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Inhibitory Effect of *Portulaca oleracea L.* Polysaccharide on Porcine Rotavirus

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Abstract **Original Research Article**

Porcine rotavirus (PoRV) infection can cause diarrhea, dehydration, vomiting and even death of piglets, which has caused great economic losses to the global pig industry. In this study, the inhibitory effect of *Portulaca oleracea L.* polysaccharide (POL-P) on porcine rotavirus (PoRV) was investigated by using porcine small intestine epithelial cells (IPEC-J2. CCK-8 method was used to detect the cytotoxicity of POL-P to IPEC-J2 cells. PoRV was infected with IPEC- J2, and a virus infection model was established in *vitro*. The maximum safe concentration of POL-P and the effect of POL-P on VP6 protein and mRNA expression were detected by CCK-8, Western blot and RT-qPCR. In addition, immunofluorescence was also used to detect the inhibition of VP6 protein expression of PoRV. The results showed that the cells treated with 100, 200, 400 μg/ml POL-P for 24 h had no toxic effect on the cells. POL-P showed inhibitory effects of protein and mRNA expression on the virus, and the concentration of 400 μg/ml displayed the best inhibitory activity. Indirect immunofluorescence detection showed that the VP6 protein expression was inhibited under POL-P treatment. In conclusion, POL-P has an obvious inhibitory effect on PoRV in *vitro*.

Keywords: *Portulaca oleracea L.* polysaccharide, porcine rotavirus, VP6.

INTRODUCTION

Porcine Rotavirus (PoRV) belongs to rotavirus genus of reovirus family. The incubation period of acute enteric infection caused by Porcine Rotavirus is usually about 12~24 hours. Pigs of all ages are infected, but most infected pigs are piglets lacking maternal antibodies. The piglets are younger, and the case fatality rate is higher [1]. In 1979, human rotavirus was detected for the first time in China, and other animal rotaviruses (water buffalo, pig, horse, sheep, monkey, monkey, rabbit, dog, giant panda and poultry) were isolated successively [2]. PoRV is one of the viruses that cause acute intestinal infectious diseases in piglets aged 10 to 60 days old. It mainly infects the small intestine of pigs through horizontal transmission of oral-fecal route [3]. In 1974, Woode and Bridge isolated the mixture of PoRV and the simple infection caused a serious impact on the productivity of the pig population, and also brought huge economic losses to the breeding industry [4]. Vaccination is an important means to prevent and control the disease, but the effectiveness of the vaccine is not good, and the virus can mutate, leading to repeated outbreaks [5]. Western medicine is mainly symptomatic treatment, but there are more adverse reactions and easy to relapse. Currently, there is no specific drug for PoRV. Therefore, it is of practical significance and urgency to find anti-porv drugs with high efficiency and low toxicity.

Portulaca oleracea L. is widely distributed in all parts of the country. It has been used for treating a variety of diseases such as anti-vomiting, diuretic, febrifuge anti-bleeding and gastric mucosal infection based on its clearing heat, detoxifying and cooling blood efficacy. It is also a high-quality livestock and poultry feed resource, with high development and utilization value [6]. Modern pharmacological studies

have also shown that *Portulaca oleracea* L. and its extracts have a variety of biological and pharmacological activities, such as anti-inflammatory, anti-tumor, antiviral, etc. [7, 8]. In addition, POL-P also has a variety of biological activities, such as anti-aging and neuroprotection [9]. Studies have demonstrated that *Portulaca oleracea* L. extract has an inhibitory effect on porcine epidemic diarrhea virus (PEDV) [10] in vitro. Although *Portulaca oleracea L.* can be used as an antiviral agent for a variety of viruses, the effect of *Portulaca oleracea L* polysaccharide (POL-P) on PoRV is unclear. In this study, we examined whether POL-P has an inhibitory effect on PoRV.

MATERIALS AND METHODS

Materials

The cells used in this experiment were porcine small intestine epithelial cells IPEC-J2. Porcine Rotavirus (PoRV) A strain HJ-2016 (GenBank entry number: HJ-2016; MH423866) was provided by the Laboratory of Porcine Infectious Disease Control, College of Veterinary Medicine, Heilongjiang Bayi Agricultural University. POL-P was provided by our laboratory.

Cytotoxicity Tests

CCK-8 method was used to detect cell viability. The cultured IPEC-J2 cells were inoculated on three identical 96-well plates with 100 μL in each well and cultured in a 5% CO_2 incubator at 37℃ for 24 h. The medium of each well was sucked out, and 10 μL POL-P of different concentrations (0,25,50,100,200,400,800,1600 μg/ml) was added to the culture plate for 12 h, 24 h and 48 h, respectively 10 μL CCK-8 and 90 ml DMEM were added to each well to continue the culture for 1 h. The absorbance (OD value) of 450 nm wavelength was detected by enzyme-labeled instrument, and cell activity was calculated according to the absorbance formula: cell viability (%) =[(As-Ab)/(Ac-Ab)] × 100% (As: absorbance of experimental group; Ac: absorbance of control group; Ab: absorbance of the blank group.).

RT-qPCR

IPEC-J2 cells infected with PoRV were added to POL-P for 24 h, washed three times with PBS, mixed with 1 ml Trizol reagent, and total RNA was extracted from the cells according to the operation method in the instructions. The qRT-PCR volume was 20 μL, consisting of 2×SYBR Premix Ex Taq (TaKaRa, Kusatsu, Japan) 10 μL, forward and reverse primers 0.5 μL (10 p mol/L), cDNA 4 μL, and sterile water 5 μL. The reaction conditions were as follows: denatured for 30 s at 95℃, 40 s at 95℃, 60 s at 60℃, and 40 s at 72℃. The primers used are as follows:

PoRV VP6-F:5'- GATTCGTGTTCCATAAGCCAAA-3';
PoRV VP6-R:5'-CTGATCCAGCGTTAATCCACATAG-3'.

Western blot detection of VP6 protein expression

VP6 is a group-specific inner shell protein of PoRV, encoded by segment 6 with 397 amino acids and a molecular weight of approximately 45 ku. VP6 is the most abundant protein in PoRV, representing about 51% of the total viral protein amount [14, 15]. It is the main structural protein to maintain the stability of viral particles and is the most effective vaccine target against PoRV [11]. It plays an important role in viral entry, replication, and maintenance of virion morphology [12]. It not only plays an important role in the replication and assembly process of viruses, but also is highly immunogenic and antigenic, and is the most important pathogen causing the pathogenesis of human and animal infections [13].

IPEC-J2 cells were scraped off the cell scraper and collected into the EP tube. 1 mL PBS was added, centrifuged 600 g for 5 min, and the supernatant was discarded. 80 μL cell lysate was added into EP tube (1 ml of cell lysate was added into 10 ml PMSF), and ice lysate was added for 20 min. After the lysis, the EP tube was centrifuged (12000 g, 10 min, 4℃). After centrifugation, the precipitation was discarded and the supernatant was retained. Add 20μL 5×buffer, blow and mix well. Place the EP tube at 100℃ for 15 min and then place it at -20℃ for storage. The extracted total protein was separated and transferred to PVDF membrane by SDS-PAGE electrophoresis, and incubated with the corresponding primary antibody. Overnight at 4℃, the membrane was washed at room temperature, and the secondary antibody was incubated for 1 h. The hypersensitive ECL chemiluminescence kit was used for color rendering, the protein expression was observed with gel imager, and the gray values of the bands were measured.

Immunofluorescence test

IPEC-J2 cells were inoculated into 12-well culture plates and cultured in a 5% CO_2 incubator at 37℃. The experiment was divided into three groups: normal control group, infected cells+POL-P (400 μg/ml) group and infected cells group. The cells were fixed with 4% PFA for 15 min, washed 3 times with PBS solution, incubated with monoclonal antibody of VP6 protein overnight, incubated with fluorescent goat anti-mouse secondary antibody at room temperature for 2 h, then added with DAPI dye solution for 15 min, cleaned with PBS solution, and observed the expression of protein under fluorescence microscope.

Data Processing

All the data were processed and analyzed by SPSS 22.0 statistical software, and the comparison of the two groups of data was conducted by the *T-test* of the comparison of the two sample means. One-way analysis of variance was used for the comparison of samples between multiple groups. All data are presented as the mean 3 standard deviations. For all

analysis, $P < 0.05$ were considered statistically significant ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$)

RESULT

The cytotoxicity of POL-P in vitro

In order to study the cytotoxicity of POL-P in vitro, CCK-8 was used to detect the relative cell viability. As shown in Figure 1, after 12 h of treatment, the cell activity of POL-P treated with different concentrations was higher than that of the control group, and the cell survival rate was higher than 90%. After 24 h of treatment, compared with the control group, the cell activity was not significantly changed after 25~400 μg/ml treatment, the cell vitality was slightly decreased after 800 μg/ml treatment, and significantly decreased after 1600 μg/ml treatment. The cell viability after 48 h treatment was similar to that after 24 h treatment. In conclusion, the cell viability decreased significantly when POL-P concentration was 800 μg/ml or higher than 800 μg/ml. Therefore, in this experiment, POL-P concentrations of 100, 200, 400 μg/ml were used to treat cells for 24 h as follow-up research conditions for further in-depth study.

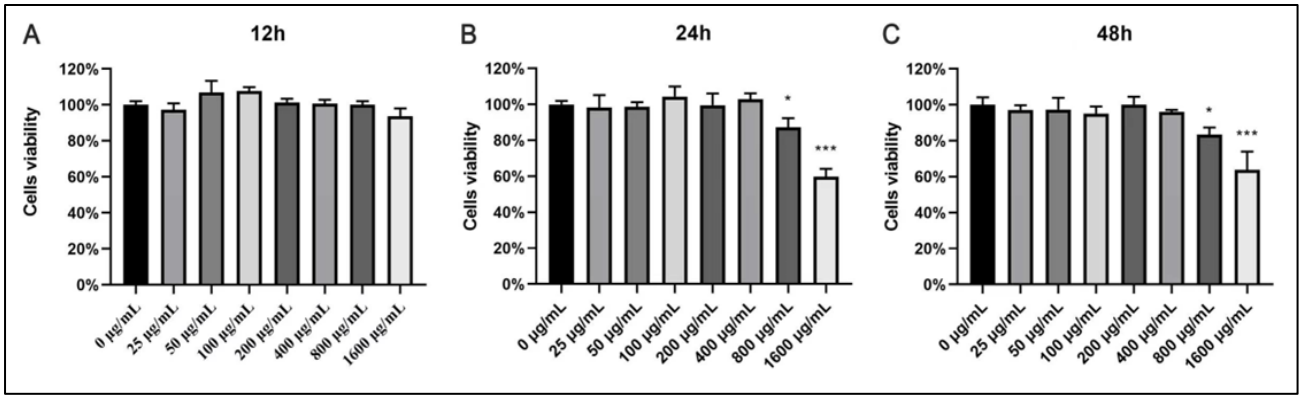

Figure 1: Effects of POL-P on cell viability1; (A) Effects of POL-P on cell viability at 12 h (B) Effects of POL-P on cell viability at 24 h (C) Effects of POL-P on cell viability at 48 h

The effect of POL-P on mRNA expression of PoRV was detected by RT-qPCR

In order to explore the influence of POL-P on the mRNA expression of PoRV, qRT-PCR was used for analysis, and the specific results were showed in Figure 2. After treatment with different concentrations of POL-P (100, 200 and 400 μg/ml) and infected cells, compared with positive control, with the increase of POL-P concentration, the mRNA expression of PoRV decreased. The results suggested that POL-P could inhibit mRNA expression of PoRV.

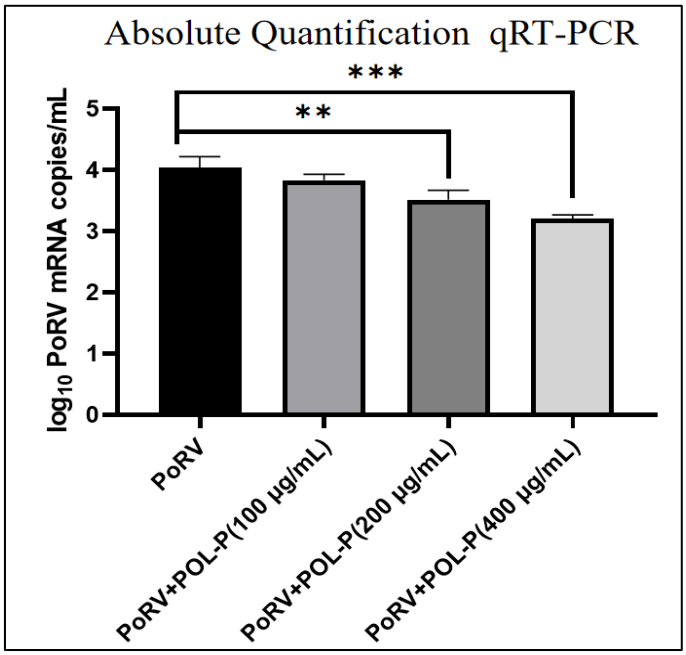

Figure 2: Effect of POL-P on the mRNA expression of PoRV

The effect of POL-P on VP6 protein expression of PoRV was detected by western blot

The effect of POL-P on the content and properties of VP6 protein was detected by Western blot. POL-P of different concentrations (100, 200, 400 μg/ml) was applied to PorV-infected cells respectively. The results showed that, compared with positive control, POL-P of three concentrations could inhibit PoRV and improve cell activity, and with the increase of concentration, the inhibition effect was more obvious (Figure. 3A, Figure. 3B). The results suggested that POL-P could inhibit VP6 protein expression of PoRV.

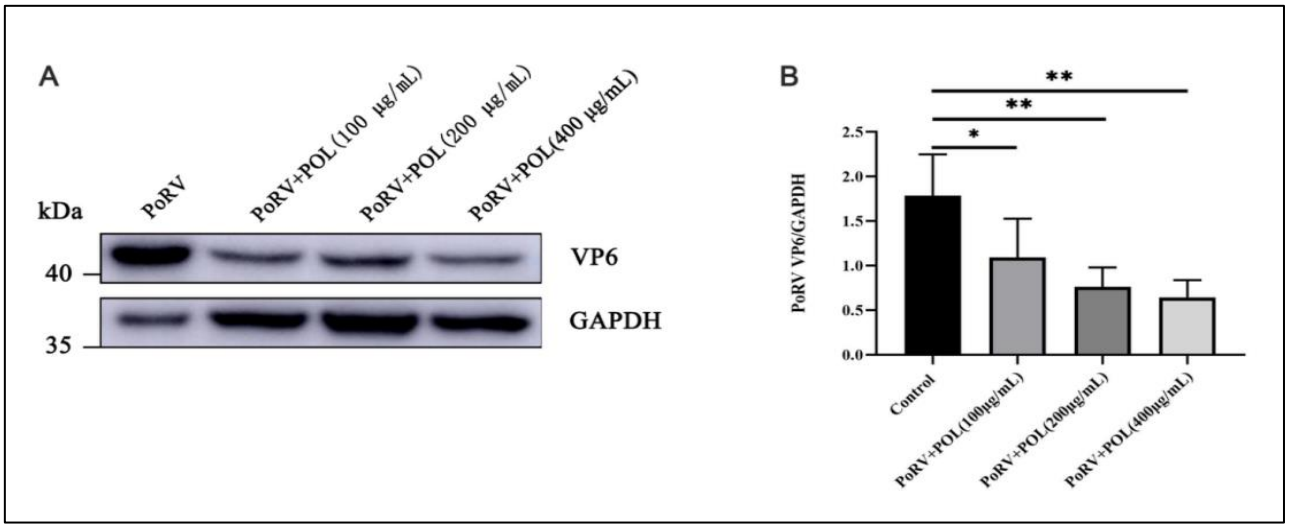

Figure 3: Effect of POL-P on the proein expression of PoRV; (A) The VP6 protein level was analyzed by western blotting; (B) Intensity represents the normalization of VP6 protein levels to GADPH levels in three independent experiments

Morphological observation of POL-P on infected PoRV cells

According to the above experimental results, the effect of 400 μg/ml POL-P was the best. We selected 400 μg/ml POL-P to treat PoRV for 24 h and observed its morphological changes. As showed in Figure 4, the protein of the normal control group was dense and granular, and the protein of the infected cells was less and large. However, the protein expression of PoRV was inhibited to a certain extent after the addition of 400 μg/ml POL-P.

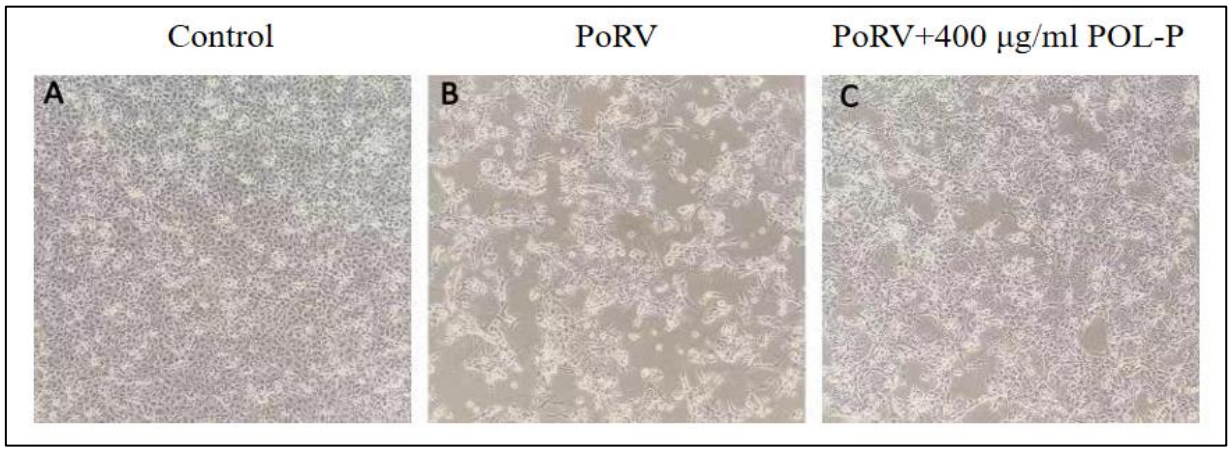

Figure 4: Morphologic results (×10); (A): normal control group; (B): group infected with PoRV cells; (C): POL-P treated group

The effect of POL-P on VP6 protein of PoRV was detected by immunofluorescence

Indirect immunofluorescence was used to detect the effect of POL-P on PoRV. As showed in Figure 5, only a very small amount of green fluorescence was observed in the control group, meaning that the VP6 protein content was very low, while the cells infected with PoRV had a large amount of green fluorescence, indicating that the VP6 protein was strongly expressed. After POL-P treatment, the green fluorescence of cells was significantly reduced. VP6 protein expression was inhibited, and the protein content was significantly decreased compared with untreated cells. This further verified the inhibitory effect of POL-P on the expression of PoRV protein.

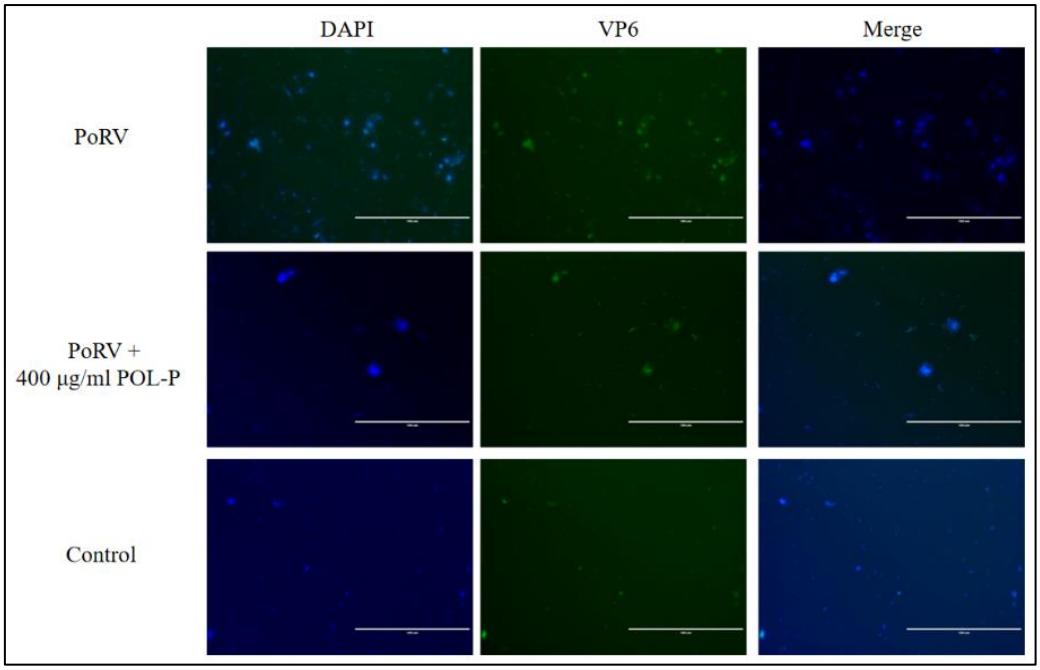

Figure 5: Fluorescence analysis of VP6 protein expression in cells

DISCUSSION

Malantha polysaccharide has several biological functions. Guo Junchao [14] *et al.*, explored the antitumor activity of POL-P by using the nude mouse model of cervical cancer, and showed that POL-P could effectively promote the apoptosis of transplanted tumor cells and inhibit the tumor growth. Niu Guangcai [15] *et al.*, showed that the anti- tumor activity of POL-P was associated with its interference with oxidative supply energy and promoting lymphocyte transformation. POL-P has anti-inflammatory function and protects the gastrointestinal tract. Aqueous extract of POL-P is protective against diabetes and related vascular complications. Bai Yu [16] *et al.*, established the diabetic rat model and explored the mechanism of POL-P in regulating blood glucose.

PoRV is widespread in many Asian countries, of which Rotavirus type A is the most common. The positive infection rate of porcine rotavirus A is 3.30%-67.30% in pigs and 61.00%-74.00% [17] in pig farms. The main target cells of PoRV were the mature epithelial cells at the end of small intestine villi. After infection with PoRV, epithelial cells are destroyed, intestinal villi become shorter and less dense, and immature cells from intestinal crypts replace mature cells, thus causing absorption disorders, dyspepsia and osmotic diarrhea.

Rotavirus particles are composed of three layers of capsid. The protein VP6, which forms the middle layer of capsid, is the antigen that determines the grouping of viruses and is the common antigen of any species of rotavirus in the same group. It shows high conserved expression in the same group and is the highest protein content in rotavirus. Studies have shown that the earliest and main antibody response after RV infection is against VP6, and VP6 is highly immunogenic and antigenic [18]. Therefore, VP6 of rotavirus is an important molecule in RV vaccine development and detection methods, and has become a hot spot in rotavirus vaccine development and virus detection methods, and the expression of VP6 protein is the basis of related research. The experimental results of this study showed that POL-P had dose-dependent effects on PoRV VP6 protein, mRNA level and virus titer. The inhibitory effect of POL-P on PoRV was greatest when the concentration of POL-P was 400 μg/ml. It is possible that POL-P could be developed into a novel antiviral drug for the control of PoRV.

CONCLUSION

POL-P, the active ingredient of *Portulaca oleracea* L. had an obvious inhibitory effect on PoRV in *vitro*. The experimental results provide a theoretical basis for developing POL-P into a new drug with high efficiency for the treatment of piglet diarrhea.

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