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Phosphorylated *Codonopsis pilosula* polysaccharide could inhibit the virulence of duck hepatitis A virus compared with *Codonopsis pilosula* polysaccharide

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ABSTRACT

To screen effective anti-duck hepatitis A virus (DHAV) drugs, we applied STMP-STPP method to prepare phosphorylated *Codonopsis pilosula* polysaccharide (pCPPS), the phosphorylation-modified product of *Codonopsis pilosula* polysaccharide (CPPS). The IR spectrum and field emission scanning electron microscope (FE-SEM) were subsequently used to analyze the structure of pCPPS. Several tests were conducted to compare the anti-DHAV activities of CPPS and pCPPS. The MTT method was used to compare the effect of the drugs on DHAV-infected duck embryonic hepatocytes (DEHs), and the Reed-Muench assay was employed to observe changes in the virulence of DHAV. We also applied real-time PCR to examine the relationship between virus replication and the expression of IFN-β. The results indicated that CPPS could not inhibit the replication of DHAV. In contrast, pCPPS increased the virus $TCID_{50}$, inhibited viral replication and, accordingly, increased the survival rate of DEHs infected with DHAV. Because DHAV induced the expression of IFN-β, and the IFN-β expression level was positively associated with the number of DHAV, the reduction of IFN-β expression levels after pCPPS treatment demonstrated a decrease in the number of virus particles. These results indicated that pCPPS, which reduces the number of DHAV, was more effective than CPPS in anti-DHAV activity.

1. Introduction

Duck hepatitis virus (DHV), a member of Avihepatovirus in the family Picornaviridae, infects young ducklings and confers duck virus hepatitis (DVH), an acute and fatal disease causing serious economic losses for the world duckling production. DHV was first isolated in 1949 and is primarily divided into three serotypes, namely, DHV-1, DHV-2 and DHV-3 [1,2]. Thereafter, DHV-1 was renamed DHAV according to the results of the sequence analysis [3]. DHAV primarily infects ducklings aged less than three weeks with a mortality rate of more than 80% [4]. So far there is no effective therapeutic antiviral drug against DHAV in clinic except for egg yolk antibodies. However, egg yolk antibodies should be preserved and transported under freezing conditions, and not all farms could meet this requirement. In addition, the antibodies should be injected individually in the clinic, consuming manpower and increasing the stress response and potential risk of transmitting other diseases. Currently, the primary strategy to control this disease is the injection of an attenuated DHAV vaccine. However, the serious economic losses resulting from DVH remain high, reflecting immune failure [5]. Therefore, it is imperative to develop new drugs to treat DHAV infection.

Codonopsis pilosula (*Franch.*) *Nannf.* is a well-known traditional Chinese herbal medicine and has been used for thousands of years. This organism is commonly used as a Tonic Chinese herbal medicine, reflecting its antiviral activity. *Codonopsis pilosula* exhibits adequate effects on invigorating spleen Qi and lung Qi. As the pathogenesis of DVH is liver meridian and wind-heat syndrome, spleen-earth injury should be prevented after treatment.

Abbreviations: DHAV, duck hepatitis A virus; CPPS, *Codonopsis pilosula* polysaccharide; pCPPS, phosphorylated *Codonopsis pilosula* polysaccharide; DEHs, duck embryonic hepatocytes; DVH, duck virus hepatitis; DMEM, Dulbecco's modified Eagle's medium; MM, maintenance medium; D-Hank's, Dulbecco's Hanks balanced salt solution; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide; STPP, sodium tripolyphosphate; STMP, sodium trimetaphosphate; PBS, phosphate-buffered saline; CC, cell control; VC, virus control; Real-time PCR, real-time polymerase chain reaction; AGE, agarose gel electrophoresis.

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Therefore, *Codonopsis pilosula* was selected as a candidate therapeutic treatment. *Codonopsis pilosula* polysaccharide (CPPS) is a primary extraction of *Codonopsis pilosula*. CPPS, with a molecular mass of 1.1×10^4 Da, comprises a backbone of (1 → 3)-linked-β-D-galactopyranosyl, (1 → 2,3)-linked-β-D-galactopyranosyl and (1 → 3)-linked-α-D-rhamnopyranosyl residues [6]. Previous studies have shown that CPPS possesses antimicrobial, antitumor, and antioxidant activities [7–9]. Polysaccharide bioactivities are enhanced after modification. The modification methods include phosphorylation, sulfation, acetylation, etc. [10]. Among these methods, phosphorylation and sulfation are two primary modification methods that significantly enhance bioactivities. Moreover, compared with sulfation, phosphorylation is secure, convenient and environmentally friendly. Polysaccharide phosphate is an important derivative of polysaccharide, possessing good antitumor, antioxidant and antiviral activities [10–12]. Furthermore, Xiong et al. reported that phosphorylated icariin reduces the mortality of ducklings infected with DHAV, indicating that the anti-DHAV activity of icariin was improved after phosphorylation [13]. Phosphorylated *Codonopsis pilosula* polysaccharide (pCPPS) is the phosphorylation-modified product of CPPS. Thus, CPPS was phosphorylated and its antiviral activity was observed.

Type I interferons (IFNs), including IFN-α and IFN-β, are cytokines with antiviral activity [14,15]. The production of interferons relies on the corresponding signaling pathway induced by viral infection or by-products of viral replication [16]. IFN-β plays an important role against RNA viral infections. In the present study, we observed the dynamic changes of IFN-β at the transcription level post DHAV infection in vitro, and evaluated the changes in IFN-β expression after treatment with different viral dilutions. We also compared the anti-DHAV activity of CPPS and pCPPS. These results will be used to screen effective anti-DHAV drugs in the clinic and provide references for research on DHAV.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with penicillin 100 IU/mL, streptomycin 100 IU/mL, and glutamine 0.75 mg/mL, and 10% fetal bovine serum was used as nutritive medium or 1% fetal bovine serum used as maintenance medium (MM). Dulbecco's Hanks balanced salt solution (D-Hank's) was used for washing embryo tissue fragments and cells. The pH levels of both DMEM and D-Hank's pH were adjusted to 7.4 using 5.6% $NaHCO_3$. Trypsin (Amresco, USA) was dissolved into 0.20% with D-Hank's, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT, Amresco, USA) was dissolved into 1 mg/mL with phosphate-buffered saline (calcium and magnesium-free). The dissolved reagents were filtered through 0.22 μm syringe filters and stored at 4 °C, and MTT was stored at 4 °C in dark bottles. The other chemicals used in the experiment were analytical grade.

Sodium trimetaphosphate (STMP, Lot no. L1226014) was purchased from Aladdin Industrial Corporation. Sodium tripolyphosphate (STPP, Lot no. 20041228) and ethylenediamine tetraacetic acid disodium salt (EDTA, Lot no. 20120703) were obtained from the Sinopharm Group Chemical Company. TRIS (CAT no. T8060) was purchased from Solarbio Science and Technology Co., Ltd. Boric acid (Lot no. 151108) was obtained from the Xilong Chemical Co., Ltd. RNAiso Plus Reagent (Lot no. 9108), the PrimeScript™ RT Master Mix Kit (Lot no. RR036A) and SYBR® Premix Ex Taq™ (Tli RNaseH Plus) Kit (Lot no. RR036A) were purchased from Takara. PCR Easy (Cat no. PE-2003) was obtained from Aogene Biotech Co., Ltd. Regular agarose (Lot no. 111860) was obtained from Biowest.

2.2. Duck embryonic hepatocytes (DEHs)

DEHs were prepared as previously described [17]. Briefly, the livers were sterilely isolated from 14 to 16-day-old duck embryos and digested into single cells. Prior to incubation in a humid atmosphere of 5% CO_2 at 37 °C, the seeding density of the cells was adjusted to 0.8×10^6–1.2×10^6/mL. The hepatocytes were taken as standby when cultured as a monolayer.

2.3. Preparation of CPPS and phosphorylated CPPS

2.3.1. Extraction of CPPS

The CPPS was extracted using water-extraction and alcohol-precipitation method [6]. Briefly, 500 g of *Codonopsis pilosula* was reflux extracted three times with 95% ethanol for 5 h each time. Subsequently, the medicine was soaked with 10-fold water, decocted and reflux extracted four times for 3 h each time. The supernatant that collected after the extraction was concentrated to 500 mL followed by centrifugation to remove the impurities. Ninety-five percent ethanol was added to the supernatant to obtain a final ethanol concentration of 80% (v/v). After 24 h, the precipitation was vacuum dried at 56 °C, and 100.40 g CPPS was obtained. The polysaccharide content was 91.33%, as determined using the phenol-sulfuric acid method [18].

2.3.2. Preparation of pCPPS

pCPPS was prepared using the STMP-STPP method [13]. The phosphorylation modification conditions included a 6 h reaction time, 70 °C reaction temperature, an 8.5 pH value and a 5:2 ratio of sodium tripolyphosphate to sodium trimetaphosphate. The polysaccharide content was determined using the phenol-sulfuric acid method [18], while the phosphate radical content was determined using the ascorbic acid method [19]. The pCPPS content was 73.37%, calculated as the sum of the polysaccharide and phosphate radical contents.

2.3.3. Infrared spectroscopy analysis

The IR spectrum of CPPS and pCPPS was recorded at a 4000–400 cm^{-1} wavenumber range using a Nicolet 200 Magna-IR spectrometer (Nicolet Instrument Corp.) [20]. OMNIC software (Nicolet Instruments Corp.) was used to analyze the major peaks (intensity and wavenumber).

2.3.4. Scanning electron microscope

The CPPS and pCPPS were coated with gold and observed with a field emission scanning electron microscope (S-4800 FE-SEM, Hitachi High-Technologies, Japan) at an accelerating voltage of 15 kV, as well as image magnifications of 40,000×.

2.4. Antiviral activity of CPPS and pCPPS in vitro

2.4.1. Antiviral effect of CPPS and pCPPS

The maximum safe concentration was 625 μg/mL for CPPS and 39 μg/mL for pCPPS, according to the results of preliminary experiments. CPPS was diluted with MM from 625 to 78 μg/mL according to its net content, and pCPPS was diluted from 39 to 4.88 μg/mL. DHAV (LQ_2 strain) solution was added at 100 μL per well to a 96-well plate containing the DEH monolayer, except for cell control (CC) group, which was treated with MM. Subsequently, the cells were incubated in a humid atmosphere of 5% CO_2 at 37 °C for 2 h. The virus solution was removed, and the cells were washed three times with D-Hank's. Thereafter, CPPS and pCPPS solutions (100 μL) at different dilutions were added to the test wells, with six repetitions per concentration. In addition, the virus control (VC) group and CC group were treated with MM. Subsequently, the cells were incubated at 37 °C till an obvious cytopathic effect was observed in