH-F	TGACAACAGCCTCAAGATCG
mGAPDH-R	GTCTTCTGGGTGGCAGT GAT
mIL-6-F	AGAGGCACTGGCAGAAAAC
mIL-6-R	TGCAGGAACTGGATCAGGAC
mTNF-α-F	TCTGTCTGCTGCACTTTGGAGTGA
mTNF-α-R	TTGAGGGTTTGCTACAACATGGGC
mIFN-β-F	GCAATTGAATGGAAGGCTTGA
mIFN-β-R	CAGCGTCCTCCTTCTGGAACT
ORF2a-F	ATGAAATGGGGTCTATGCAAAG
ORF2a-R	TCACCATGAGTTCAAAAGAAAAGTT
ORF3-F	ATGGCTAATAGCTGTACATTCC
ORF3-R	TCGCCGTGCGGCACTG
ORF4-F	ATGGCTGCGTCCTTTCTTTTCCTCT
ORF4-R	AAGCACTTCCCCAACATACTTGAAC
ORF5-F	TGCTTCYGAGATGAGTGAAA
ORF5-R	CAAAAGGTGCAGAAGCCCTA
ORF6-F	ATGGGGTCGTCTCTAGATG
ORF6-R	TTTGGCATATTTAGCAAGG
ORF7-F	ATGCCAAATAACAACGGC
ORF7-R	TGCTGAGGGTGATGCTGT

[a] F, forward primer; R, reverse primer. The letter “m” indicates that it is for a monkey gene.
[b] Green monkey gene sequences and PRRSV gene sequences were downloaded from GenBank.

2.4. Western blot

Six-well-plate cell samples (2×10^6/well) were harvested in cell lysis buffer (Beyotime, China) containing PMSF (Beyotime, China). Processed samples was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinyl difluoride (PVDF) membrane (Roche, USA). Then the membranes were blocked with 3% BSA (Ruishu, China) in TBST (20 mM Tris-HCl PH8.0, 150 mM NaCl, 0.05% Tween 20) for 1 h at 37 °C. After blocking they were incubated with an anti-PRRSV N protein monoclonal antibody (1:1000 dilution, Jeno Biotech, Inc., Republic of Korea), anti-glyceraldehyde phosphate dehydrogenase (GAPDH) antibody (1:1000 dilution, Cell Signaling Technology, USA) overnight at 4 °C. After washing four times with TBST buffer, membranes were incubated with anti-rabbit IgG, HRP-linked antibody or anti-rabbit IgG, HRP-linked antibody (1:1000 dilution, Cell Signaling Technology, USA) for 1 h at 37 °C. The antibody signals were exposed using a chemiluminescence (ECL) reagent (Fdbio Science, China).

2.5. Flow cytometry (FCM)

We used flow cytometry to detect the expression of PRRSV-EGFP, which indicated the viral replication. PRRSV-EGFP-infected cells were harvested and resuspended in PBS at a concentration of 1×10^6 cells/ml. Flow cytometry analyzed the ratio of GFP-positive cells and

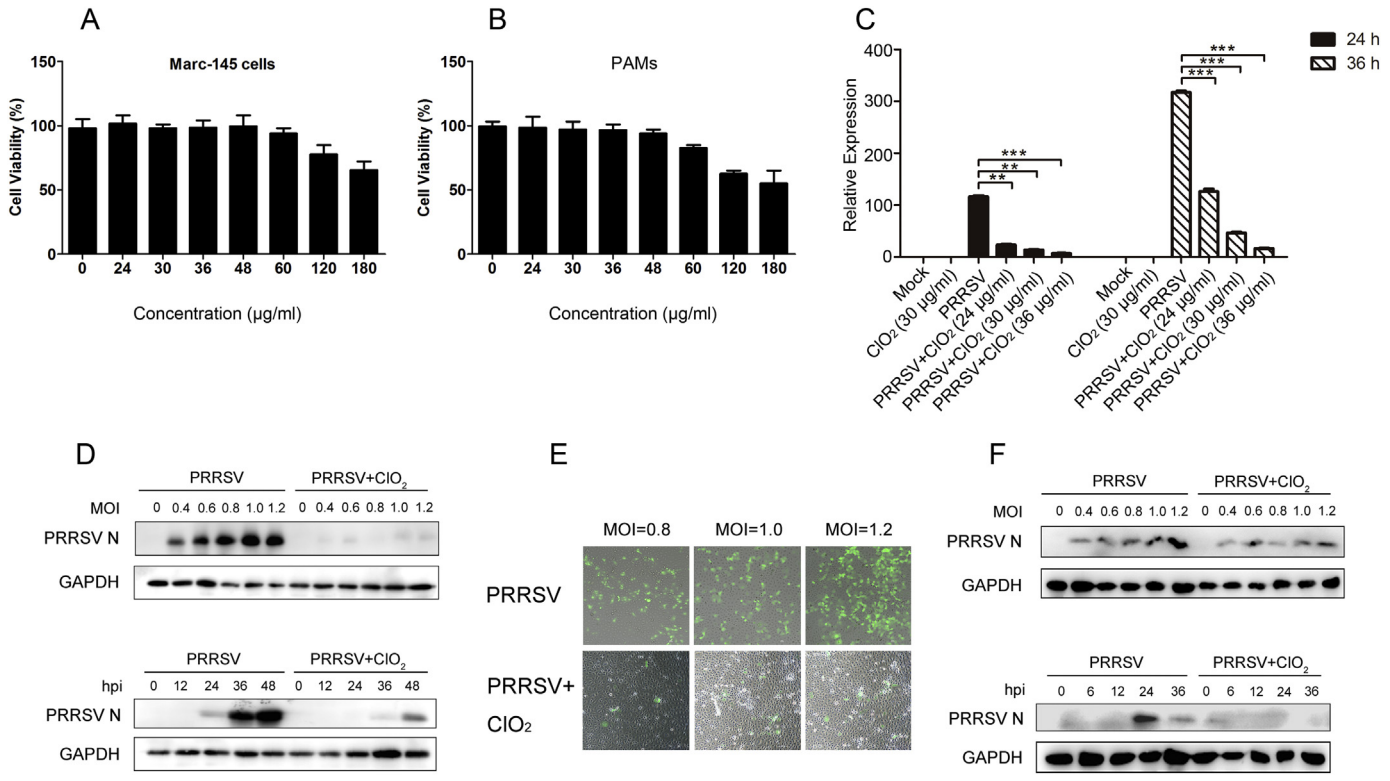


Fig. 1. ClO_2 effectively suppresses the infection and replication of PRRSV both in Marc-145 cells and PAMs. (A) The cytotoxicity of ClO_2 was measured by the alamarBlue assay. Marc-145 cells were treated with ClO_2 at indicated concentrations for 48 h and cell viability assay was performed. (B) The cytotoxicity of ClO_2 on PAMs was also measured using alamarBlue assay. (C) Marc-145 cells were mock infected or infected with PRRSV-EGFP (MOI = 0.6) in the presence of different concentrations of ClO_2 (0, 24, 30, and 36 μg/mL) for 24 or 36 h. The expression of viral ORF7 (PRRSV N) was detected by qRT-PCR. (D and E) Marc-145 cells were infected with PRRSV-EGFP at different MOIs in the presence (30 μg/mL) or absence of ClO_2. N protein was determined by Western blot analysis (D) and GFP fluorescence of PRRSV was detected by immunofluorescence microscopy (E). (F) The same treatments were performed in porcine alveolar macrophages (PAMs), and Western blot was used to analyze the viral N protein at different MOIs and different infection periods. Data are representative of the results of three independent experiments (means ± SE). Significant differences compared with control group are denoted by * ($P < .05$), ** ($P < .01$) and *** ($P < .001$).

fluorescence intensity, which represents the viral load. Typically, 10,000 labeled cells were acquired using a FACSCalibur (BD Bioscience, USA) and analyzed by FlowJo software.

2.6. Antiviral assay

Cells were seeded in six-well plates and grown to 70%–80% confluence. There were four approaches to analyze the antiviral effects of ClO_2. (I) Virucidal assay: PRRSV-EGFP (MOI = 0.6) was pre-incubated with different concentrations of ClO_2 (0, 24, 30, and 36 μg/mL) for 2.5 h at 37 °C. The mixtures were then added to Marc-145 cells. After incubation for 4 h at 37 °C, the mixtures were removed. Cells were supplied with fresh DMEM containing 2% FBS and cultured for another 36 h. The supernatants were collected to detect viral titers. The cells were harvested for flow cytometry and immunoblotting analysis. (II) Pre-treatment: Cells were treated with different concentrations of ClO_2 (0, 36 and 48 μg/mL) for 4 h, PRRSV-EGFP was then added and cultured for 36 h. (III) Co-treatment: Various concentrations of ClO_2 (0, 36 and 48 μg/mL) were added to cells and cultured with PRRSV-EGFP together for 36 h. (IV) Post-treatment: Cells were inoculated with PRRSV-EGFP for 8 h, then the inoculum was removed and ClO_2 (0, 36 and 48 μg/mL) was added to cells for another 36 h at 37 °C. In all above antiviral assays, the supernatants were collected to detect viral titers. In order to detect viral load and the expression of PRRSV capsid protein, cells were harvested for flow cytometry and immunoblotting analysis.

2.7. Viral attachment, entry and release assays

For attachment assay, cells were cooled for 2 h at 4 °C, and then infected with PRRSV-EGFP (MOI = 0.6) in the presence of different concentrations of ClO_2 (0, 24, 30, and 36 μg/mL) for 4 h at 4 °C. After rinsing with cold PBS for three times, cells were replenished with fresh DMEM containing 2% FBS for 36 h at 37 °C. The supernatants were harvested and titrated as $TCID_{50}$ and the cells were collected for flow cytometry and Western blot analysis so that we could determine the effects of ClO_2 on viral attachment.

As for entry assay, cells were inoculated with PRRSV-EGFP (MOI = 0.6) for 4 h at 4 °C. After binding to cell surface, cells were washed with PBS three times and cultured at 37 °C for 6 h in the presence of various concentrations of ClO_2 (0, 24, 30, and 36 μg/mL). When the temperature of culture medium switched to 37 °C, ClO_2 was added to the cells at 0, 2, or 4 h (The moment that the temperature shift to 37 °C was regarded as 0 h). After incubation for total 6 h, cells were washed with PBS and incubated for another 36 h at 37 °C. Cells were collected to detect the viral burden by using flow cytometry and the supernatants were harvested and titrated as $TCID_{50}$ that implied virus infectivity.

For release assay, cells were incubated with PRRSV-EGFP (MOI = 0.6) for 36 h at 37 °C. After that, cells were rinsed with PBS three times and ClO_2 at different concentrations was added to the cells for 6 h at 37 °C. At last, cells were collected for flow cytometry and Western blot analysis to detect the viral load and PRRSV N protein expression, respectively.

2.8. RT-PCR amplification of ORFs of viral genome

Viral RNA were abstracted using RaPure Viral RNA/DNA Kit (Magen, China). Titanium® One-Step RT-PCR Kit (TaKaRa, Japan) was

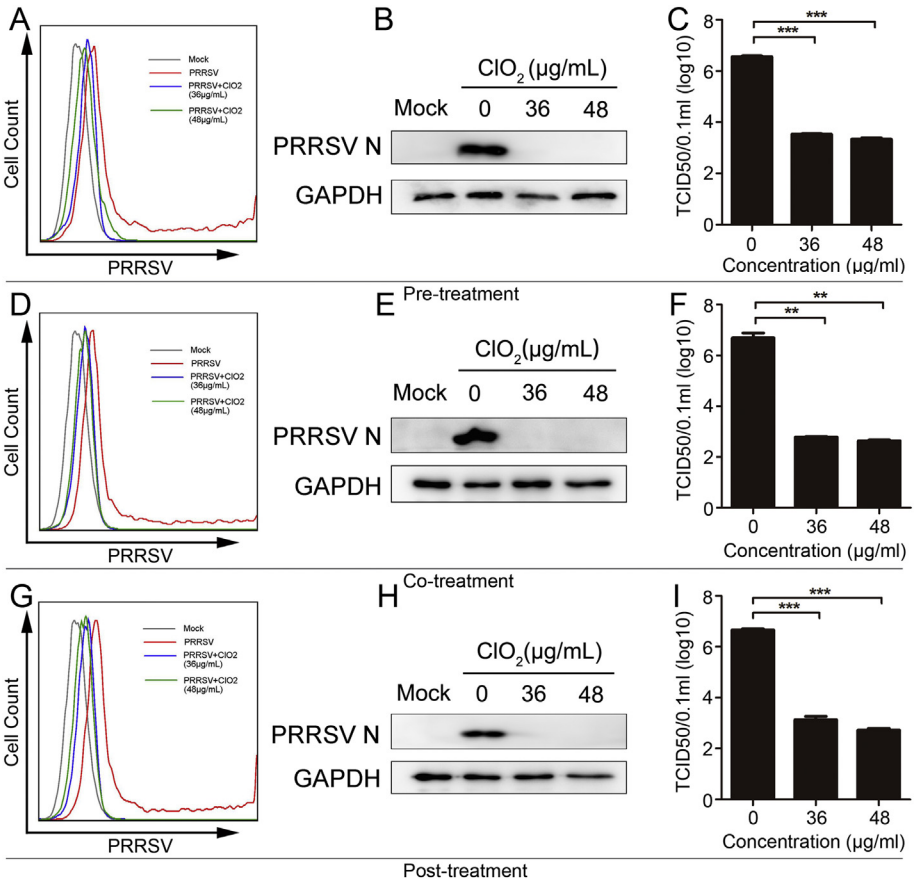


Fig. 2. Viral protein translation and PRRSV production were inhibited by ClO_2 treatment. (A–C) Marc-145 cells were pre-treated with different concentrations of ClO_2 (0, 36 and 48 μg/mL) for 4 h, then inoculated with PRRSV-EGFP at an MOI of 0.6 for 36 h, PRRSV-EGFP were detected by flow cytometry analysis (A). The protein levels of PRRSV N were determined by Western blot analysis (B). The supernatants were collected to detect viral titers (C). (D–F) Various concentrations of ClO_2 (0, 36 and 48 μg/mL) and PRRSV at an MOI of 0.6 were added to Marc-145 cells for 36 h. Cells were harvested for flow cytometry analysis (D) and immunoblotting analysis (E), and viral yields were detected in the supernatants (F). (G–I) Marc-145 cells were mock infected or infected with PRRSV-EGFP at an MOI of 0.6 for 8 h, and then ClO_2 (0, 36 and 48 μg/mL) was added. After incubation for another 36 h, PRRSV-EGFP was detected by flow cytometry (G), PRRSV N protein was determined by Western blot (H). Simultaneously, the supernatants were harvested to measure viral titers as $TCID_{50}$ (I). Data are representative of the results of three independent experiments (means ± SE). Significant differences compared with control group are denoted by * ($P < .05$), ** ($P < .01$) and *** ($P < .001$).

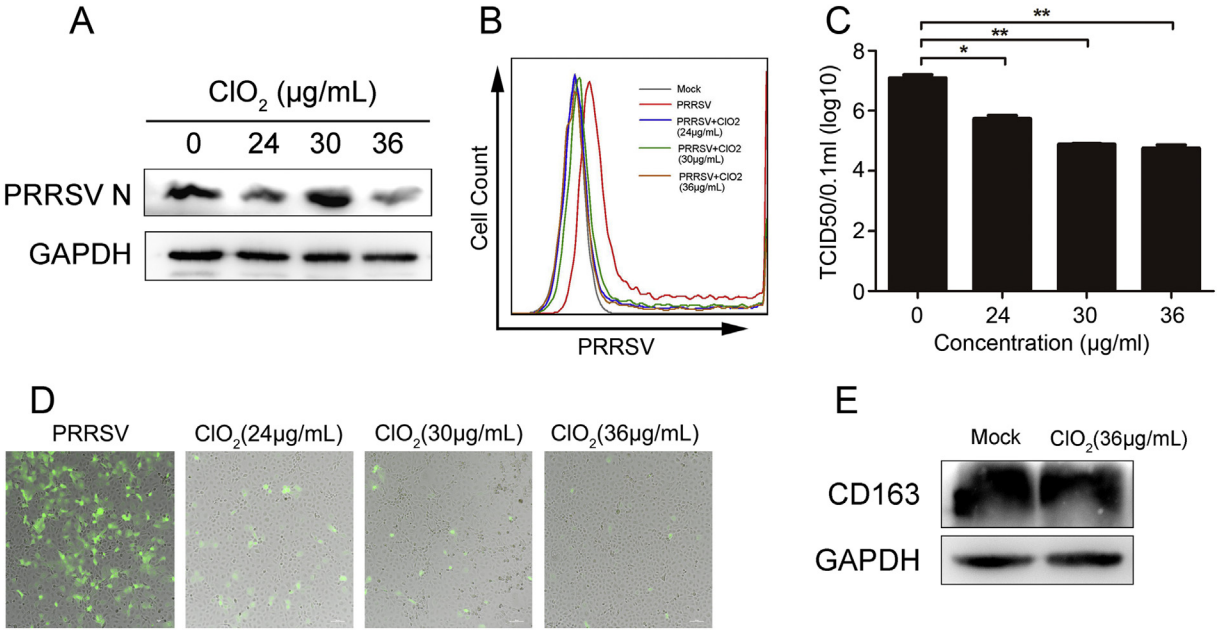


Fig. 3. Analysis of ClO_2 treatment effects on PRRSV attachment to Marc-145 cells. (A–D) Marc-145 cells were incubated with PRRSV-EGFP at an MOI of 0.6 in the presence of different concentrations of ClO_2 (0, 24, 30, and 36 μg/mL) for 4 h at 4 °C. After removing the unbound viral particles, cells were replenished with fresh DMEM and switched to 37 °C for 36 h. The expression of the viral N protein was detected by Western blot (A). Viral replication were determined by flow cytometry analysis (B) and viral production was measured and shown as $TCID_{50}$ (C). The fluorescence of PRRSV-EGFP was visual using immunofluorescence microscopy (Bar, 100 μm) (D). The expression of CD163 receptor in PAMs was detected by western blot in the absence and presence of ClO_2 (36 μg/mL) (E). Data are representative of the results of three independent experiments (means ± SE). Significant differences compared with the control group are denoted by * ($P < .05$), ** ($P < .01$) and *** ($P < .001$).

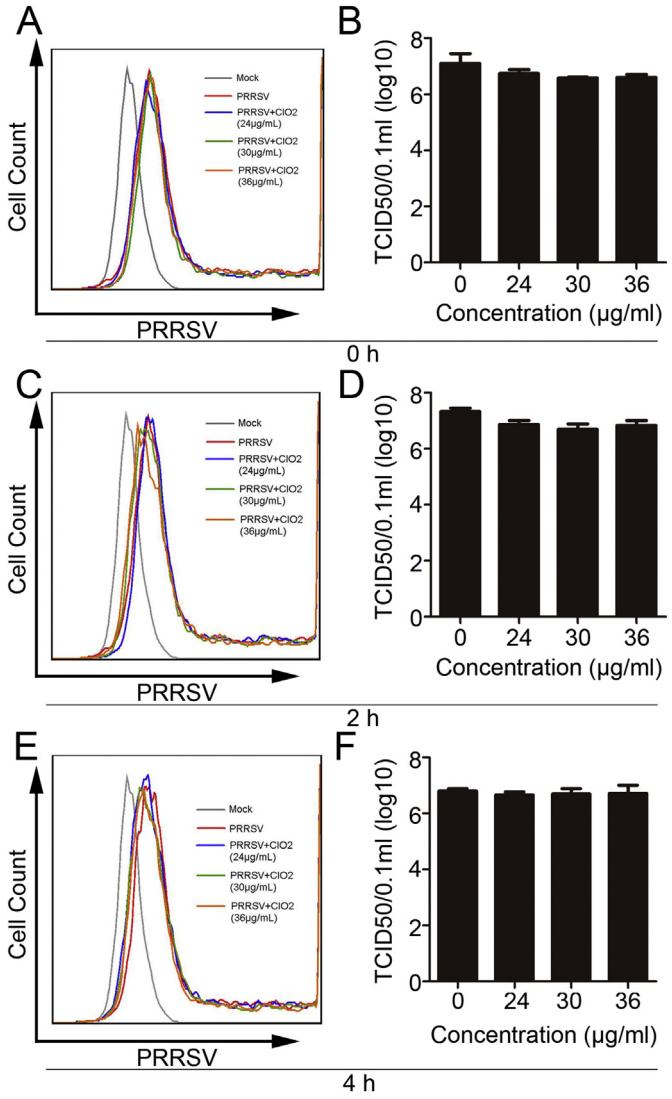


Fig. 4. Effects of ClO_2 treatment on PRRSV entry. (A–F) Marc-145 cells were inoculated with PRRSV-EGFP at an MOI of 0.6 for 4 h at 4 °C, unbounded virus particles were removed, ClO_2 (0, 24, 30, 36 μg/mL) was added accordingly at 0, 2, or 4 h after switching to 37 °C in a time-of-addition manner. After incubation for total 6 h, cells were washed with PBS and incubated for another 36 h. At the indicated time 0 (A and B), 2 (C and D) or 4 h (E and F), cells were harvested. Positive cells infected with PRRSV-EGFP were tested by flow cytometry (A, C and E). Cultured supernatants were also collected to detect viral titers (B, D and F). Data are representative of the results of three independent experiments (means ± SE). Significant differences that PRRSV infected cells in the presence and absence of ClO_2 are denoted by * ($P < .05$), ** ($P < .01$) and *** ($P < .001$).

used for RNA reverse transcription. The primers of ORFs are listed in Table 1. The cycler program was consisted with 94 °C for 4 min, then 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min using 2 × SuperTaq PCR Mix with Loading Dye (GenStar, China).

2.9. Statistical analysis

All experiments were performed with at least three independent replicates. Student's *t*-test and one-way ANOVA were used to analyze the data. Statistical analysis was performed using SPSS 17.0 and GraphPad Prism 5.0. $P < .05$ was considered to be significant.

3. Results

3.1. ClO_2 effectively suppresses the replication of PRRSV

To identify the antiviral activity of ClO_2 against PRRSV, we first examined whether ClO_2 can inhibit the replication of PRRSV on Marc-145 cells. Based on a cytotoxicity assay, concentrations of ClO_2 that were non-cytotoxic were lower than 60 μg/mL in Marc-145 cells after treatment for 48 h (Fig. 1A). PAMs cultured in medium containing 48 μg/mL ClO_2 retained approximately relative viability of 100% compared with controls after treatment for 48 h (Fig. 1B). Marc-145 cells were mock infected or infected with PRRSV-EGFP (MOI = 0.6) in the presence of different concentrations of ClO_2 (0, 24, 30, and 36 μg/mL) for 24 or 36 h. The mRNA relative expression of the viral ORF7 was reduced compared with the control group that PRRSV infected Marc-145 cells without ClO_2, which also exhibits its inhibiting effects in a dose-dependent manner (Fig. 1C). Next we investigated its effects on PRRSV infection, different MOIs of PRRSV-EGFP (at MOIs of 0.4, 0.6, 0.8, 1.0 or 1.2) were used to infect Marc-145 cells in the absence or presence of ClO_2 (30 μg/mL). As shown in Fig. 1D and E, ClO_2 significantly inhibited the replication of PRRSV regardless of different multiplicity of infections. Moreover, ClO_2 suppressed PRRSV infection in a time-dependent manner when we evaluated its effects on PRRSV (MOI = 0.6) at 12, 24, 36, and 48 h post-infection (hpi) (Fig. 1D). To investigate whether the same antiviral effects can be observed on PAMs, which are susceptible cells of PRRSV in vivo, we did the same experiments with CHR6 strain in PAMs. Consistent with the findings obtained with Marc-145 cells, ClO_2 showed a potent antiviral activity against PRRSV in dose- and time-dependent manners (Fig. 1F).

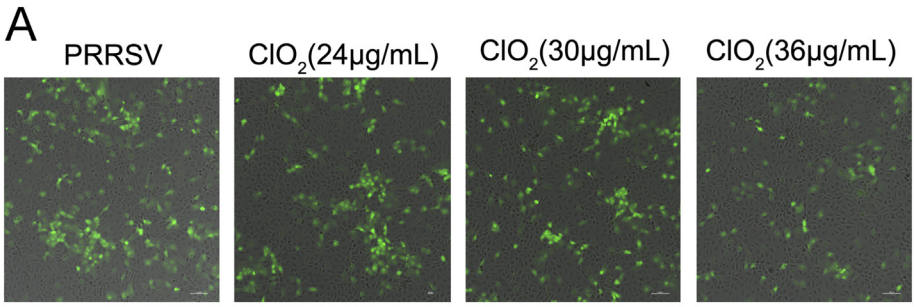
3.2. Viral protein translation and PRRSV production were inhibited by ClO_2 treatment

To further demonstrate that ClO_2 has great ability to influence the infection of PRRSV, the effects of treatment with ClO_2 were analyzed with three different approaches as described in the materials and methods. Compared with control group, the virus titers and viral load were significantly decreased following the pre- or co-treatment of ClO_2 in Marc-145 cells infected with PRRSV (Fig. 2A, C, D and F). The N protein of PRRSV were also reduced by the pre- or co-treatment of ClO_2 (Fig. 2B and E). Consistent with the findings obtained with pre-treatment and co-treatment methods, the expression of PRRSV and viral titers were decreased by ClO_2 when administered with post-treatment in Marc-145 cells (Fig. 2G–I). Taken together, ClO_2 exerts the efficiency on the suppression of PRRSV infection in Marc-145 cells.

3.3. ClO_2 blocks PRRSV binding to cell surface

We have identified that ClO_2 can inhibit the replication of PRRSV, but the underlying molecular mechanism that the antiviral activity of ClO_2 against PRRSV remains unknown. Virus invasion mainly has 4 processes: binding, entry, replication and release. To characterize which stage of the viral life cycle is interrupted by ClO_2, an attachment assay was initially used to test whether ClO_2 can block PRRSV binding to Marc-145 cells. PRRSV infected Marc-145 cells with absence of ClO_2 regarded as control group. As shown in Fig. 3A, ClO_2 significantly reduced the N protein level of PRRSV during the period of viral attachment. The number of PRRSV-EGFP-infected cells, fluorescent yield and viral titer were all decreased in ClO_2-treated cells as well (Fig. 3B, C, and D). To identify whether ClO_2 could destroy the cell receptors, we also detected the expression of CD163 receptor in PAMs, which was the most important receptor for PRRSV infection. As shown in Fig. 3E, ClO_2 had no effect on the expression of CD163 receptor. These data suggest that ClO_2 is able to observably reduce the virus particles attaching to the receptors of cell surface.

Next we investigated whether ClO_2 can block viral entry. We added



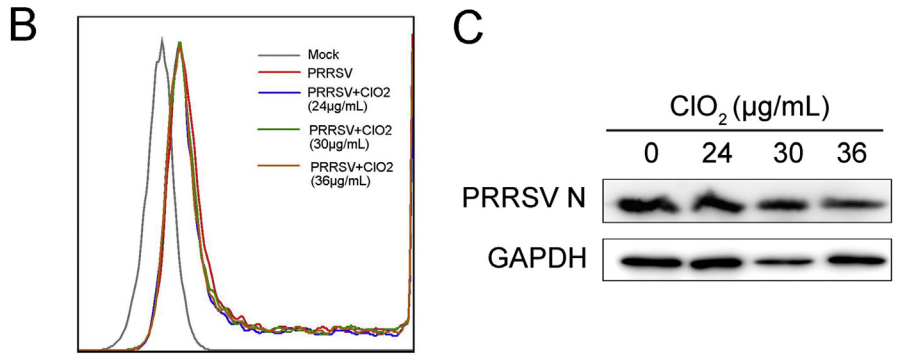


Fig. 5. ClO_2 has no influence on the release of PRRSV. (A–C) Cells were incubated with PRRSV-EGFP at an MOI of 0.6 for 36 h at 37 °C. After that, ClO_2 at different concentrations (0, 24, 30, and 36 μg/mL) was added to the cells for 6 h. Cells were harvested to identify viral load using immunofluorescence analysis (Bar, 100 μm) (A) and flow cytometry analysis (B) respectively. PRRSV N protein levels were detected by Western blot (C). Data are representative of the results of three independent experiments (means ± SE). Significant differences compared with control group are denoted by * ($P < .05$), ** ($P < .01$) and *** ($P < .001$).

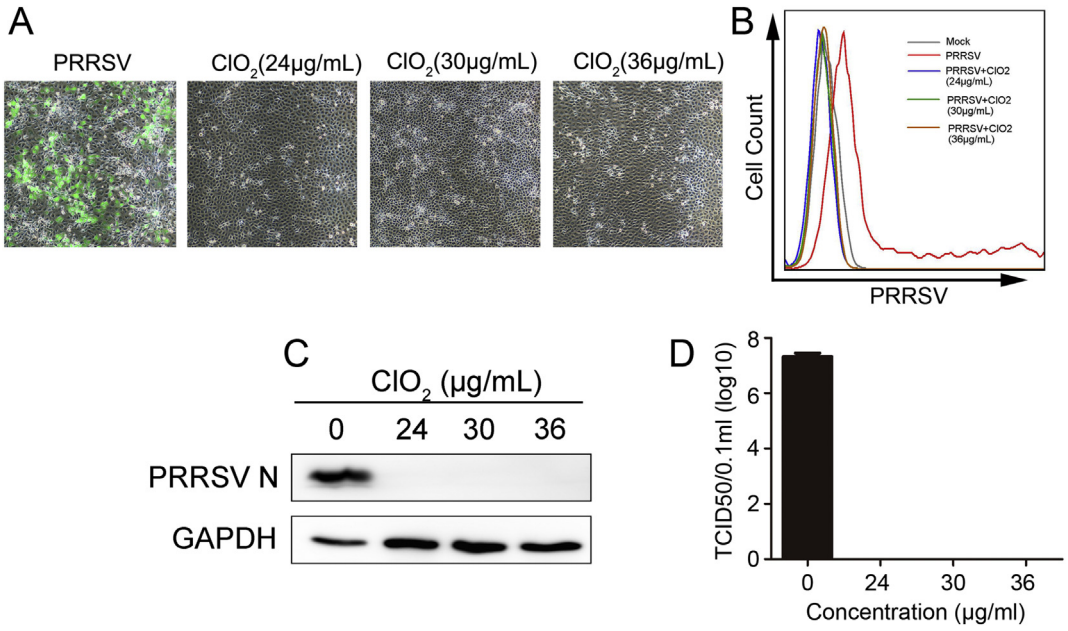


Fig. 6. ClO_2 displays extracellular virucidal activity against PRRSV. (A–D) PRRSV-EGFP was pre-incubated with different concentrations of ClO_2 (0, 24, 30, and 36 μg/mL) for 2.5 h at 37 °C. The mixtures was added to Marc-145 cells for 4 h. Afterwards, cells were supplied with fresh DMEM and cultured for another 36 h. PRRSV-EGFP were detected by immunofluorescence microscopy (Bar, 100 μm) (A). Flow cytometry was used to detect PRRSV-EGFP positive cells (B) and the expression of viral N protein was determined by Western blot (C). The virus titers ($TCID_{50}$) were also analyzed (D). The data represent the means ± SE from three independent experiments (*, $P < .05$; **, $P < .01$; ***, $P < .001$; ND means non-detectable).

ClO_2 to the infected Marc-145 cells at the entry stage of the viral life cycle. As shown in Fig. 4, there was no significant change in viral titer and viral fluorescence intensity when ClO_2 was added at 0 (Fig. 4A and B), 2 (Fig. 4C and D) or 4 h (Fig. 4E and F) after the medium temperature shifting to 37 °C, indicating that ClO_2 has little effect during the period of viral entry.

Finally, we examined the effects of ClO_2 on the release process of PRRSV life cycle in Marc-145 cells. As shown in Fig. 5, there was no effect on the GFP fluorescence of PRRSV (Fig. 5A and B). ClO_2 could not reduce the N protein of PRRSV during the stage of viral release (Fig. 5C). Taken together, ClO_2 blocks PRRSV binding to cell membrane rather than the processes of entry and release in Marc-145 cells.

3.4. ClO_2 displays extracellular antiviral activity against PRRSV

ClO_2 acts as a new broad spectrum high effect disinfectant, which has strong inactivated effect on viruses, bacteria, fungi and mycotic spores (Sigstam et al., 2014). To investigate the direct inactivation effect of ClO_2 against PRRSV, a virucidal assay described in the materials and methods was performed on Marc-145 cells. PRRSV pre-incubated with absence of ClO_2 regarded as control group. We could not find any viral fluorescence in PRRSV-infected cells with ClO_2 treatment using

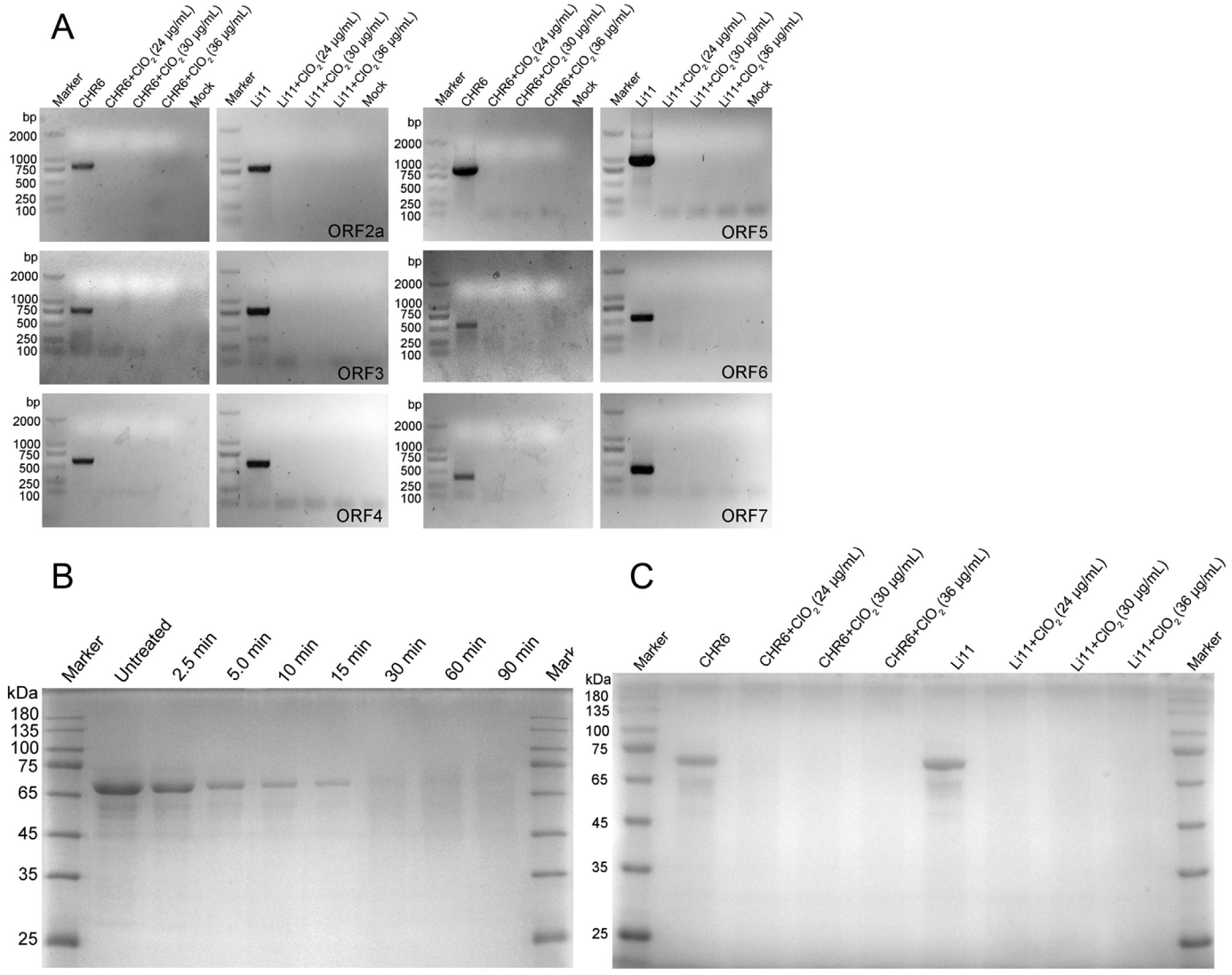


Fig. 7. The inactivation of PRRSV by ClO_2 is mainly driven by genome and protein damage. (A–C) CHR6 or Li11 were incubated with different concentrations of ClO_2 (0, 24, 30, and 36 μg/mL) for 2 h. ORF2a (771 bp), ORF3 (762 bp), ORF4 (564 bp), ORF5 (914 bp), ORF6 (522 bp), and ORF7 (369 bp) of CHR6 and Li11 were amplified using RT-PCR (A). The degradation process of CHR6 proteins at the indicated times after treatment with ClO_2 was examined (B). Meanwhile, the mixtures of CHR6, Li11 and ClO_2 were subjected to SDS-PAGE analysis (C). Data are representative of the results of three independent experiments.

fluorescence microscope (Fig. 6A). On the contrary, there was full of GFP fluorescence which represents viral burden in visual field following the treatment of PRRSV only (Fig. 6A). Compared to PRRSV control group, the amount of GFP fluorescence were significantly reduced by the treatment of ClO_2 (Fig. 6B). Consistently, N protein and viral titers were undetectable in cells treated with ClO_2 (Fig. 6C and D). All these data indicate that PRRSV is completely inactivated by the treatment of ClO_2 in Marc-145 cells.

3.5. The inactivation of PRRSV by ClO_2 is mainly driven by genome and protein damage

We demonstrated that ClO_2 inhibits PRRSV replication, but how it works is still unknown. Nucleocapsid is the basic structure of the virus, which contains nucleic acids and proteins (Doan and Dokland, 2003; Dokland, 2010). To investigate whether ClO_2 destroys the structure of proteins and genome, ClO_2 at the indicated concentrations (0, 24, 30, 36 μg/mL) was incubated with classical strain CHR6 or highly pathogenic strain Li11 for 2 h at 37 °C, and then the mixtures were harvested for viral proteins and ORFs analysis by SDS-PAGE (Coomassie brilliant blue staining) and RT-PCR respectively. As shown in Fig. 7A, RNA fragments of ORF2a, ORF3, ORF4, ORF5, ORF6, ORF7 were all undetectable when CHR6 and Li11 were treated with ClO_2 (0, 24, 30, 36 μg/mL), suggesting that ClO_2 destroys the structure of viral genome. Next, we explored the dynamic change of viral proteins at the indicated times after treatment with ClO_2. The degradable functions of ClO_2 on CHR6 worked in a time-dependent manner. At the time of 5 min, viral proteins started to break down and PRRSV was absolutely degraded at 30 min (Fig. 7B). Moreover, the structural proteins were degraded both in CHR6 and Li11 (Fig. 7C). These data demonstrate that ClO_2 can degrade the structural proteins of PRRSV and destroy viral genome resulting in the inactivation of PRRSV.

3.6. ClO_2 decreases the level of inflammatory cytokines induced by PRRSV

PRRSV causes acute interstitial pneumonia after infection because of the accumulation of a large number of inflammatory factors (Thanawongnuwech et al., 2004). To investigate the effect of ClO_2 on the expression of cytokines in Marc-145 cells, Marc-145 cells were mock infected or infected with CHR6 (MOI = 0.1) in the presence of different concentrations of ClO_2 (0, 24, 30, and 36 μg/mL) for 24 or 36 h. qRT-PCR was conducted to evaluate the expression of pro-

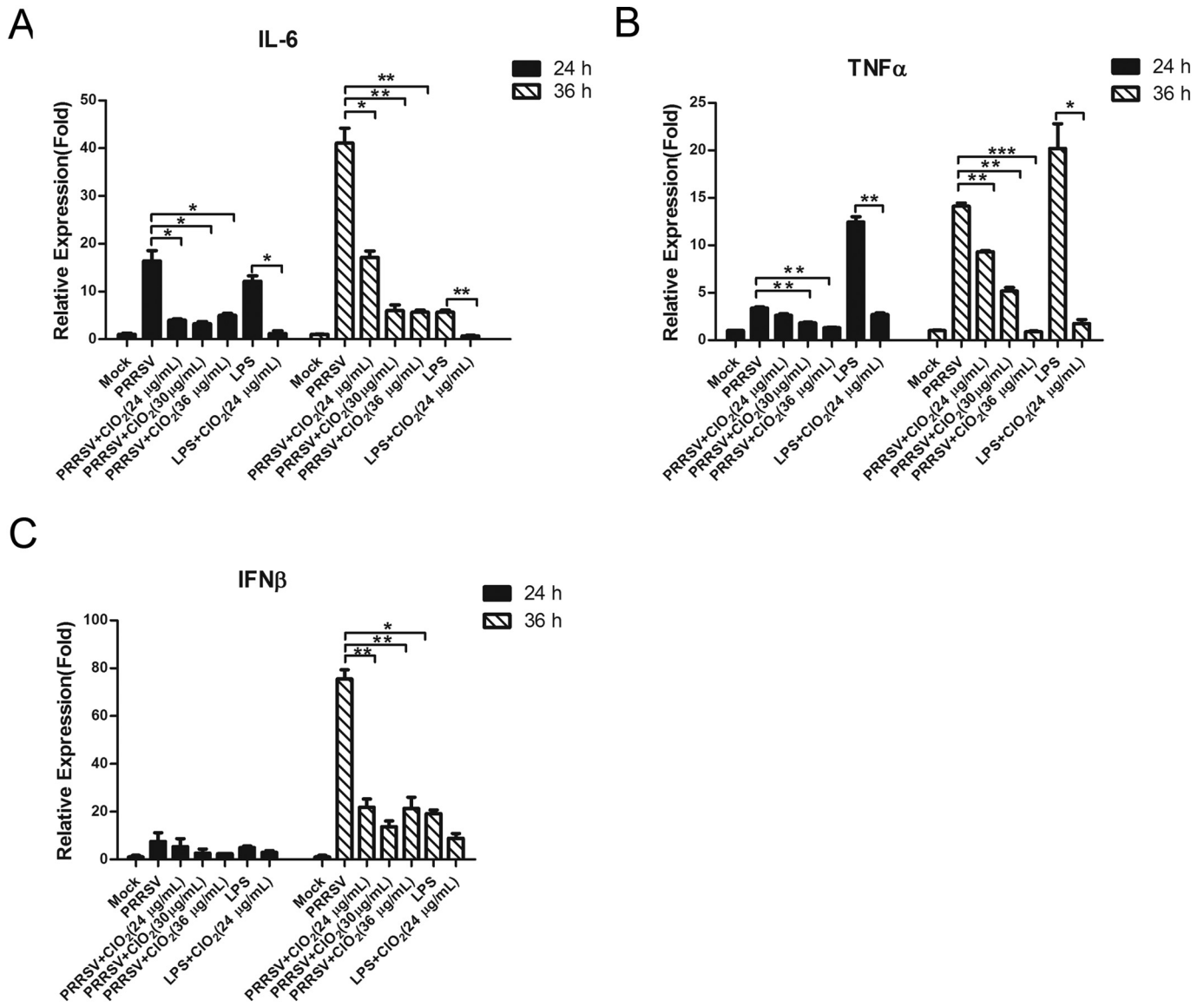


Fig. 8. Expression of cytokines in Marc-145 cells treated with ClO_2. (A–C) Marc-145 cells were mock infected or infected with CHR6 (MOI = 0.1) in the presence of different concentrations of ClO_2 (0, 24, 30, and 36 μg/mL) for 24 or 36 h. The expression of pro-inflammatory cytokines IL-6 (A), TNF-α (B), and IFN-β (C) were analyzed using qRT-PCR at 24 and 36 hpi. LPS was served as positive control. Relative expression (fold) in comparison with mock infected cells (set up as 1) is shown. Data are the results of three independent experiments (means ± SE). Significant differences between PRRSV infected cells and those treated with ClO_2 are denoted by * ($P < .05$), ** ($P < .01$) and *** ($P < .001$).

inflammatory cytokines interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and IFN-β. LPS was served as positive control. As shown in Fig. 8A and B, there was a significant decrease on the expression of IL-6 and TNF-α in cells treated with ClO_2 and PRRSV compared to those treated with PRRSV alone, and this phenomenon caused by ClO_2 was in a dose-dependent manner at 24 and 36 hpi. Consistently, the expression of IFN-β was significantly reduced by ClO_2 in cells at 36 hpi (Fig. 8C). Taken together, these data suggest that ClO_2 reduces the mRNA level of pro-inflammatory cytokines induced by PRRSV, maintaining a steady state for the growth of cells.

4. Discussion

PRRSV is one of the most economically important disease of pigs worldwide. PRRS is initially reported in North America in late 1980s (Neumann et al., 2005). PRRSV has quickly spread many countries and has become a major problem in preventing swine diseases in China (Murtaugh et al., 2010). Besides, its high variation and persistent infection make it difficult to control (Murtaugh et al., 2010; Stadejek et al., 2013). To prevent PRRSV infection, many methods are applied to interrupt the different stages of viral life cycle. Current PRRS vaccines such as MLVs fail to effectively prevent and control this disease (Kimman et al., 2009; Murtaugh and Genzow, 2011). Some novel vaccines with specific adjuvants are emerged in recent years, but they provide little protection (Du et al., 2017a). Some drugs, for example herbal extracts, chemical compounds, siRNA, microRNA and neutralizing antibodies have been verified that they can inhibit the replication of PRRSV in vitro (Du et al., 2017a). However, it is not sure whether they still maintain the antiviral activity but do no harm to animal bodies in vivo. Therefore, these agents are far from being ready for practical use in anti-PRRSV therapy. Despite the sustained effort, the prevention and treatment of PRRSV are still unavailable. In this study, we identified that ClO_2 has a strong inhibitory effect against PRRSV infection and replication with no cytotoxicity in both Marc-145 cells and PAMs (Fig. 1).

In antiviral assay, ClO_2 strongly inhibited PRRSV replication when

Marc-145 cells were infected with PRRSV-EGFP and pre-, co-, or post-treated with ClO_2 for 36 h (Fig. 2), indicating that ClO_2 might directly affect virus by impairing RNA and protein synthesis. PRRSV enters host cells through receptor-mediated viral entry and endocytosis, CD163 has been determined to be an essential receptor for viral internalization (Shi et al., 2015; Zhang and Yoo, 2015). The first step of viral invasion needs the attachment of PRRSV towards cellular receptor. Previous studies have demonstrated that GP2a and GP4 are viral attachment proteins interacting with CD163, suggesting that viral GPs play an important role in host-cell binding even entry (Wang et al., 2011; Welch and Calvert, 2010). Then we carried attachment, entry and release assays at multiple stages of the viral life cycle, we found that ClO_2 inhibited the process of PRRSV binding to Marc-145 cells (Fig. 3). Meanwhile our results demonstrated that ClO_2 could inactivate virus through breaking down the genome and structural proteins of PRRSV (Figs. 6 and 7). Presumably, the underlying mechanisms may be the destruction of GPs so that the interaction between PRRSV and cell receptors is possibly blocked and the life cycle of PRRSV is terminated by ClO_2. On the contrary, there is no influence on viral entry and release when treated with ClO_2 (Figs. 4 and 5). Thus ClO_2 mainly blocked attachment stage of virus life cycle, indicating that PRRSV inactivation by ClO_2 leads to the inability of the virus to bind to its host. However, whether ClO_2 can alter the structure of cell receptors is still unclear and accordingly would be our next issue to be addressed in future.

PRRSV infection induces the release of pro-inflammatory factors, such as IL-1, IL-6, TNF-α, which may contribute to the pathogenesis and inflammation response (Hao et al., 2015; Thanawongnuwech et al., 2004). Persistent and increased production may lead to cell apoptosis. Our study demonstrated that ClO_2 inhibited inflammatory response in PRRSV-infected Marc-145 cells via decreasing the mRNA levels of the pro-inflammatory cytokines (Fig. 8). ClO_2 moderates PRRSV-induced inflammatory responses associated with viral replication and promotes host resistance against PRRSV infection. Many signal transduction pathways can modulate pro-inflammatory gene expression, such as NF-κB, MAPK and so on. More studies are required to verify whether ClO_2 has the influence on signal transduction pathways.

In conclusion, our study demonstrates that ClO_2, the purity is 99%, inhibits the infection and replication of PRRSV by targeting attachment process in the viral life cycle. ClO_2 has strong inactivated effects on PRRSV through degrading viral genome and proteins. It is possible that ClO_2 can serve as a potential agent against PRRSV infection.

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Conflicts of interests

The authors declare that they have no conflict of interests.

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