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Antiviral activity and underlying molecular mechanisms of Matrine against porcine reproductive and respiratory syndrome virus in vitro

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS), caused by porcine reproductive and respiratory syndrome virus (PRRSV), is an acute infectious disease. The prevalence of PRRS has made swine industry suffered huge financial losses. Matrine, a natural compound, has been demonstrated to possess anti-PRRSV activity in Marc-145 cells. However, the underlying molecular mechanisms were still unknown. The main objective of our study was to discuss the effect of Matrine on PRRSV N protein expression and PRRSV induced apoptosis. Indirect immunofluorescence assay (IFA) and Western blot were used to assess the effect of Matrine on N protein expression. Apoptosis was analyzed by fluorescence staining. In addition, the effect of Matrine on caspase-3 activation was investigated by Western blot. Indirect immunofluorescence assay and Western blot analysis demonstrated that Matrine could inhibit N protein expression in Marc-145 cells. And Matrine was found to be able to impair PRRSV-induced apoptosis by inhibiting caspase-3 activation.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a devastating swine disease worldwide. The causative agent, porcine reproductive and respiratory syndrome virus (PRRSV), is a single-stranded RNA virus in the Arteriviridae family, order Nidovirales. Its genome with the size of approximately 15 kb encodes at least eight open reading frames (ORFs). ORF7 encodes the nucleocapsid (N) protein which is the most abundant and immunogenic viral protein among all proteins in PRRSV (Dokland, 2010). Porcine reproductive and respiratory syndrome was first discovered in China in 1996 and a severe outbreak of this syndrome occurred in 2006 which affected more than two million pigs across more than ten provinces in China (Tian et al., 2007). The currently available measure for controlling and preventing the syndrome mainly depends on vaccination. The vaccines suffer from problems in either controlling complicated outbreaks or genetic variants of the virus. Moreover, PRRS can suppress immune system. Therefore, finding safe, effective and inexpensive ways to control PRRSV infection is impetus.

Traditional Chinese medicines, science antiquity to date, have been widely used to treat infectious diseases. Matrine, a major quinolizidine alkaloid purified from the dried roots of *Sophora flavescens* Ait (Chinese Pharmacopoeia 2005), has been found to display multi-pharmacological effects including antivirus, anti-inflammation, and immunity-regulation (Azzam et al., 2007; Cao et al., 2011; Jiang et al., 2007; Ma et al., 2008). Our previous research data showed that Matrine could inhibit PRRSV infection on Marc-145 cells and the mode of antiviral action of Matrine on PRRSV infection may be able to directly inactivate PRRSV and interfere with its replication within cells (Zhao et al., 2013). All these results were encouraging to further explore the exact molecular mechanisms on anti-PRRSV of Matrine. In this study, effects of Matrine on PRRSV N protein expression and PRRSV induced apoptosis were evaluated to further clarify the molecular mechanism of Matrine against PRRSV in vitro.

2. Materials and methods

2.1. Test compounds

Matrine and Ribavirin were purchased from National Institutes For Food and Drug Control (Beijing, PR China). The batch numbers were respectively 110805-200508 and 140629. The purity of

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Matrine was greater than 99%. These compounds were dissolved and diluted in DMEM (Dulbecco's Modified Eagle Medium, Sigma, USA) with 2% FCS (fetal calf serum, Hyclone, USA). Ribavirin was used as a positive control (Kim and Lee, 2013). In the previous study, cytotoxicity and anti-PRRSV activity of Matrine were measured with MTT test and the analyses of cytopathogenic effect (CPE) in Marc-145 cells. These results showed that the maximum non-cytotoxic concentration (MNTC) of Matrine and Ribavirin were 0.75 and 0.125 mg/ml, respectively. And the maximum inhibition ratio of Matrine and Ribavirin were 93.6 and 61.4, respectively. When the concentration of Matrine was lower than 0.1875 mg/ml, the inhibition ratio was lower than 20% (Zhao et al., 2013). In order to display dose-dependent and ensure non-cytotoxicity, the first three concentrations of Matrine (0.75, 0.375 and 0.1875 mg/ml) were used to evaluate the effect on PRRSV N protein expression in the following experiment.

2.2. Cells and virus

Marc-145 cells, a subclone of the African green monkey kidney epithelial cell line, obtained from China Institute of Veterinary Drug Control (Beijing, PR China), were grown in DMEM supplemented with 10% FCS, 100 IU/ml penicillin G and 100 μg/ml streptomycin at 37 °C in a humidified cabinet containing 5% CO_2 atmosphere.

PRRSV vaccine (JXAI-R, No. 1012001, Guangdong Dahuanong Animal Health Products Co., Ltd., PR China) was propagated in Marc-145 cells. Virus titers, calculated by the method of Reed and Muench (Reed and Muench, 1938), was $10^{7.5}$ $TCID_{50}$/ml. Virus liquids were stored at −80 °C till use.

2.3. Indirect immunofluorescence assay (IFA)

Procedures for IFA were carried out as previously reported (Sun et al., 2012) with some modifications. In one procedure, the confluent monolayer of Marc-145 cells in 384-well culture plates were infected with 20 μl of each of three different concentrations of Matrine and a constant amount of 100 $TCID_{50}$ PRRSV. In the other procedure, cells were pre-infected with 20 μl PRRSV for 2 h at 37 °C, virus medium was then removed and washed thrice in PBS, and finally each of three different concentrations of Matrine were added. All these plates were incubated at 37 °C in a 5% CO_2 humidified for 48 h. All cells were then fixed with cold mixtures of acetone and methanol (1:1) for 30 min at −20 °C and washed thrice in PBS. The infected cells were incubated with monoclonal antibodies against N protein (1:50) for 2 h at 37 °C and washed thrice in PBS. Afterwards fixed cells were incubated for 1 h at 37 °C in FITC-conjugated goat anti-mouse IgG (Bioss, Beijing, PR China) at 1:30 dilution and washed thrice in PBS. The infected cells treated with antibodies were immediately visualized with fluorescence microscope. Cells were also stained with DAPI (4′,6-diamidino-2-phenylindole, 1:20, KeyGen, Nanjing, PR China).

2.4. N protein determination by Western blot

In parallel, N protein expression in the cells was determined using Western blot. Above-mentioned cell samples (described in Section 2.3) were prepared. The total protein was extracted using a total protein extraction kit (Applygen, PR China) according to the manufacturer's instructions. Protein concentration was estimated by NanoDrop spectrophotometer (ND-1000, USA). After heat denaturation at 95 °C for 5 min. 20 μg of protein were loaded on sodium dodecyl sulfate polyacrylamide gel (SDS–PAGE) with a 4% stacking gel and a 12% gradient gel. At the end of the electrophoresis, proteins were transferred to a PVDF membrane and then blocked with 5% skim milk in TBST with gentle shaking at room temperature for 2 h. The membrane was incubated with anti-N protein antibody (diluted 1:1000) at room temperature for 2 h. After washing the membrane with TBST thrice, membrane strips were incubated with a 1:5000 dilution of HRP (horseradish peroxidase) conjugated anti-mouse IgG at 37 °C for 1 h with gentle shaking and then washed with TBST thrice again. Detection was performed with enhanced chemiluminescence (eECL kit, Cowin Biotech Co., Ltd., China) and recorded on an X-ray film (Kodak). β-Actin was determined as a loading control to normalize samples.

2.5. Cell apoptosis determination with fluorescence microcopy

Cell apoptosis was analyzed using fluorescence microscope as previously described with some modifications (Sui et al., 2010). Briefly, the confluent monolayer of Marc-145 cells in 384-well plates was prepared, and then Matrine and PRRSV were added (the finally concentration of Matrine was 0.75 mg/ml, and virus concentration was 100 $TCID_{50}$). Cells control and PRRSV control were set up simultaneously. After 24, 48 and 60 h incubation at 37 °C in a 5% CO_2 humidified atmosphere, cells were separately treated according to the protocol of the AnnexinV-EGFP Kit (Nanjing KeyGen Biotech. Co., Ltd., Nanjing, PR China) and then analyzed by fluorescence microscope.

2.6. Detection of caspase-3 using Western blot

Cell samples were prepared at 48 h post-infection as described in the above section. The protein samples (50 μg) were separated by SDS–PAGE with a 4% stacking gel and a 15% gradient gel. The proteins in SDS–PAGE were transferred onto PVDF membranes and blocked with TBST containing 5% skim milk at room temperature for 2 h. Then the membranes were incubated with rabbit anti-caspase-3 (diluted 1:1000, Cell Signaling Technology, #9662) and mouse anti-β-actin (diluted 1:1000, CWBIO, #CW0096) at 4 °C overnight with gentle shaking. After washing the membrane with TBST thrice, membrane strips were incubated with different secondary antibodies matching to their respective primary antibodies at 1:5000 dilutions at 37 °C for 1 h with gentle shaking and then washed with TBST thrice again. Protein detection was performed with enhanced chemiluminescence (eECL kit, Cowin Biotech Co., Ltd., PR China) and recorded on an X-ray film (Kodak).

3. Results

To determine the inhibitory effect of Matrine on N protein, Marc-145 cells were simultaneously infected with PRRSV and Matrine or pre-incubated with PRRSV followed by Matrine. The expression of the N protein of these treated cell samples were then analyzed by IFA and Western blot. The data showed that Matrine inhibited N protein expression in a dose dependent manner. As shown in Fig. 1, with the lower concentration of Matrine, the more N protein (green florescence) was detected. From Fig. 1D and G, or 1E and H, even if at the same concentration of Matrine, the N protein expression levels were different according to the modes of adding the compound. Obviously, the expression of N protein was less in the mode of adding PRRSV and Matrine simultaneously onto cells. And the results of Western blot were also consistent with the data from the IFA, demonstrating that Matrine could both inactive PRRSV directly and interfere with PRRSV replication. In addition, from Fig. 1E and G, we observed a characteristic pattern of small foci of positive cells, suggesting the drug may inhibit PRRSV dissemination.

The cell apoptosis was analyzed with Annexin V-EGFP and PI double staining. The results showed that PRRSV induced early and late apoptosis (Fig. 2). As indicated in Fig. 3A, PRRSV-infected