

Porcine epidemic diarrhea virus uses cell-surface heparan sulfate as an attachment factor

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Abstract It is well known that many viruses use heparan sulfate as the initial attachment factor. In the present study, we determined whether porcine epidemic diarrhea virus (PEDV), an emerging veterinary virus, infects Vero cells by attaching to heparan sulfate. Western blot analysis, real-time PCR, and plaque formation assay revealed that PEDV infection was inhibited when the virus was pretreated with heparin (an analogue of heparan sulfate). There was no inhibitory effect when the cells were pre-incubated with heparin. We next demonstrated that enzymatic removal of the highly sulfated domain of heparan sulfate by heparinase I treatment inhibited PEDV infection. We also confirmed that sodium chlorate, which interferes with heparan sulfate biosynthesis, also inhibited PEDV infection. Furthermore, we examined the effect of two heparin derivatives with different types of sulfation on PEDV infection. The data suggested de-N-sulfated heparin, but not N-acetyl-de-O-sulfated heparin, inhibits PEDV infection. In summary, our studies revealed that heparan sulfate acts as the attachment factor of PEDV in Vero cells.

Introduction

Porcine epidemic diarrhea (PED), an acute and highly contagious enteric disease of swine, was first discovered in feeder pigs and fattening swine in England in 1971. Since October 2010, severe PED characterized by a high mortality rate affected pigs of all ages in China, resulting in enormous economic losses [1–7]. In 2013, PEDV began to spread quickly in swine farms in the United States [8].

Porcine epidemic diarrhea is caused by porcine epidemic diarrhea virus (PEDV), which belongs to the genus *Alphacoronavirus* within the family *Coronaviridae* and subfamily *Coronavirinae* [9]. PEDV is a single-stranded, enveloped, positive-sense RNA virus with an approximately 28-kb genome, including 5' and 3' untranslated regions (UTR) and seven known open reading frames (ORFs) [10].

Binding of the virus to its receptor(s) is the first critical step for virus infection. The recognition and interaction with specific receptors determine the host range and tissue tropism of the virus [11]. Heparan sulfate proteoglycans are glycoproteins composed of several covalently attached heparan sulfate (HS) chains, a form of glycosaminoglycan (GAG) [12]. Glycosaminoglycan can provide docking sites for the attachment of various viruses to the surface of eukaryotic cells [13]. GAGs possess negative charges because of N- and O-sulfation of the carbohydrate moieties [14]. Interactions between GAG and its ligand may be due to either electrostatic force or specific interactions [15]. Viruses that exploit GAGs for their attachment to cells include human papillomavirus (HPV) [16], Sindbis virus [17], noroviruses [18], dengue virus [19], hepatitis C virus [13], and human immunodeficiency virus type 1 (HIV-1) [20]. Heparan sulfate (HS) is the most ubiquitous moiety in glycosaminoglycan. Heparan sulfate is a complex

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polysaccharide that is present on the cell surface and in the extracellular matrix and regulates various cellular activities, including cell growth and differentiation, embryogenesis, angiogenesis, and homeostasis [21].

In the present study, we demonstrate that PEDV utilizes heparan sulfate for its attachment to Vero cells. Western blot, real-time PCR and plaque formation assays revealed that pretreatment of virus with heparin could inhibit PEDV infection. We next found that both N- and O-linked sulfate groups within the heparan sulfate carbohydrate structure are functionally important for PEDV binding to cells. We also showed that the binding ability of PEDV to Vero cells was reduced after the enzymatic removal of cell-surface heparan sulfate or the inhibition of heparan sulfate biosynthesis by treatment with chlorate. Our results thus reveal that cell-surface heparan sulfate serves as an attachment factor for PEDV infection.

Materials and methods

Cell culture and virus

Vero cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen, China) supplemented with 10 % fetal bovine serum (FBS, Invitrogen, China) and 1 % penicillin-streptomycin solution (Invitrogen, China). Porcine epidemic diarrhea virus (strain HLJBY) was propagated in Vero cells cultured in DMEM supplemented with 10 % fetal bovine serum and 60 μ g of trypsin per ml.

Reagents

Heparin sodium salt was purchased from Santa Cruz Biotechnology (USA). N-acetyl-de-O-sulfated heparin sodium salt and de-N-sulfated heparin sodium salt were purchased from Sigma (China). Heparinase I from *Flavobacterium heparinum* was purchased from NEB (USA). Sodium chlorate was purchased from TCI (Japan).

Evaluation of the role of GAGs in PEDV infection of Vero cells

Virus inactivation experiments were performed by preincubation of PEDV particles with various concentrations of heparin, de-N-sulfated heparin or N-acetyl-de-O-sulfated heparin for 1 h at 37 °C before inoculation of Vero cells. Vero cells were incubated with treated virus for 1 h at 4 °C. Unbound virus was removed by aspiration. The cells were washed three times with PBS and maintained at 37 °C in a humidified atmosphere with 5 % CO₂. Virus

infectivity was evaluated by western blot analysis, qRT-PCR and plaque formation assay.

Heparinase I treatment

Cells were seeded at 2×10^5 per well in 6-well plates 24 h prior to infection. The cells were incubated with heparinase I in 20 mM Tris-HCl (pH 7.5), 4 mM CaCl₂, 50 mM NaCl, and 0.01 % bovine serum albumin (BSA) for 1 h at 37 °C and then washed with PBS. Vero cells were subsequently infected with PEDV for 1 h at 4 °C. The cells were incubated at 37 °C for 24 h after washing with PBS. Infectivity was determined by western blot analysis, qRT-PCR and plaque formation assay. To measure the effect on attachment, the cells were subjected to qRT-PCR immediately after incubation at 4 °C.

Inhibition of cellular GAG sulfation by sodium chlorate

The sodium chlorate inhibition experiment was performed in DMEM with 10 % FBS. Vero cells were cultured for 24 h in the presence of sodium chlorate at concentrations ranging from 0 mM to 100 mM, followed by PEDV infection.

Western blot analysis

The cells in a 6-well plate were washed with cold PBS and scraped from the culture plate. The cells were lysed for 2 h at 4 °C with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 % Triton X-100, 2 mM EDTA, 0.1 % SDS, and 5 mM sodium orthovanadate) containing a protease inhibitor cocktail (Roche, USA) and 0.1 mM PMSF, and centrifuged at 14,000 *g* for 30 min at 4 °C. The protein concentration was determined using the Bradford protein assay. Equal amounts of protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins in the gel were transferred to polyvinylidene fluoride membrane (Bio-Rad, USA), which was then blocked with 5 % BSA in phosphate-buffered saline with Tween-20 (PBST) at 4 °C overnight and probed for 2 h with different primary antibodies. The membrane was incubated for 1 h with the corresponding HRP-conjugated secondary antibodies. Reactive bands were visualized by an enhanced chemiluminescence system (Vazyme, China).

qRT-PCR

Total RNA was extracted and purified from Vero cells using TRIzol Reagent according to the manufacturer's instruction (Vazyme). Intracellular PEDV and Vero cell genome levels

were quantified using a SYBR Green Probe 3-Step qRT-PCR Kit (Vazyme, China) and an ABI PRISM 7300 sequence detection system (Applied Biosystems, USA). To prepare cDNA, total RNA was reverse transcribed using a HiScript™ Q RT SuperMix Kit (Vazyme, China) according to manufacturer's instruction. Triplicate cDNA samples were amplified using an RT-PCR kit and the following primers: PEDV ORF3: F, TTTGCACTGTTTAGAGCGTCT; R, AGTAAAAGCAGACTAAACAAAGCCT. GAPDH: F, AGGTCGGAGTCAACGGATTT; R, TAGTTGAGGTCATGAAGGG. The amplification conditions included a pre-denaturation step of 95 °C for 5 min; then 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 95 °C for 15 s; followed by 60 °C for 60 s and a final step of 95 °C for 15 s. The data were analyzed by the $2^{-\Delta\Delta C_t}$ method, and target gene expression was normalized to GAPDH mRNA in the same sample.

Plaque formation assay

Tenfold serial dilutions (10^2 to 10^5) of virus-containing culture supernatant were incubated with a confluent monolayer of Vero cells at 37 °C for 2 h with gentle agitation every 20 minutes. Unbound virus was removed by washing with cold PBS. An overlay medium (2 % low-melting-point agarose in DMEM medium containing 2 % FBS) was added to each well, and the plate was incubated at 37 °C with 5 % CO₂ for 3-4 days. The cells were stained with 0.5 % crystal violet.

Cytotoxicity assay

Approximately 2×10^4 Vero cells in each well of a 96-well cell culture plate were incubated at 37 °C for 20 h in the presence of 5 % CO₂. The cells were then cultured in DMEM medium containing 2 % FBS and 5-100 mM sodium chlorate for 24 h. The cytotoxicity was assayed by measuring lactate dehydrogenase release from cells, using a CytoTox-ONE Homogeneous Membrane Integrity Assay Kit (Promega, USA) according to the manufacturer's instruction.

Statistical analysis

All data were determined in triplicate and were representative of at least two substantive experiments. The results are given as mean \pm standard deviation of triplicate determinations. The differences between means are considered significant at $p < 0.05$ and very significant at $p < 0.01$. We analyzed all data by one-way ANOVA using the SPSS 17.0 software package (version 17.0, SPSS Inc., Chicago, IL, USA).

Results

Effect of heparin on PEDV infection of Vero cells

In vitro studies on glycosaminoglycan-protein interactions usually rely on soluble heparin as a GAG model [22]. Heparin is a linear polysaccharide composed of α 1-4-linked disaccharide repeating units [23]. The negatively charged heparin is a structural analogue of heparan sulfate [24]. In addition, there are many studies of viruses using heparin as a competitive inhibitor of heparan sulfate, including PRRSV, filoviruses, enterovirus 71, Sindbis virus, dengue virus type 2 and yellow fever virus [25–29].

To evaluate the effect of heparin treatment on PEDV infection, various concentrations of heparin were preincubated with PEDV at MOI of 0.1 for 1 h at 37 °C before infection of Vero cells. The expression of PEDV N protein was analyzed 24 h postinfection (hpi) by western blot. Significant inhibition of PEDV infection by heparin was observed at different concentrations (20-100 μ g/ml) (Fig. 1A). We further evaluated the RNA level of the PEDV ORF3 gene in Vero cells 24 hpi by quantitative real-time PCR. As shown in Fig. 1B, the level of PEDV ORF3 RNA also decreased in a concentration-dependent manner. A similar inhibitory effect was observed in the plaque formation assay (Fig. 1C). In addition, we infected the cells with PEDV preincubated with heparin at 4 °C for 1 h and measured the level of ORF3 RNA after the unbound virus was washed away with cold PBS. As shown in Fig. 1D, the level of ORF3 RNA decreased as the concentration of heparin increased, suggesting that heparin inhibits virus attachment to the cells.

When Vero cells were incubated with different concentrations of heparin before virus infection, we did not find any inhibitory effect in western blot analysis (Fig. 1E). Collectively, these data suggested that the inhibitory effect was due to the interaction of heparin with PEDV.

Enzymatic digestion of cell-surface HS

Heparinase I can digest highly sulfated heparan-like structures on HS [30] and is commonly applied to determine the effect of removal of HS on ligand binding to the cell surface [31]. To confirm that heparan is involved in the infection of Vero cells by PEDV, we examined PEDV infection after the cells were treated with heparinase I. Different concentrations of heparinase I were pre-incubated with the cells for 1 h at 37 °C prior to inoculation with virus. Western blot analysis at 24 hpi showed that the expression level of the PEDV N protein decreased in a dose-dependent manner (Fig. 2A). The level of ORF3 RNA assayed by qRT-PCR also confirmed that enzymatic treatment resulted in a maximum

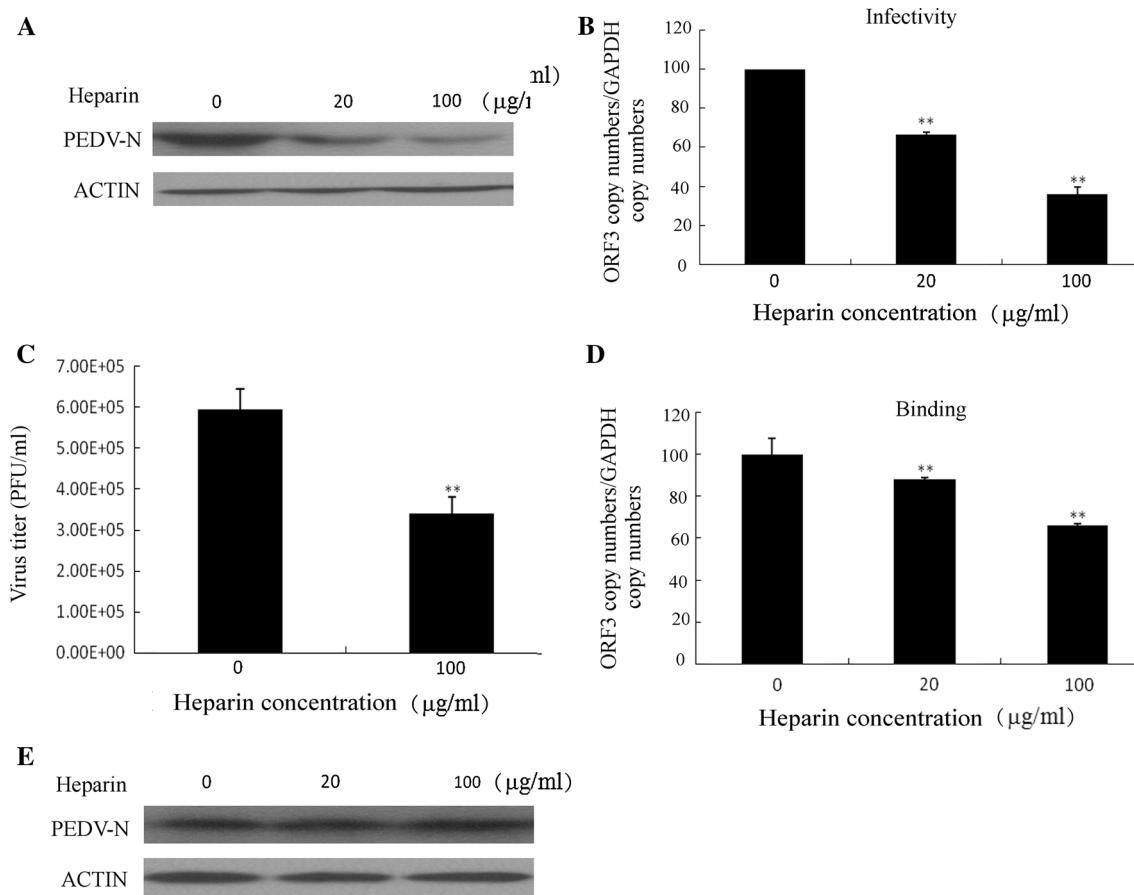


Fig. 1 Effect of heparin on PEDV infection of Vero cells. (A) Heparin was preincubated with PEDV at MOI of 0.1 for 1 h at 37 °C before infection of Vero cells. The N protein of PEDV was analyzed by Western blot. Actin was used as the sample loading control. (B) The level of ORF3 RNA in the infected cells was measured by qRT-PCR, normalized to GAPDH. (C) PEDV titers were measured by plaque formation assay. (D) Inhibition of virus attachment to the cells by heparin treatment. Vero cells were incubated with heparin-treated

PEDV for 1 h at 4 °C. The level of PEDV ORF3 RNA in Vero cells was determined by qRT-PCR, normalized to GAPDH mRNA. (E) Heparin was preincubated with Vero cells for 1 h at 37 °C before the cells were infected. The N protein of PEDV was analyzed by Western blot at 24 hpi. Actin was used as the sample loading control. The results are representative of at least two different experiments. Statistical significance was determined by one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$

reduction of PEDV infectivity of approximately 90 % at 20 mU/ml (Fig. 2B). A similar inhibitory trend was observed in the plaque formation assay (Fig. 2C). We also treated the cells with heparinase I before incubating with the virus at 4 °C for 1 h. After extensive washing with cold PBS, we immediately measured the level of ORF3 RNA and found that it decreased in proportion to the concentration of heparinase I (Fig. 2D).

Our data demonstrated that HS significantly affected the binding ability of PEDV to the cells, indicating that cell-surface HS contributes greatly to PEDV attachment to Vero cells.

Effect of sodium chlorate on PEDV infection of Vero cells

Chlorate competitively inhibits the formation of 3-phosphoadenosine 5-phosphosulfate, which can reduce the

degree of sulfation of heparan sulfate by as much as 60 % [32]. Therefore, chlorate treatment is used to examine the role of heparan sulfate on virus attachment to cells [16, 33].

After Vero cells were grown in medium containing various concentrations of sodium chlorate for 24 h, the cells were inoculated with PEDV (MOI = 0.1) for 1 h at 4 °C. The cells were incubated at 37 °C for 24 h after washing with PBS. Western blot analysis at 24 hpi showed that NaClO₃ treatment (5–100 mM) decreased the amount of the bound PEDV on Vero cells significantly (Fig. 3A). qRT-PCR results suggested the treatment reduced the viral RNA levels about 18–77 % (Fig. 3B), which was consistent with the results of the plaque formation assay (Fig. 3C).

To exclude the possibility that the reduction of the infection was due to cytotoxicity, the cytotoxicity of NaClO₃ was determined by LDH assay. The data indicated that sodium chlorate showed no cytotoxicity when the concentration was as high as 100 mM (data not shown).

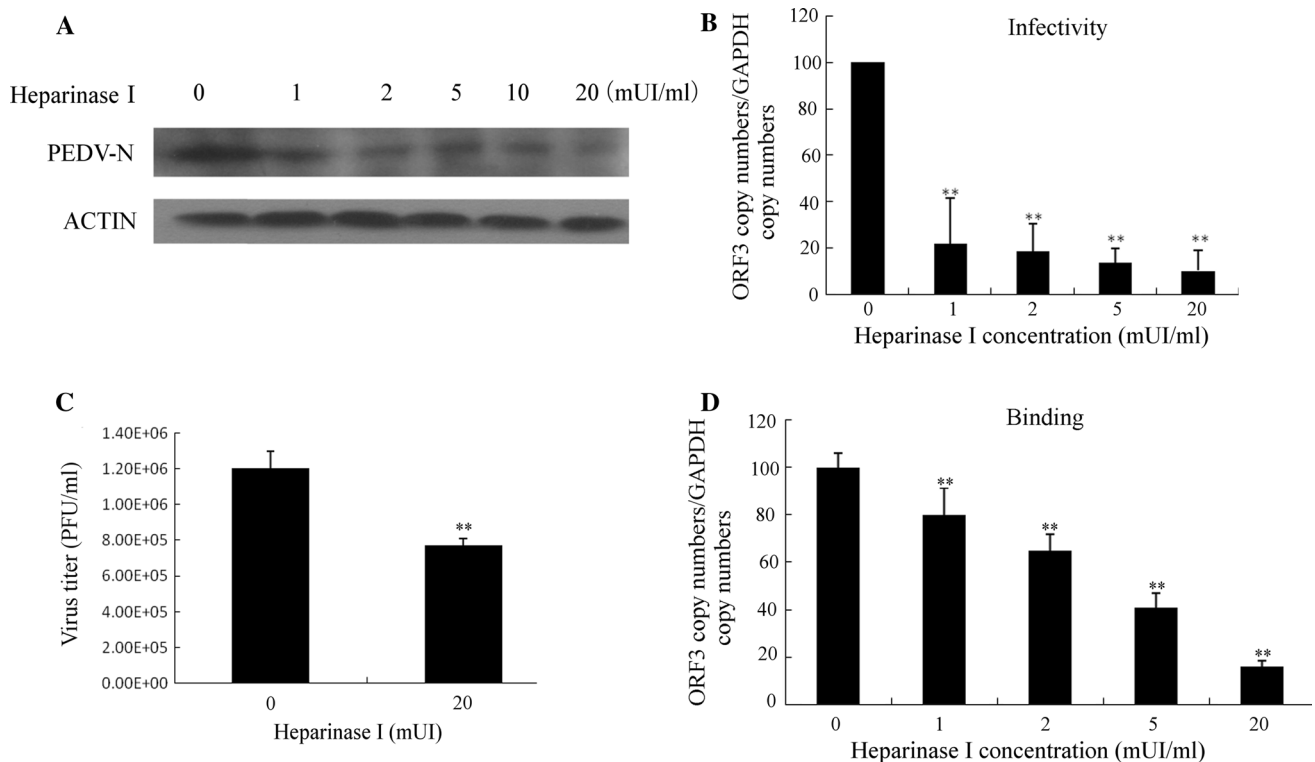


Fig. 2 Inhibition of PEDV infection by treatment of cells with heparinase I. Vero cells were pretreated with different amounts of heparinase I for 1 h at 37 °C prior to inoculation. (A) The N protein of PEDV was analyzed by Western blot. Actin was used as the sample loading control. (B) The level of ORF3 RNA was determined by qRT-PCR, normalized to GAPDH mRNA. (C) PEDV titers were measured by plaque formation assay. (D) Inhibition of virus attachment to the

cells by heparinase I treatment. The level of ORF3 RNA was determined by qRT-PCR, normalized to GAPDH mRNA, after the treated cells were incubated with PEDV for 1 h at 4 °C. The results are representative of at least two different experiments. Statistical significance was determined by one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$

N- and O-sulfation on heparan sulfate are critical for PEDV infection

GAGs possess negative charges because of N- and O-sulfation on the carbohydrate moieties [14]. Compared with heparin, de-N-sulfated heparin and de-O-sulfated heparin have the same size but less negative charges. N-acetyl-de-O-sulfated heparin (de-O) has no N- or O-sulfate groups, while de-N-sulfated heparin (de-N) completely lacks N-sulfation.

To study the effect of the sulfation pattern of heparin on viral inhibition, de-O and de-N heparins were tested. Various concentrations of heparin variants were pre-incubated with PEDV at MOI of 0.1 for 1 h at 37 °C before virus infection. Western blot analysis at 24 hpi showed that de-O heparin failed to abolish PEDV infection. On the contrary, it enhanced virus infection, although not significantly at lower concentrations (<500 µg/ml). The infection was significantly increased at 1000 µg/ml (Fig. 4A). qRT-PCR analysis of ORF3 RNA also showed that de-O treatment of the virus increased its infectivity in a concentration-dependent manner, with an 80.7 % increase

at 1000 µg/ml (Fig. 4B). This result was consistent with a previous study on enterovirus 71 [28]. A similar phenomenon was found in plaque formation assay (Fig. 4C). Meanwhile, de-N-sulfated heparin treatment appeared to moderately inhibit virus infection (Fig. 4D). De-N treatment also decreased ORF3 RNA levels and virus titers (Fig. 4E and F). These data indicated that both N- and O-sulfation within HS are functionally important for PEDV infection.

Discussion

Interaction of a virus with its host-cell receptor results in attachment to, entry into, and infection of target cells. The virus receptor/coreceptor is one of the critical factors that determine the host range and tissue tropism of the virus [11]. The association with glycosaminoglycans on the cell surface is one of the attachment mechanisms used by viruses, bacteria and protozoa for infection. Although porcine aminopeptidase N (pAPN) has been shown to be the functional receptor for PEDV infection [34], the initial

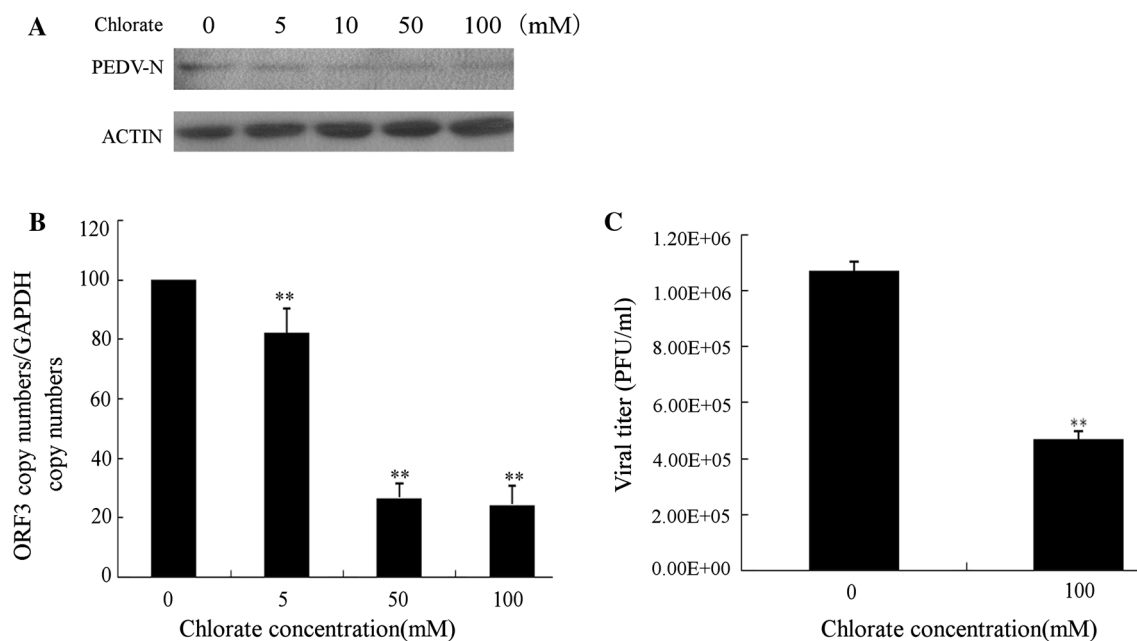


Fig. 3 Inhibitory effect of sodium chlorate on PEDV infection. Vero cells were pretreated with different concentrations of sodium chlorate for 24 h at 37 °C prior to inoculation. (A) The N protein of PEDV was analyzed by Western blot. Actin was used as the sample loading control. (B) The level of ORF3 RNA from the infected cells was

determined by qRT-PCR, normalized to GAPDH mRNA. (C) PEDV titers were measured by plaque formation assay. The results are representative of at least two different experiments. Statistical significance was determined by one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$

attachment process in PEDV infection has not been described in detail.

Heparin is a highly sulfated polysaccharide with multiple negatively charged groups. Most of the disaccharide units of heparin include 2-O-sulfated iduronic acid and 6-O-sulfated and N-sulfated glucosamine [35]. In this study, we provide evidence that cell-surface heparan sulfate is required for attachment of PEDV to Vero cells by using heparin as a competitive agent. We found that PEDV infection of Vero cells was strongly inhibited when PEDV was pretreated with heparin.

The importance of heparan sulfate in PEDV infection of target cells was further demonstrated in a heparinase I treatment experiment. In addition, sodium chlorate treatment experiment also showed a clear dose-dependent inhibition of PEDV binding to cells, indicating the crucial importance of sulfation of heparan sulfate in virus attachment.

The importance of the sulfation pattern for viral attachment has been demonstrated previously for other GAG-dependent viruses, such as dengue virus and RSV [19, 36]. To investigate the involvement of different sulfate groups of heparin on the binding of PEDV to Vero cells, specifically desulfated heparins were used to test their ability to prevent PEDV infection. Our data showed that N-acetyl-de-O-sulfated heparin, which lacks both N- and O-sulfate groups, moderately enhanced virus

infection when compared with heparin, but replacement of N-sulfate groups with N-acetyl groups (de-N-sulfated heparin) resulted in a moderate reduction in PEDV infection. A comparison of the inhibitory effects of heparin and two heparin variants indicated that N-sulfation and O-sulfation are both required for the inhibitory effects on PEDV infection, which is consistent with a previous study on ecotropic murine leukemia virus [35]. Previous studies have revealed that the O-sulfate group of heparin exerts an important inhibitory effect on herpes simplex virus [37], pseudorabies virus [38], and HIV [39] infection. N-sulfation of heparin is required for inhibition of respiratory syncytial virus infection [40]. Therefore, the specific pattern of sulfate groups on heparin, and not just its net negative charge, determines its ability to inhibit virus infection. The distribution of sulfate groups on heparin and the complex mode of interaction between the virus and the cell are also critical for determining the antiviral activity of heparin and its derivatives. Our result demonstrate that N-acetyl-de-O-sulfated heparin (de-O) treatment enhances virus infection, which is consistent with a previous study on enterovirus 71 [28]. Treatment with de-O had no effect on PEDV attachment to Vero cells, but it moderately enhanced virus internalization (data not shown). A recent study has demonstrated that heparosan can be internalized into several cell types (bovine lung microvascular endothelial cells, Chinese

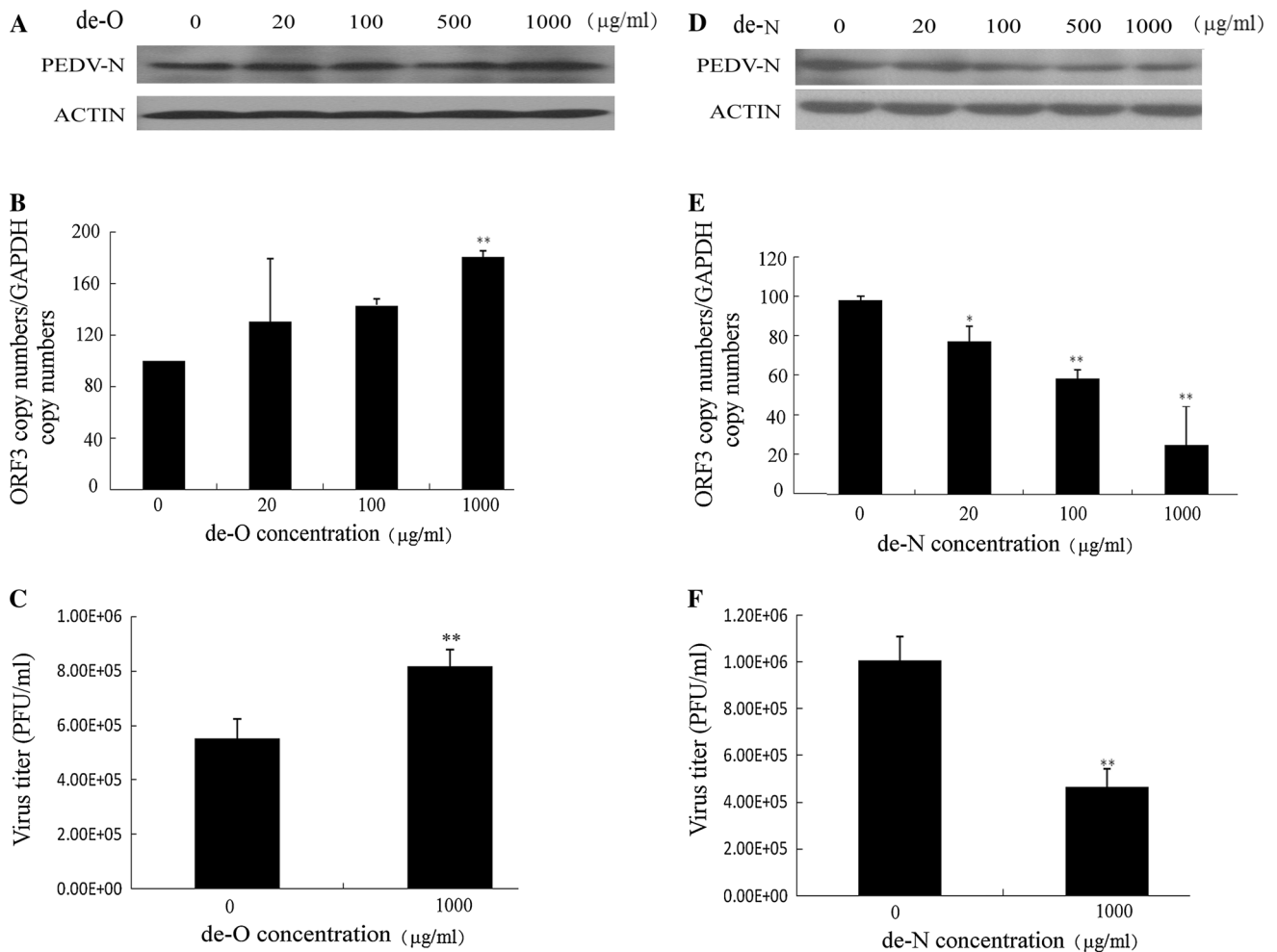


Fig. 4 The effect of the sulfation pattern of heparin on PEDV infection. (A) N-acetyl-de-O-sulfated heparin (de-O) was pre-incubated with PEDV at MOI of 0.1 for 1 h at 37 °C before infection of Vero cells. The N protein of PEDV was analyzed by Western blot. Actin was used as the sample loading control. (B) The level of ORF3 RNA in the infected cells was determined by qRT-PCR, normalized to GAPDH mRNA. (C) PEDV titers were measured by plaque formation assay. (D) De-N-sulfated heparin (de-N) was preincubated

with PEDV at MOI of 0.1 for 1 h at 37 °C before infection of Vero cells. The N protein of PEDV was analyzed by Western blot. Actin was used as the sample loading control. (E) The level of ORF3 RNA in the infected cells was determined by qRT-PCR, normalized to GAPDH mRNA. (F) PEDV titers were measured by plaque formation assay. The results are representative of at least two different experiments. Statistical significance was determined by one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$

hamster ovary K1 cells, BXPC-3 human pancreatic cancer cells, HT-29 human colorectal cancer cells, and U87-Mg human glioma cells) significantly more efficiently than heparin [41]. Considering that the structure of de-O heparin is similar to that of heparosan, we speculate that de-O treatment might enhance virus infection by increasing virus internalization through an unknown mechanism that remains to be investigated.

In summary, our data suggest that PEDV utilizes cell-surface heparan sulfate as an attachment factor. Our findings contribute new information about the mechanism of the PEDV entry process and provide important insights for the design and development of heparin-based agents to inhibit PEDV infection.

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