Antiviral activity of sulfated *Chuanmingshen violaceum* polysaccharide against Newcastle disease virus

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Newcastle disease virus (NDV) is a member of *Paramyxovirinae* subfamily and can infect most species of birds causing severe economic losses. The current control measure is vaccination, but infections cannot be completely prevented. It remains a constant threat to the poultry industry and new control measures are urgently needed. This study demonstrates that sulfated *Chuanmingshen violaceum* polysaccharides (sCVPSs) were potent inhibitors of NDV, with 50 % inhibitory concentrations (IC50) ranging from 62.55 to 76.31 μg ml⁻¹ in Baby hamster kidney fibroblasts clone 21 (BHK-21) and from 101.57 to 125.90 μg ml⁻¹ in chicken embryo fibroblasts (CEF). sCVPS is more effective than heparan sulfate (HS; as a positive control) with IC50 values of 99.28 μg ml⁻¹ in BHK-21 and 118.79 μg ml⁻¹ in CEF. sCVPSs and HS exhibit anti-NDV activity by prevention of the early stages of viral life. The mechanism of action study indicated that virus adsorption in BHK-21, and both virus adsorption and penetration in CEF were inhibited by sCVPSs. When the number of viruses was increased to an m.o.i. of 0.1 in the immunofluorescence study and to an m.o.i. of 1 in the fluorescent quantitative PCR study, viral infection was also significantly suppressed; the antiviral activity of sCVPSs was independent of the m.o.i. sCVPSs also prevented the cell-to-cell spread of NDV. *In vivo* tests carried out on specific pathogen-free (SPF) chickens showed that sCVPSs also inhibited virus multiplication in heart, liver, spleen, lung and kidney. These results indicated that sCVPSs perform more effectively than HS as antiviral agents against NDV, and can be further examined for their potential as an alternative control measure for NDV infection.

INTRODUCTION

Paramyxoviruses belonging to the *Paramyxoviridae* family are enveloped, negative-sense single-stranded RNA viruses responsible for many important human and animal diseases (Bhella *et al*., 2002). In recent years, the newly emerging paramyxoviruses have caused some of the deadliest zoonoses; other old paramyxoviruses have also emerged and become a constant threat to humans and animals (Aguilar & Lee, 2011). Newcastle disease virus (NDV), a member of the *Paramyxovirinae* subfamily, is the pathogen for Newcastle disease (ND) which is one of the most devastating infectious diseases of poultry and causes enormous economic loss because of its worldwide distribution and high flock mortality (Miller *et al*., 2010; Ravindra *et al*., 2009; Zhang *et al*., 2011). Despite various vaccines having been widely used to prevent and control NDV infections, outbreaks of ND in vaccinated poultry flocks still continue due to the significant differences between the current vaccine strains and prevailing NDV strains (Zhang *et al*., 2010, 2011). Moreover, there are no antiviral agents available against NDV in poultry so new alternative control measures are urgently needed.

The antiviral activity of sulfated polysaccharides was first reported in 1958 (Gerber *et al*., 1958), and since then a large number of sulfated polysaccharides, both chemically synthesized and naturally extracted, have been found to possess a broad spectrum of antiviral activity (Baba *et al*., 1988; Gonzalez *et al*., 1987; Han *et al*., 2010; Mastromarino *et al*., 1997; Pujol *et al*., 2007). To date, a number of sulfated polysaccharides have been demonstrated to be potent inhibitors of paramyxoviruses, including parainfluenza virus, respiratory syncytial virus, NDV, mumps virus and measles virus (Table 1). The mechanism of action is mainly attributed

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to interference with the binding of virus to the host cell membrane, produced by mimicking the sugar-rich virus receptors present on the cell surface leading to the interaction of sulfated polysaccharides with positively charged domains of the viral envelope glycoproteins involved in the attachment to host cells (Damonte et al., 2004; Pujol et al., 2007).

Sulfation has proved to be a potent way to enhance the antiviral activity of polysaccharides (Huang et al., 2008; Lu et al., 2008; Wang et al., 2010a; Zhao et al., 2011). Chuanmingshen violaceum extract is used as a tonic in Chinese medicine. We have previously sulfated polysaccharides extracted from Chuanmingshen violaceum (CVPSs) and found that sulfated CVPSs (sCVPSs) exhibited significant antiviral activity against duck enteritis virus (Song et al., 2013b). Here, we have characterized their antiviral activities against NDV in two different host cells and in NDV-infected chickens for the purpose of developing a new candidate anti-NDV drug.

**RESULTS**

**Cytotoxicity and 50 % inhibitory concentration of CVPS, sCVPSs and HS in vitro**

The anti-NDV activity of CVPSs, sCVPSs and heparan sulfate (HS; sulfur content: 5–7 %; Sigma-Aldrich) were evaluated for their ability to inhibit plaque formation using a plaque reduction assay where the test polysaccharides were present during virus infection of both chicken embryo fibroblasts (CEF) and baby hamster kidney fibroblasts clone 21 (BHK-21). As summarized in Table 2, whilst CVPSs exhibited no anti-NDV activity, sulfated derivatives exhibited inhibitory activity with varying levels of effectiveness according to the degree of sulfation (DS). The sCVPSs showed a higher activity in BHK-21 with the 50 % inhibitory concentration (IC50) ranging from 62.55 to 76.31 µg ml⁻¹ than that in CEF with IC50 ranging from 101.57 to 125.90 µg ml⁻¹. In contrast to the sCVPSs, the positive control agent (HS) showed lower antiviral activity against NDV. Moreover, the inhibitory effect of HS was the same as those of sCVPSs; the anti-NDV efficacy of HS was higher in BHK-21 with an IC50 of 99.28 µg ml⁻¹ compared to evaluation in CEF with IC50 ranging from 118.79 to 138.2 µg ml⁻¹. Both BHK-21 and CEF viability was slightly affected by sCVPSs as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay; the 50 % cytostatic concentration (CC50) was >1382 µg ml⁻¹ in BHK-21 and 1124 µg ml⁻¹ in CEF. However, HS exhibited no cytotoxicity with the test maximum concentration (2000 µg ml⁻¹). The selectivity index (CC50/IC50) for sCVPSs was approximately 23 in BHK-21 and 12 in CEF.

### Table 1. Summary of sulfated polysaccharides with activity against paramyxoviruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Sulfated polysaccharides</th>
<th>Sources</th>
<th>IC50 (µg ml⁻¹)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parainfluenza virus</td>
<td>Sulfated galactan</td>
<td>Aghardiella tenera</td>
<td>50</td>
<td>Witvrouw et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>Sulfated dextran</td>
<td>Sigma (MW 5000)</td>
<td>&gt;400</td>
<td>Witvrouw et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>Sulfated dextran</td>
<td>Pfeifer and Langen (MW 1000–70 000)</td>
<td>&gt;200</td>
<td>Hosoya et al. (1991)</td>
</tr>
<tr>
<td>Pentosan polysulfate</td>
<td>Sulfated galactan</td>
<td>Aghardiella tenera</td>
<td>4</td>
<td>Witvrouw et al. (1994)</td>
</tr>
<tr>
<td>Respiratory syncytial virus</td>
<td>Sulfated galactan</td>
<td>Aghardiella tenera</td>
<td>14</td>
<td>Witvrouw et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>Sulfated dextran</td>
<td>Sigma (MW 5000)</td>
<td>0.14–12</td>
<td>Hosoya et al. (1991); Witvrouw et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>Sulfated xylomannan</td>
<td>Norhogenia fustigiata</td>
<td>1.2</td>
<td>Hasui et al. (1995);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.8–3</td>
<td>Hasui et al. (1995);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.8–3</td>
<td>Hasui et al. (1995);</td>
</tr>
<tr>
<td>Newcastle disease virus</td>
<td>Fucoidian</td>
<td>Cladosiphon okamuranus</td>
<td>58</td>
<td>Elizondo-Gonzalez et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Sulfated dextran</td>
<td>Pfeifer and Langen company (MW 1000–70 000)</td>
<td>&gt;200</td>
<td>Hosoya et al. (1991)</td>
</tr>
<tr>
<td>Measles virus</td>
<td>Sulfated dextran</td>
<td>Ueno Fine Chemicals</td>
<td>0.86</td>
<td>Hosoya et al. (1991)</td>
</tr>
<tr>
<td></td>
<td>Sulfated xylomannan</td>
<td>Norhogenia fustigiata</td>
<td>0.9</td>
<td>Damonte et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>Sulfated xylomannan</td>
<td>Norhogenia fustigiata</td>
<td>104</td>
<td>Bourgeois et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>Hepean sulfate</td>
<td>Sigma (bovine kidney)</td>
<td>0.8–3</td>
<td>Hasui et al. (1995);</td>
</tr>
<tr>
<td></td>
<td>Sulfated polysaccharides</td>
<td>Spirulina platensis</td>
<td>17–39</td>
<td>Hayashi et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>(composed of mannos, galactose, glucose and uronic acid)</td>
<td></td>
<td>23–92</td>
<td>Hayashi et al. (1996)</td>
</tr>
</tbody>
</table>
**Table 2. Antiviral activity of various sCVPSs, HS and CVPS against NDV in CEF and BHK-21**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>BHK-21</th>
<th>CEF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>CC50 (µg ml⁻¹)</strong></td>
<td><strong>IC50 (µg ml⁻¹)</strong></td>
</tr>
<tr>
<td>sCVPS₀.37</td>
<td>1382 ± 29</td>
<td>62.55 ± 3.02</td>
</tr>
<tr>
<td>sCVPS₀.95</td>
<td>1647 ± 22</td>
<td>71.02 ± 3.47</td>
</tr>
<tr>
<td>sCVPS₆.9</td>
<td>1808 ± 40</td>
<td>76.31 ± 1.32</td>
</tr>
<tr>
<td>HS</td>
<td>&gt;2000</td>
<td>99.28 ± 2.76</td>
</tr>
<tr>
<td>CVPS</td>
<td>&gt;2000</td>
<td>–</td>
</tr>
</tbody>
</table>

*Fifty per cent cytotoxic concentration (CC₅₀), the concentration required to reduce DEF viability by 50%, was measured by the MTT method.
†Fifty per cent inhibitory concentration (IC₅₀) is the concentration required to reduce virus plaques by 50%.
§SI, selectivity index is defined as the ratio of CC₅₀ to IC₅₀ (SI = CC₅₀/IC₅₀).

**sCVPSs and HS inhibited early stages of viral life**

The plaque reduction study revealed that the sulfated polysaccharides (sCVPSs and HS) possessed inhibitory activity against NDV. Therefore, a time of treatment study was employed to analyse the influence of sCVPSs and HS on the virus replication cycle; polysaccharides were added at different times following virus infection. Cells were infected with NDV, after which sCVPSs and HS (200 µg ml⁻¹) were added at different times post-infection (p.i.): 0, 15, 30 and 60 min, and 2, 3 and 5 h. Inhibition was evaluated by plaque reduction assay. As shown in Fig. 1, the test polysaccharides exhibited significant antiviral activity at early phases of infection (0, 15 and 30 min p.i.) in both BHK-21 (Fig. 1a) and CEF (Fig. 1b), with higher activity in BHK-21 than in CEF. Inhibition was greatest when sCVPS was added to the cells at 0 min p.i., the rate being about 55% in CEF and 70% in BHK-21. When sCVPS was added at 30 min p.i., inhibition decreased slightly and remained at 43% in CEF and 54% in BHK-21. In contrast, the inhibitory effect of HS on the two host cells was about 50% when it was added at 0 min p.i., and decreased significantly to about 25% when it was added at 30 min p.i. There was little difference in inhibition when the four test polysaccharides were added at different time points within 15 min (0 and 15 min) p.i. In addition, sCVPSs and HS exhibited poor antiviral activity when they were added 1 h p.i.: the rate was <6% in CEF and 10% in BHK-21. The sCVPSs showed better activity than HS in the two host cells at early treatment times (0, 15, 30 and 60 min), and sCVPS₀.37 was the most active of the three. However, no activity was detected when the test polysaccharides were added at 2, 3 and 5 h p.i. (data not shown).

**Virus adsorption and penetration were prevented by sCVPSs and HS**

The time of addition study indicated the antiviral activity of sCVPSs and HS was exerted mainly by interference with early step of virus infection. Here, five tests, each at a separate phase of the viral life cycle, were performed to elucidate which step was prevented by the polysaccharides; the results are summarized in Table 3. A pretreatment assay in which the polysaccharides were incubated with cells before virus infection did not detect any activity in the two host cells (Table 3), suggesting that the test polysaccharides cannot bind to virus receptors on the cell surface involved in the initial attachment of the virus. However, when added to the monolayer during virus adsorption, all the sCVPSs and HS showed a markedly enhanced inhibitory effect in the two host cells (Table 3), with the sCVPSs exhibiting higher activity than HS. Little difference in inhibitory effect was observed when the test compounds were evaluated in BHK-21 and CEF, based on IC₅₀ values which ranged from 61.87 to 85.05 µg ml⁻¹ in BHK-21 and from 64.73 to 78.01 µg ml⁻¹ in CEF. For HS, the IC₅₀ values were 109.02 µg ml⁻¹ in BHK-21 and 97.41 µg ml⁻¹ in CEF. In the penetration experiment, the virus was incubated with a CEF monolayer at 4°C for 1 h to allow adsorption before addition of test polysaccharides. Compound dilutions were then added during a 1 h penetration step at 37°C. All sCVPSs inhibited virus penetration, with IC₅₀ ranging from 102.97 to 146.51 µg ml⁻¹ in CEF, but when evaluated in BHK-21, no inhibition was observed. In addition, HS did not prevent NDV penetration in the two host cells. After penetration into host cells, the virus began its replication by using host resources. Therefore, experiments were conducted to measure the effect of test polysaccharides on virus replication. No compound exhibited an inhibitory effect during virus replication (Table 3). In the antiviral trials of test polysaccharides designed according to the viral life cycle, polysaccharides were at times directly incubated with the virus during infection. To test whether the observed antiviral effects of sCVPSs were related to their inactivation activity, concentrated NDV were incubated with dilutions of test compounds in vitro before infection of cells. Inactivation efficacy was then measured, but no activity was detected (Table 3).
virus had penetrated host cells. However, after complete virus replication, mature progeny virions were released into extracellular spaces and subsequently spread laterally between adjacent cells. The spread of virus correlates with plaque size, so the ability of sCVPSs to inhibit progeny virions from infecting adjacent cells was determined. Plaque size in sCVPS-treated groups was significantly reduced ($P$, 0.05) by almost 35% in BHK-21 and 30% in CEF compared with that in the untreated group (Fig. 2). In the two host cells, a significant difference ($P$, 0.05) in plaque size was observed between sCVPS groups with concentrations of 200 and 50 mg ml$^{-1}$, but there was no significant difference between concentrations of 200 and 100 mg ml$^{-1}$.

Viral protein expression was inhibited by sCVPSs

Indirect immunofluorescence assay was performed to determine the effect of sCVPSs on viral protein expression. BHK-21 and CEF were infected with NDV at an m.o.i. of 0.1 with or without sCVPS$_{1.37}$ (200 mg ml$^{-1}$). Viral protein expression was detected by immunofluorescence assay 24 h after infection. As shown in Fig. 3, high fluorescence of viral proteins was detected in NDV-infected cells without sCVPS treatment (Fig. 3a, d). After sCVPS$_{1.37}$ treatment, the amount of virus protein expression in NDV-infected cells drastically decreased and only very low fluorescence was seen (Fig. 3b, e). No fluorescence was observed in non-infected cells (Fig. 3c, f).

sCVPSs and HS significantly inhibited virus infection

To confirm the potent antiviral action of the test polysaccharides, inhibitory activity was evaluated in an experiment performed at an m.o.i. higher than that allowable in the plaque reduction assay. Thus, real-time reverse-transcription PCR (rt RT-PCR) was used to quantify the amount of virus inhibited by sCVPSs and HS during the early stage of infection when the number of infectious viruses increased to an m.o.i. of 1. The test polysaccharides were added to BHK-21 and CEF monolayers during virus infection. After incubation for 1 h at 37°C, the number of copies of infective viral genomes was quantified by rt RT-PCR. A standard curve was also established with detection ranging from $1 \times 10^2$ to $1 \times 10^8$...
copies/reaction ($R^2=0.997$; data not shown). Both sCVPSs and HS within the test concentrations could significantly ($P<0.05$) reduce the number of infective viruses in the two host cells (Fig. 4). One hundred-fold fewer copies of viral genomes were produced in the sCVPS$_{1.37}$ ($400$ μg ml$^{-1}$) and sCVPS$_{0.95}$ ($400$ μg ml$^{-1}$) treated groups compared with the untreated group. In both host cells, the inhibitory activity of sCVPSs was increased both with DS and concentration, and exceeded that of HS; however, the sCVPSs exhibited higher activity in BHK-21 than in CEF.

**In vivo antiviral activity of sCVPSs**

sCVPSs exhibited inhibitory activity against NDV *in vitro*, as demonstrated by the studies described above. The anti-NDV activity of sCVPSs *in vivo* was also evaluated in specific pathogen-free (SPF) chickens that had been inoculated with NDV and simultaneously given sCVPS$_{0.95}$. As shown in Fig. 5, virus multiplication in each of the test organs of the sCVPS-treated group was lower than in the untreated group. Moreover, the virus titres of spleen, heart and lung in the sCVPS-treated group were significantly ($P<0.05$) lower than that in the untreated group.

**DISCUSSION**

Many sulfated polysaccharides, derived from natural extraction and chemical synthesis, have exhibited antiviral activity against paramyxoviruses, with their efficacy varying with the sources of both sulfated polysaccharides and viruses (Table 1). In the present study, non-sulfated CVPS exhibited no inhibitory effect, but sCVPSs were demonstrated to be potent inhibitors of NDV, and antiviral activity was confirmed in two host cell lines (Table 2). The results are consistent with the theory that sulfation is an effective method by which to enhance the antiviral activity of polysaccharides. Moreover, this study also found that the ability of sCVPSs to inhibit NDV infection was independent of m.o.i. (Figs 3 and 4). This important property that can block virus infection in the presence of high initial virus count provides support for potential therapeutic usage (Talarico & Damonte, 2007). HS and its analogues have long been known to have a broad antiviral spectrum (Lee et al., 2006; Nyberg et al., 2004; Pourianfar et al., 2012). Therefore, HS was selected as positive control for further evaluation of the anti-NDV potency of sCVPSs. It was shown that the efficacy of HS was approximately the same as that of sCVPS$_{0.69}$, but lower than that of sCVPS$_{1.37}$ and sCVPS$_{0.95}$ (Table 2), indicating that sulfur content and structural variation of polysaccharides affected their antiviral activity (Harden et al., 2009).

The antiviral potency of sulfated polysaccharides has always been associated with DS; higher DS usually accompanies higher activity within a certain range (Damonte et al., 2004). This study concurs with this conclusion: the anti-NDV activity of sCVPSs increased...
with DS in both BHK-21 and CEF (sCVPS_{1.37} > sCVPS_{0.95} > sCVPS_{0.69}) (Table 2). Similar results were also observed in an investigation of sCVPSs versus duck enteritis virus (Song et al., 2013b).

In recent years, the inhibitory effect of some sulfated polysaccharides against NDV has been studied. Hu and co-workers prepared the sulfated derivatives of polysaccharides extracted from *Lycium barbarum* (Wang et al., 2010b), *Tremella fuciformis* (Zhao et al., 2011) and *Auricularia auricula* (Nguyen et al., 2012), and the activity to inhibit cellular infectivity of NDV to CEF was ascertained by determining the number of living cells after NDV infection. It was also revealed that sulfation could increase the anti-NDV activity of polysaccharides. However, we could not make a comparison with these reports because the IC_{50} values were not calculated by standard plaque reduction assay. Another study showed that fucoidan from *Cladosiphon okamuranus* (sulfur content 13.6 %) exhibited anti-NDV activity with an IC_{50} value of 58 μg ml^{-1} in Vero cells (Elizondo-Gonzalez et al., 2012). When compared with fucoidan, the inhibitory activity of sCVPSs was slightly less because the IC_{50} value of sCVPS_{1.37} (sulfur content 14.55 %) was 62.55 μg ml^{-1} in BHK-21. There were no differences in the sulfur content between sCVPS_{1.37} and fucoidan, so the differences in efficacy were mainly due to virus, host cells and the characteristics of the polysaccharides themselves.

![Figure 2](http://vir.sgmjournals.org)

**Fig. 2.** The relative plaque size of untreated and sCVPS-treatment groups. BHK-21 and CEF monolayers grown in six-well plates were infected with about 100 p.f.u. of NDV per well. After virus penetration, MM containing 1 % methylcellulose and sCVPS_{1.37} (50, 100, 200 μg ml^{-1}) was then added and plates were incubated at 37 °C for 60 h. The size of plaques was determined and values were obtained from three independent experiments. Significant differences (P<0.05) among groups exist when there are not the same letters on each column (P<0.05).
including sugar composition, molecular mass, and molecular conformation (Harden et al., 2009; Wijesekara et al., 2011).

The antiviral activity exerted by sulfated polysaccharides mainly results from its ability to effectively block early stages of viral life, including virus binding to the target cell and viral entry (Damonte et al., 2004; Harden et al., 2009). For development of antiviral drugs, inhibition of viral infection at this early stage is considered less toxic than chemotherapy (Liu & Thorp, 2002). Our results obtained from time of addition assays indicated that sCVPSs had the same effects (Fig. 1). Moreover, the mode of action experiments demonstrated that sCVPSs interfered with NDV infection, not by direct inactivation of virus, but by prevention of the virus attachment and penetration (Table 3). Despite sCVPSs being unable to inhibit NDV replication, cell-to-cell spread of NDV was prevented (Fig. 2), which may be attributed to the interference with virus adsorption and penetration.

Infection of NDV was achieved via its two envelope proteins, haemagglutinin-neuraminidase (HN) and the fusion protein (Connaris et al., 2002). HN binds to sialic acid-containing receptors, leading to virus adsorption, and promotes membrane fusion causing virus penetration into host cells (Crennell et al., 2000; Connaris et al., 2002). There are two sialic acid binding sites on the globular head region of HN (Crennell et al., 2000; Zaitsev et al., 2004). In the present study, the mechanism of sCVPS blocking of virus attachment was thought to result from the interactions of anionic charges in sCVPS with positively charged domains in or nearby the two binding sites, inducing occupancy or a shielding effect. After binding to cells through the two HN sites, fusion was triggered (Mahon et al., 2011). Then, a series of conformational changes of fusion protein occurred, causing fusion of the viral envelope with the host cell membrane (Porotto et al., 2012). Interestingly, our study found that sCVPSs could only prevent NDV penetration in CEF. These results suggest that the fusion protein domains involved in interaction with sCVPSs were essential for NDV fusion with CEF membrane, but not for fusion with BHK-21 membrane. Moreover, the results also indicate that NDV fusion with different host membranes involves different interaction sites.

To date, antiviral treatments for NDV infection are not available because of their cost and toxicity (Elizondo-Gonzalez et al., 2012). Although the potent chemotherapeutic antiviral drug ribavirin inhibited many paramyxoviruses in vitro, it showed very poor anti-NDV activity and high toxicity (Elizondo-Gonzalez et al., 2012). In recent years, sulfated polysaccharides have become more attractive for development of new antiviral therapeutics because of their numerous advantages over other classes of antiviral drugs, such as relatively low production costs, a broad spectrum of antiviral properties and low cytotoxicity (Wijesekara et al., 2011). The present study also investigated the antiviral activity of various sCVPSs in vivo and found that they could inhibit virus multiplication in heart, liver, spleen, lung and kidney of NDV-infected chickens (Fig. 5). Given their potent anti-NDV activity both in vitro and in vivo, sCVPSs show potential for the control of NDV infection in poultry.
Fig. 4. Anti-NDV effect of concentration of various sCVPSs and HS measured by rt RT-PCR assay. (a) BHK-21 and (b) CEF monolayers grown in six-well plates were infected with NDV at an m.o.i. of 1 in the presence or absence of different sCVPSs (400, 200 and 100 µg ml⁻¹) and HS (100 µg ml⁻¹). After 1 h incubation at 37 °C, the total number of viruses were quantified by rt RT-PCR with reference to a standard curve, with detection ranging from 10² to 10⁸ copies/reaction ($R^2=0.997$, data not shown). Significant differences ($P<0.05$) among groups exist when there are not the same letters on each column ($P<0.05$).

Fig. 5. In vivo antiviral activity of sCVPS₀.₉₅. SPF chickens were inoculated with $10^6$ EID₅₀ NDV and simultaneously given sCVPS₀.₉₅ once a day for four successive days. After 4 days of infection, the mean virus titres [log₁₀TCID₅₀ (g tissue)⁻¹] of heart, liver, spleen, lung and kidney were calculated. * $P<0.05$ vs untreated group.
METHODS

Extraction and sulfation of CVPS. CVPS was extracted as previously described (Song et al., 2013a). Briefly, dried *Chuanmingshen violaceum* powder was defatted with 95% ethanol under reflux for 6 h and then decocted three times with distilled water. The decoction was collected and concentrated. crude CVPS was precipitated by adding alcohol to the decoction (v/v, 3:1). The polysaccharides were deproteinized with Sevag reagent, filtered by column chromatography and dialysed against distilled water for 3 days. Finally, CVPS was obtained by lyophilization.

sCVPSs with three different degrees of sulfation (1.37, 0.95 and 0.69) were prepared in our laboratory by the chlorosulfonic acid–pyridine method (Song et al., 2013b). The sulfur content of the three sCVPs measured by the barium chloride–gelatin method (Dodgson & Price, 1962) was 14.55%, 11.71% and 9.48%, respectively. Briefly, chlorosulfonic acid was added dropwise to pyridine (v/v, 1:4, 1:6 and 1:8) in an ice-water bath, with stirring. CVPS (300 mg) dispersed in dry *N,N*-dimethylformamide (20 ml) was then added. After stirring in a water bath at 60 °C for 2 h, the mixture was neutralized with NaOH, dialysed and lyophilized to yield sCVPSs.

Cell culture and virus infection. CEF prepared as previously described (Zhao et al., 2011) were grown in Eagle’s minimum essential medium (MEM) supplemented with 10% (v/v) bovine calf serum (Gibco), 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin. Baby hamster kidney fibroblasts clone 21 (BHK-21) provided by the Institute of Prevention Veterinary Medicine, Chengdu, China, were grown in MEM supplemented with 10% (v/v) FCS (Hangzhou Tianhang Biological Technology), 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin. For maintenance medium (MM), the serum concentration was reduced to 2%.

NDV (Mukteswar strain) was provided by the Institute of Prevention Veterinary Medicine, Chengdu, China. Viruses were propagated in 9-day-old chicken embryo eggs and titrated by plaque formation on both CEF and BHK-21.

Cytotoxicity assay. The cytotoxicity of CVPS, sCVPSs and HS (H7640; sulfur content: 5–7%; Sigma-Aldrich) was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma). CEF and BHK-21 monolayers grown in 96-well plates were incubated with MM containing twofold dilutions of test polysaccharides. After 72 h incubation at 37 °C, 10 μl of PBS containing MTT (final concentration: 0.5 mg ml⁻¹) was added to each well. The plates were re-incubated for 4 h at 37 °C, the supernatant in each well was then aspirated and 200 μl of DMSO was added to solubilize the formazan crystals. After vigorous shaking, absorbance values were measured in a microplate reader (Bio-Rad) at 570 nm. The 50% cytotoxic concentration (CC50) was calculated as the concentration of compound required to reduce cell viability by 50%.

Antiviral activity assay. Antiviral activity was evaluated by plaque reduction assay as described before (Talarico & Damonte, 2007). CEF and BHK-21 monolayers grown in 24-well plates were infected with about 50 p.f.u. NDV per well. The plates were incubated at 37 °C for 2 h to allow adsorption and penetration. After being washed twice with PBS, MM containing 1% methylocellulose and dilutions of test polysaccharides were then added into each well and plates were incubated at 37 °C for 60 h. Plaques were counted as described above and IC50 was calculated.

Virus adsorption assay. CEF and BHK-21 monolayers grown in 24-well plates were infected with about 50 p.f.u. NDV per well in the absence or presence of various concentrations of test polysaccharides. After 1 h incubation at 4 °C, the compounds were washed three times with cold PBS to remove un-adsorbed viruses and then overlaid with MM containing 1% methylocellulose. The plates were incubated at 37 °C for 60 h. Plaques were counted as described above and IC50 was calculated.

Virus penetration assay. CEF and BHK-21 monolayers grown in 24-well plates were infected with about 50 p.f.u. NDV per well. The plates were incubated at 37 °C for 2 h to allow adsorption and penetration. After washing twice with PBS, MM containing 1% methylocellulose and dilutions of test polysaccharides were then added into each well and plates were incubated at 37 °C for 60 h. Plaques were counted as described above and IC50 was calculated.

Virus replication assay. CEF and BHK-21 monolayers grown in 24-well plates were infected with about 50 p.f.u. NDV per well. The plates were incubated at 37 °C for 60 h. Plaques were counted as described above and IC50 was calculated.

Virus inactivation assay. MM containing dilutions of test polysaccharides was incubated with an equal volume of virus suspension (5 × 10^4 p.f.u.) at 37 °C for 1 h. The solution was then diluted 10^4 times and the titre of residual infectious virus was quantified by plaque assay in both CEF and BHK-21.

Plaque size assay. CEF and BHK-21 monolayers grown in six-well plates were infected with about 100 p.f.u. per well. The plates were then incubated at 37 °C for 2 h to allow penetration. After the inocula had been removed, MM containing 1% methylocellulose and sCVPSs (50, 100 and 200 μg ml⁻¹) was added. The plates were incubated at 37 °C for 60 h and plaque size was calculated through Nikon Imaging Software Elements. The mean plaque size was obtained by measuring 20 plaques from each group (Antoine et al., 2012).

Indirect immunofluorescence assay. CEF and BHK-21 monolayers grown in coverslips were infected with NDV at an m.o.i. of 0.1 in the presence or absence of sCVPSs (200 μg ml⁻¹). After 24 h incubation, monolayers were washed with PBS and fixed in
paraformaldehyde for 30 min at room temperature. After washing three times with PBS, monolayers were permeabilized using 0.5% (v/v) Triton X-100 in PBS for 5 min before incubation with 2% BSA in PBS for 1 h at 37 °C. Monolayers were washed and incubated with anti-NDV polyclonal antibody (produced in our laboratory according to the methods of Talarico & Damonte, 2007) overnight at 4 °C. Then monolayers were washed and incubated with fluorescein (FITC)-labelled goat anti-rabbit IgG (Wuhan Boster Bio-engineering) for 1 h at 37 °C. Finally, cells were washed and directly observed using a fluorescence microscope (Eclipse 80i, Nikon) (Wang et al., 2011).

Real-time reverse-transcription PCR assay. CEF and BHK-21 monolayers grown in six-well plates were infected with NDV at an m.o.i. of 1 in the presence or absence of sCVPS (400, 200 and 100 μg ml⁻¹) and HS (100 μg ml⁻¹). After 1 h incubation at 37 °C, monolayers were washed three times with cold PBS to remove uninfected viruses and then total RNA was extracted from cells using RNAiso reagent (D9108; Takara) according to the manufacturer’s instructions. The forward and reverse primers based on the F gene of NDV (Mukteswar; GenBank accession number JF950509.1) were 5'-GCTATTGCGAGTACATGATC-TAMRA-3' (positions 5717–5739) and 5'-CGGCTCTCTCATATATTGTTG-3' (positions 5769–5789). A 24 bp probe, 5'-5' Carboxyfluorescein (FAM) -AGATGGCACTACCCCGGTTATC-TAMRA-3', complementary to an internal region between the two primers was also designed.

Standard NDV RNA was prepared by transcription in vitro. Briefly, primers with T7 promoter sequence (underlined) were 5'-GATTC-ACATATGACTGCACATATAGGGAGCCGACATGACCC-3' (positions 5670–5688) and 5'-GTCAGATGCTCGGTGCACAC T-3' (positions 5911–5930), which amply a 287 bp fragment by RT-PCR. Then the fragment was purified and in vitro transcribed by T7 kit (D2540; Takara) to yield standard RNA which was subsequently quantified by measuring the optical density at 260 nm. Serial 10-fold dilutions of standard NDV RNA from 1 x 10⁵ to 1 x 10⁸ copies/reaction tube were prepared to construct a standard curve by using the one step primeScript RT-PCR kit (Perfect Real-time) (DRR064; Takara). The rt RT-PCR assay was performed at 42 °C for 5 min, 95 °C for 10 s, 40 cycles of 95 °C for 5 s, 56 °C for 10 s and 72 °C for 10 s with the CFX96 Real-time PCR detection system (Bio-Rad).

In vivo antiviral activity tests. SPF fertile eggs were purchased from the Beijing Laboratory Animal Centre, Beijing, China, and hatched at our laboratory. When 14 days old, 60 SPF chickens were randomly divided into three groups. In sCVPS-treated and non-treated groups, chickens were challenged with 0.1 ml NDV suspension at a dose of 10⁵ 50% embryo infectious dose (EID₅₀) though nasal drip and eye-drop. Meanwhile, chickens in the sCVPS-treated group were administered 0.1 ml sCVPS₉₀₀ at a dose of 4 mg (kg body mass)⁻¹ through intramuscular injection, once a day for four successive days; in the untreated group, chickens were administered 0.1 ml physiological saline. After 4 days, three chickens from each group were killed and hearts, livers, spleens, lungs and kidneys were collected and homogenized in PBS (Dortmans et al., 2011). Then, virus titres were determined on CEF by the Reed–Muench method (Reed & Muench, 1938) and presented as the mean [log₁₀TCID₅₀ (g tissue)⁻¹] values.

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