Short Communication

Suppression of porcine reproductive and respiratory syndrome virus proliferation by glycyrrhizin

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ABSTRACT

Glycyrrhizin is a natural component extracted from the roots of *Glycyrrhiza glabra*. In this study, we investigated the antiviral activity of glycyrrhizin against porcine reproductive and respiratory syndrome virus (PRRSV), an *Arterivirus* that has been devastating the swine industry worldwide since the late 1980s. Our results showed that treatment with glycyrrhizin significantly reduced PRRSV proliferation and PRRSV-encoded protein expression in a dose-dependent manner. Mechanistically, glycyrrhizin mainly inhibits the penetration stage, and has little effect on the steps of adsorption or release of PRRSV in its life cycle. Furthermore, we were able to exclude a direct inhibitory action of glycyrrhizin on PRRSV particles. Given these results, glycyrrhizin may be a candidate component for a novel porcine reproductive and respiratory syndrome (PRRS) control strategy.

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Porcine reproductive and respiratory syndrome (PRRS) is an economically important infectious disease characterized by severe reproductive failure in sows and respiratory distress in piglets and growing pigs (Lunney et al., 2010; Meng, 2012). The causative agent, PRRS virus (PRRSV), is a single-stranded positive-sense RNA virus classified within the *Arteriviridae* family (Nelsen et al., 1999; Snijder et al., 2013). Since its emergence in the late 1980s, PRRS has been a threat to the global swine industry (Neumann et al., 2005).

At present, vaccination is the most prevalent way of controlling PRRSV infection. Unfortunately, the current commercially vaccines fail to provide sustainable disease control and prevention (Huang and Meng, 2010; Murtaugh and Genzow, 2011; Yoo et al., 2004). Antiviral therapeutics provide a pivotal tool for combating viral infections, especially for cases in which existing vaccines fail to match the circulating virus. Therefore, an alternative measure to control PRRSV, such as pharmacological intervention, is needed. Traditional Chinese medicines provide a valuable source for promising antiviral drugs. Many natural compounds and compositions have been proven to have antiviral activities, including

against PRRSV, such as flavaspidic acid AB (Yang et al., 2013), ouabain, valinomycin, bufalin (Karuppannan et al., 2012), and morpholino oligomer (Opriessnig et al., 2011).

Glycyrrhizin, a triterpene saponine, is a constituent of licorice root. Previous studies have demonstrated that glycyrrhizin has the ability to inhibit the proliferation of many viruses, such as hepatitis B virus (HBV) (Sato et al., 1996), hepatitis C virus (HCV) (Matsumoto et al., 2013), herpes simplex virus (HSV) (Huang et al., 2012), SARS coronavirus (Cinatl et al., 2003), and influenza viruses (Wolkerstorfer et al., 2009). In the present study, we aimed to assess the antiviral activity of glycyrrhizin against PRRSV and further elucidate the exact phase of the viral reproductive cycle that is affected by glycyrrhizin.

We first tested the cytotoxicity of glycyrrhizin (glycyrrhizic acid ammonium salt, Sigma–Aldrich) to MARC-145 cells, the permissive cells for PRRSV infection *in vitro*, by MTT assay as described previously (Yang et al., 2013). As shown in Fig. 1A, no appreciable cytotoxicity in MARC-145 cells could be observed at concentrations ranging from 200 to 800 μM. To test whether glycyrrhizin has antiviral activity against PRRSV, MARC-145 cells were pretreated with glycyrrhizin at the indicated concentrations and then infected with PRRSV strain WUH3 (at 0.1 PFU/cell). Plaque assays were performed to determine virus titers of the infected cells at the indicated times post-infection. As shown in Fig. 1B, treatment with glycyrrhizin resulted in a notably significant reduction of PRRSV
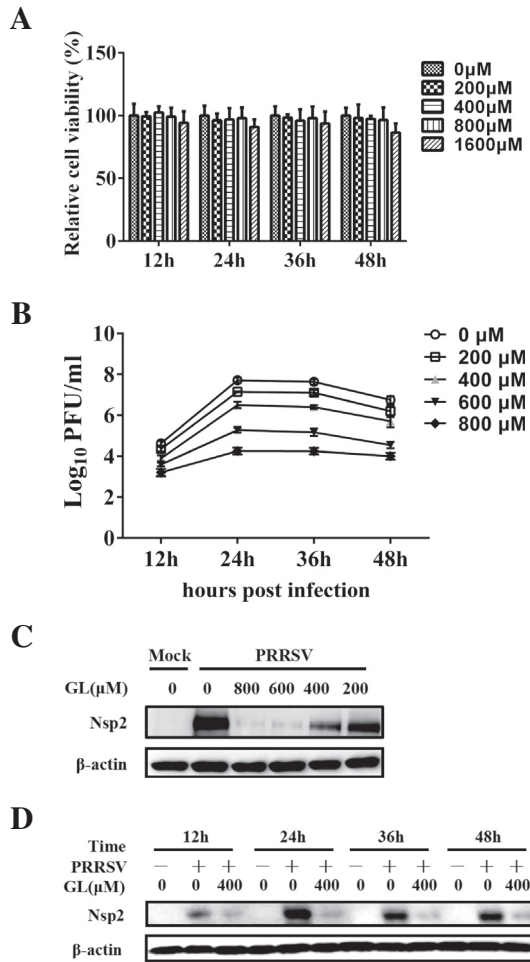
Fig. 1. Glycyrrhizin has antiviral activity against PRRSV. (A) Determination of cytotoxicity of glycyrrhizin by MTT assay. MARC-145 cells were incubated with various concentrations of glycyrrhizin or the control PBS for 48 h prior to the MTT assay. (B) MARC-145 cells were pretreated with glycyrrhizin at the indicated concentrations for 2 h prior to PRRSV infection (0.1 PFU/cell). The infected cells were cultured in the presence of different concentrations of glycyrrhizin, and collected at 12, 24, 36 and 48 hpi for plaque assays. Plaque forming units were counted and the viral titer was determined as plaque forming units per ml (PFU/ml). Differences between glycyrrhizin treated groups and PBS treated control group were statistically significant ($P < 0.01$). (C) MARC-145 cells were treated with glycyrrhizin (GL) at the indicated concentrations for 2 h, then mock-infected or infected with PRRSV (0.1 PFU/cell) and cultured in the presence of glycyrrhizin. At 24 hpi, cells were collected for western blot analysis using a specific antibody against PRRSV Nsp2 protein, with β-actin used as a loading control. (D) MARC-145 cells were pretreated with 400 μM of glycyrrhizin for 2 h prior to PRRSV infection (0.1 PFU/cell), then cultured in the presence of glycyrrhizin. At 12, 24, 36 and 48 hpi, cells were harvested for western blot analysis using a monoclonal antibody against PRRSV Nsp2. The data in this figure are representative of three independent experiments.

titer in a dose-dependent manner at all tested time points. To further confirm the reduction of virus titer induced by treatment with glycyrrhizin, we also tested the expression of PRRSV nonstructural protein 2 (Nsp2) using western blot with monoclonal antibody against the Nsp2 of PRRSV strain WHU3 (Wang et al., 2013a). The results showed that the expression of PRRSV Nsp2 was notably significantly reduced following treatment with glycyrrhizin, and this inhibition also occurred in a dose-dependent manner (Fig. 1C). Moreover, the inhibition of Nsp2 expression by glycyrrhizin could be observed at all examined time points (Fig. 1D). These results indicate that treatment with glycyrrhizin inhibits PRRSV proliferation.

Suggested mechanisms of glycyrrhizin antiviral activity include direct effects on the adsorption, penetration and particle maturation steps of the virus reproductive cycle. To further explore the possible mechanism of action through which glycyrrhizin inhibits PRRSV infection, we first attempted to investigate the growth kinetics of PRRSV. MARC-145 cells were infected with PRRSV at 0.1 PFU/cell. Both the cells and culture medium were titrated by plaque assay at 0 hpi and every 2 h between 6 and 26 hpi, as well as 36 and 48 hpi. The results showed that infectious PRRSV could be detected in the supernatant at 10 hpi (Fig. 2A), suggesting that progeny virus particles were released after 8 hpi. It should be noted that there is slight difference between the virus titers of Figs. 1B and 2A. We speculated that this difference was resulted from the different cell treatment methods because cells were washed three times with PBS in the experiment of Fig. 1B when compared to Fig. 2A.

Subsequently, a plaque reduction assay was applied to evaluate the effect of glycyrrhizin on PRRSV adsorption. Briefly, MARC-145 cells cultured in 6-well plates were pre-chilled at 4 °C for 1 h, and then the medium was replaced by a mixture of glycyrrhizin (800 μM) and PRRSV (0.001, 0.0001 PFU/cell). After incubation at 4 °C for another 2 h (to permit binding, but prevent viral internalization), the free virus was removed. The cell monolayer was washed with ice-cold phosphate-buffered saline (PBS) three times, covered with overlay medium, incubated at 37 °C under 5% CO_2 for a further 72 h, and examined by a plaque assay. The results of Fig. 2B showed that glycyrrhizin reduced virus infection by 15% at 0.001 PFU/cell and approximately 30% at 0.0001 PFU/cell through inhibiting PRRSV attachment to MARC-145 cells.

Next, we evaluated the effect of glycyrrhizin on PRRSV internalization. MARC-145 cells cultured in 6-well plates were pre-chilled at 4 °C for 1 h, then were incubated for another 2 h at 4 °C with different concentrations of PRRSV (1.0, 0.1, 0.01, 0.001, 0.0001 PFU/cell). The virus-containing medium was replaced by fresh medium containing glycyrrhizin (800 μM) or PBS, and the temperature was shifted to 37 °C to allow virus entry. Three hours later, the cells were carefully washed with PBS to remove the non-internalized virus particles and glycyrrhizin or PBS and the cells were overlaid with 1.8% (w/v) Bacto agar mixed 1:1 with 2× DMEM containing 0.05 mg/ml neutral red. After incubation at 37 °C for 72 h, plaques were counted directly. The results showed that infectious virus titers were reduced by approximately 1000-fold when glycyrrhizin was added during the penetration stage (Fig. 2C), demonstrating that glycyrrhizin inhibited PRRSV internalization to MARC-145 cells. The titers of three PBS control groups (0.01, 0.1, and 1.0 PFU/cell) are not shown because of the countless viral plaques, while the viral plaques of GL-treated groups are zero at 0.001 and 0.0001 PFU/cell. Previous work has shown that glycyrrhizin suppresses virus penetration by modifying the fluidity of cellular membranes, and through this mechanism reduces HIV-1 and IAV infection (Harada, 2005; Wolkerstorfer et al., 2009). It has been demonstrated that PRRSV makes entry into MARC-145 cells through a microfilament-dependent endocytic mechanism (Kreutz and Ackermann, 1996). Thus, glycyrrhizin most likely inhibits PRRSV penetration by interfering with the cellular membranes, eventually leading to a reduction in endocytosis. Further experiments will be needed to confirm this hypothesis.

To determine whether glycyrrhizin inhibits PRRSV at the replication step, MARC-145 cells were incubated with PRRSV (0.1 PFU/cell) for 6 h, then the cell-free virus particles were removed and the cells were cultured in fresh medium containing glycyrrhizin (800 μM). At 7, 8, 9 and 10 hpi, the infected cells were collected and total RNA was isolated using Tri-Zol reagent following the manufacturer's instructions (Invitrogen). PRRSV negative-sense RNA was quantified by real-time RT-PCR as previously described (Zhang et al., 2006). No noticeable differences in PRRSV RNA levels could be detected between cells treated with glycyrrhizin and the control cells treated with PBS, leading to the