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Matrine and icariin can inhibit bovine viral diarrhoea virus replication by promoting type I interferon response *in vitro*

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Abstract

Introduction: Bovine viral diarrhoea virus (BVDV) can cause diarrhoea (BVD) in an animal herd, leading to heavy economic losses. There are limited drugs available for treating and controlling BVD. This research aims to investigate the antiviral and immunoregulatory effects of two traditional Chinese herb extracts against BVDV infection. The extracts are matrine and icariin, which have been proved to have immunostimulant and antiviral effects. **Material and Methods:** A cell counting kit-8 assay was used to analyse the toxicity of matrine and icariin to Madin–Darby bovine kidney (MDBK) cells. The model of MDBK cells infected with BVDV was utilised to uncover the antiviral mechanism of matrine and icariin, which along with their immunoregulatory ability was evaluated by quantitative reverse-transcription PCR and ELISA. **Results:** The results showed that matrine and icariin can significantly inhibit the gene expression level of the BVDV 5′ untranslated region through various pathways. Both matrine and icariin can statistically upregulate the gene expression level of interferon alpha, interferon beta (IFN-β), toll-like receptor 3, retinoic acid–inducible gene I and interferon regulatory factor 3, and raise the concentration of IFN-β after BVDV infection. **Conclusion:** This study proves that both matrine and icariin have inhibitory effects on BVDV replication by activating IFN production and the IFN signalling pathway. The finding is promising and should open up the possibility of larger-scale *in vitro* research followed by *in vivo* experiments evaluating matrine and icariin as therapeutic agents in BVD cases.

Keywords: bovine viral diarrhoea virus, matrine, icariin, antiviral effect, immunoregulation.

Introduction

Bovine viral diarrhoea virus (BVDV) belongs to the *Flaviviridae* family and *Pestivirus* genus. It is a single-stranded, positive-sense RNA virus that contains a large open reading frame (ORF) and 5′ and 3′ untranslated regions (UTR) (9, 28). The virus can be divided into cytopathogenic and non-cytopathogenic biotypes, which respectively have and do not have an ability to cause apoptotic changes in cultured cells *in vitro* (18). It is the causative agent of bovine viral diarrhoea (BVD), a disease that affects a range of domestic animals including pigs, cattle and sheep. The clinical signs of BVDV infection include diarrhoea, fever, dehydration, nasal discharge, leukocytosis, gastrointestinal mucosal erosion and spontaneous abortion. Additionally, BVDV infection can be persistent, and if it becomes so host immunotolerance can develop, which have a significant impact on livestock farming (27). Current prevention and treatment measures for BVDV infection have limited effectiveness. Therefore, the development of new treatment strategies is urgently needed. One possible approach is the use of antiviral drugs to contain viral infection, immune-enhancing drugs being highly favoured for this purpose. However, there are currently limited drugs available for the treatment of BVDV infections (5).

Matrine, a natural alkaloid compound, is found in the roots of *Sophora flavescens*, which possesses various pharmacological functions, including anti-inflammatory, antioxidant, anti-tumour, anti-fibrosis and immune regulatory activity. Additionally, it has been shown to protect against damage in the heart, liver, lung, kidney, brain, and other tissues and organs (3, 6). Moreover,

matrine exhibits significant antiviral activity with minimal adverse effects (10). *Epimedium* is a well-known traditional Chinese herb, from which the flavonoid compound icariin is extracted. Studies have revealed that icariin exhibits various biological activities and pharmacological effects, including anti-oxidative, anti-aging, anti-tumour and anti-osteoporosis properties (21, 24). Additionally, it improves cardiovascular function, promotes hematopoietic function and protects against neuronal damage (7).

The multiple beneficial properties of matrine and icariin have only partially been confirmed in non-human species and their antiviral activity against BVDV has not been thoroughly investigated. The potential antiviral mechanism of matrine and icariin against BVDV infection was evaluated in this study, which provides new insights into the potential use of matrine and icariin for BVD containment.

Material and Methods

Cells, virus and reagents. Madin–Darby bovine kidney (MDBK) cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F12, 1:1) (Gibco Life Technologies, Grand Island, NY, USA) with 10% foetal bovine serum (FBS; Gibco-BRL, Carlsbad, CA, USA) and 1% penicillin–streptomycin. The cells were cultured in a humidified atmosphere at 37°C and 5% CO_2. The NADL BVDV reference strain was procured from the China Institute of Veterinary Medicine (Nangjing, China). The tissue culture infective dose ($TCID_{50}$) for NADL on MDBK cells was determined using the Spearman–Kärber method and was found to be $l0^{-4.5}$/0.1 mL. Matrine (catalogue No. S2322; purity 99.72%) and icariin (catalogue No. S2312; purity 99.05%) were purchased from Selleck Biotechnology (Houston, TX, USA). Icariin was diluted in cell maintenance solution to final concentrations of 128 nM, 64 nM, 32 nM, 16 nM, 8 nM, 4 nM, 2 nM, and 1 nM. Matrine was diluted to 1 mg/mL in dimethyl sulphoxide (DMSO), and further diluted in cell maintenance medium to achieve final concentrations of 512 ng/mL, 256 ng/mL, 128 ng/mL, 64 ng/mL, 32 ng/mL, 16 ng/mL, 8 ng/mL and 4 ng/mL. The final solution was passed through a 0.22 μm filter membrane and stored at 4°C.

Viral infection. The virus was diluted with DMEM/F12 medium and incubated with cells at 37°C for 1.5 h, then the cells were washed three times with phosphate-buffered saline (PBS) to remove the residual virus solution, and DMEM/F12 containing 2% FBS and 1% penicillin–streptomycin was added.

Cytotoxicity test. Ninety-six-well culture plates were inoculated with approximately 4×10^4 MDBK cells. Once the degree of cell confluence reached 70–90%, the culture medium was discarded, and the cells were washed with PBS three times. The concentration gradients of matrine (512 ng/mL, 256 ng/mL, 128 ng/mL, 64 ng/mL, 32 ng/mL, 16 ng/mL, 8 ng/mL and 4 ng/mL) and icariin (128 nM, 64 nM, 32 nM, 16 nM, 8 nM, 4 nM, 2 nM and 1 nM) were added to the culture medium, and the cells were incubated at 37°C with 5% CO_2. After 24 h, 10 μL of cell-counting kit-8 solution (Beyotime Biotechnology, Shanghai, China) was added to each well, and the plates were incubated at 37°C for 3 h. Dimethyl sulphoxide was the control in the matrine-treated test. The half-maximal inhibitory concentrations (IC_{50}) of matrine and icariin on MDBK cells were calculated based on the optical density values at 450 nm. For the calculation of half-maximal effective antiviral concentration (EC_{50}), the same procedure was followed but with BVDV at 100 × the 50% tissue culture infectious dose ($TCID_{50}$) added to the cells in the presence of matrine and icariin. Finally, the therapeutic index (TI) of matrine and icariin was calculated based on the IC_{50} and EC_{50} using the formula: $TI = IC_{50}/EC_{50}$.

Extraction of RNA and performance of quantitative reverse-transcription PCR. The total RNA was extracted from MDBK cells using Trizol reagent (TransGen Biotech, Beijing, China) and following the manufacturer's instructions. A PrimeScript II 1st Strand cDNA Synthesis Kit was used to conduct the reverse transcription reaction (TaKaRa, Kusatsu, Japan). The primers were BVDV 5′UTR (forward primer 5′-ATG CCCWGTAGGACTAGCA-3′ and reverse primer 5′-TCA ACTCCATGTGCCATGTAC-3′), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward primer 5′-GATTGT CAGCAATGCCTCCT-3′ and reverse primer 5′-GGTCAT AAGTCCCTCCACGA-3′), IFN-α (forward primer 5′-GTG AGGAAATACTTCCACAGACTCACT-3′ and reverse primer 5′-TGARGAAGAGAAGGCTCTCATGA-3′), IFN-β (forward primer 5′-ACAGAGTCACCCACCTCACC-3′ and reverse primer 5′-GACCAGGTGTGTGTCAGTCC-3′), IRF3 (forward primer 5′-GGCTTGTGATGGTCAAGGTT-3′ and reverse primer 5′-TGCAGGTCGACAGTGTTCTC-3′), RIG-I (forward primer 5′-CCTCAGTTGGCGTTGGAGAT-3′ and reverse primer 5′-GAGCCTCTGTCTCTGCCATC-3′) and TLR3 (forward primer 5′-TCTGTCCCTGAGCAG CAACC-3′ and reverse primer 5′-AAGGTCCAGCGT GGTGAGAT-3′). The threshold cycle (Ct) values of the target genes were normalised to GAPDH and compared to the BVDV positive control (without drugs) using the $2^{-\Delta\Delta Ct}$ method. The protocol of the quantitative reverse-transcription (RT-q) PCR was described in a previous study (25). The experiment was conducted in triplicate for each reaction.

Evaluation of the different functions of matrine and icariin against BVDV in MDBK cells. To perform the viral replication cycle test, the monomer concentrations based on the results of cytotoxicity test and on the morphological observations of cells were selected. Specifically, matrine was used at 128 ng/mL and icariin at 8 nM.

Virucide group. In this group the virus and medicine were co-incubated before being added to the cells. When the MDBK cells grew into monolayers on six-well plates, the culture medium was discarded and

the cells were washed with PBS three times. The matrine and icariin were incubated separately and simultaneously mixed with equal volumes of BVDV (100 $TCID_{50}$) at 4°C for 1 h. The mixture was added to the cells and incubated at 37°C for 1.5 h with full shaking every 15 min. Then the liquid was discarded, the cells were washed with PBS three times and 2 mL of maintenance solution was added, after which the cells and solution were placed in the incubator for 24 h at 37°C and 5% CO_2. After washing with PBS three times, whole cell lysates were collected for RT-qPCR detection.

Co-treatment group. In this group the medicine and virus were added simultaneously to the cells. Madin–Darby bovine kidney cell monolayers grown on six-well plates were precooled at 4°C for 30 min and then washed three times with PBS at 4°C. Matrine and icariin solutions were added separately to each well, along with an equal volume of BVDV (100 $TCID_{50}$) and incubated at 4°C for 1.5 h, with full shaking every 15 min. Then the liquid was discarded and 2 mL of maintenance solution was added to the cells, which were cultured in the incubator for 24 h at 37°C and 5% CO_2. After washing with PBS three times, the cell lysates were collected for the RT-qPCR assay.

Pre-treatment group. In this group the cells were incubated with the extracts first and infected with the virus next. When the MDBK cell grew into monolayers on six-well plates, the culture medium was discarded and the cells were washed with PBS three times. A 500 μL volume of icariin or matrine solution at the maximum safe concentration (maximum concentration with no effect on cell viability) was added to each well and incubated at 37°C for 3 h. The solution was then discarded, and 500 μL of 100 $TCID_{50}$ BVDV solution was added and incubated at 37°C for 1.5 h, with full shaking every 15 min. The residual viruses were discarded, 2 mL of maintenance solution was added, and the cells were placed in the incubator at 37°C for 24 h. The cell lysates were collected for the RT-qPCR.

Post-treatment group. In this group the cells were infected by virus first and treated with the extracts next. When the MDBK cells grew into monolayers on six-well plates, the culture medium was discarded and the cells were washed with PBS three times. Then, 500 μL of 100 $TCID_{50}$ BVDV solution was added to each well. The cells were placed in the incubator at 37°C for 1.5 h, with full shaking every 15 min. The liquid was then discarded, 2 mL of matrine or icariin of different concentrations were added, and the cells were placed in the incubator at 37°C for 24 h. The cell precipitations were collected and analysed with the RT-qPCR.

Analysis of matrine and icariin on secretion of IFN-I in BVDV-infected MDBK cells. Similar to the pre-treatment group method described above, cell supernatants were collected from MDBK cells at 24 h after BVDV infection. The cell supernatants were centrifuged to remove particulates and aggregates. The concentrations of IFN-β in MDBK cells were measured using bovine IFN-β ELISA kits (Enzyme-linked Biotechnology, Shanghai, China) according to the manufacturer's instructions, and the cell precipitation was collected at 24 h, 48 h and 72 h for RT-qPCR detection. All samples were measured in triplicate.

Statistical analyses. Data are expressed as the mean and standard deviation of the mean (SD), and the significance of differences between groups was evaluated using Student's *t*-test or the one-way analysis of variance followed by Tukey's post-hoc test. Results with P-values of $< 0.05 < 0.01 < 0.001$ and < 0.0001 were considered to be statistically significant. All experiments were repeated at least three times individually. Graphs were plotted and analysed using GraphPad Prism software, version 8.0 (GraphPad Software, La Jolla, CA, USA).

Results

Effects of matrine and icariin on MDBK cell viability. Matrine had no significant effect on MDBK cell viability when the concentration was 4, 8, 16, 32, 64 or 128 ng/mL compared with the DMSO control (Fig. 1A), but it significantly decreased the activity of MDBK cells at 256 and 512 ng/mL (P-value < 0.0001). Therefore, 128 ng/mL was considered the maximum concentration of matrine which was non-toxic to MDBK cells and was adopted as the safe concentration. The dose–effect relationship between the logarithm of matrine concentration (X) and the inhibition rate of MDBK (Y) is shown in Fig. 1B. The IC_{50} of matrine was calculated as 294.9 ng/mL. Icariin had no significant effect on MDBK cell activity when the concentration was 1, 2, 4 or 8 nM compared with the control group (Fig. 1C), but it significantly decreased the activity of MDBK cells at 16, 32, 64 and 128 nM (P-value < 0.0001). Therefore, 8 nM was regarded as the maximum concentration of icariin which was non-toxic to MDBK cells and was adopted as the safe concentration. The dose–effect relationship between the logarithm of the concentration (X) and the inhibition rate (Y) of MDBK is shown in Fig. 1D. The IC_{50} of icariin was calculated as 124.8 nM.

The dose–effect relationship of matrine and icariin against BVDV. At the safe concentrations, matrine and icariin had inhibitory effects on BVDV, and the viral inhibition rate gradually increased with the rise in concentration, indicating that the anti-BVDV effects of matrine and icariin were dose-dependent (Figs 2A and 2C). Furthermore, matrine at 128 ng/mL had a very highly significant anti-BVDV effect compared to this extract at 4, 8, 16 and 32 ng/mL (P-value < 0.0001), and had a significant anti-BVDV effect compared to 64 ng/mL (P-value < 0.05). Icariin of 8 nM had a very highly significant anti-BVDV effect compared to this extract at 0.25 and 0.5 nM (P-value < 0.0001), a highly significant anti-BVDV effect compared to 1 and 2 ng/mL (P-value < 0.001), and a significant anti-BVDV effect compared to 4 ng/mL (P-value < 0.05). The EC_{50}s of matrine and

icariin against BVDV were 43.55ng/mL and 4.48 nM (Figs 2B and 2D), and their TIs were 6.772 and 27.857, respectively.

Effects of different function modes of matrine and icariin against BVDV in MDBK cells. As shown in Figs 3A and 3B, the BVDV 5′UTR gene level in the virucide group was very highly significantly decreased from that in the BVDV-positive group (P-value < 0.0001), illustrating that both matrine and icariin had a direct virucidal effect on BVDV. The co-treatment group was established to identify whether matrine and icariin could inhibit the process of virus adsorption. The results showed that the BVDV 5′UTR gene level in the co-treatment group decreased very highly significantly from that in the BVDV model group (P-value < 0.0001) (Figs 3A and 3B), suggesting that both matrine and icariin could block the replication process of BVDV by inhibiting adsorption. Furthermore, the BVDV 5′UTR gene level in the pre-treatment and post-treatment groups was very highly significantly downregulated from that in the BVDV model group (P-value < 0.0001), suggesting that the two herbal extracts had preventive and therapeutic effects. Therefore, these results indicated that matrine and icariin could inhibit BVDV replication through multiple mechanisms.

It is well known that preventing disease is preferable to treating it. In order to explore whether matrine and icariin could be used as feed additives for the prevention of BVD, the pre-treatment group of 24 h was set as the benchmark, and the 5′UTR gene level of BVDV increased with the continuous replication of BVDV at 48 h and 72 h (Fig. 3C). The levels of BVDV 5′UTR in MDBK cells pretreated with matrine were very highly significantly lower at 24 h, 48 h and 72 h than that in MDBK cells infected with BVDV only (the BVDV-positive group) (P-value < 0.0001) (Fig. 3C). Similarly, the levels of BVDV 5′UTR in MDBK cells pretreated with icariin were significantly lower at 24 h, 48 h and 72 h than that in MDBK cells infected with BVDV only (the BVDV-positive group) (P-value < 0.0001) (Fig. 3C).

Effect of matrine and icariin on BVDV's inhibition of IFN-α/β expression. At 24 h, BVDV infection downregulated the secretion of IFN-β as compared to the control group without BVDV infection (P-value < 0.01) (Fig. 4A). Compared with the BVDV-only group, there was an increase in IFN-β secretion in the group treated with matrine alone (P-value < 0.01), where it was higher than in the control group (P-value < 0.05). Furthermore, again compared to the BVDV-only group, there was a significant increase in IFN-β secretion in the matrine pretreatment group (P-value < 0.01), where it was higher than the control group (P-value < 0.05). There was no statistical difference between the icariin-treated group and the BVDV group (P-value > 0.05), nor was there a significant difference between the icariin-treated group and the control group (P-value > 0.05). However, the level of IFN-β in the icariin pretreatment group was significantly higher than those in the BVDV group (P-value < 0.01) and the control group (P-value < 0.05). It demonstrated that matrine and icariin could block BVDV's inhibition of the secretion of IFN-β and significantly upregulate the expression of IFN-β in BVDV infection (Fig. 4A).

Regarding the messenger RNA (mRNA) levels of IFN-α and IFN-β, their expression levels in the matrine-treated group increased very highly significantly over those in the control group and the BVDV group at 24 h after BVDV infection (P-value < 0.0001) (Figs 4B and 4C). The expression level of IFN-α in the matrine pretreatment group was significantly higher than that in the BVDV group at 24 h (P-value < 0.01), while the level of IFN-β in the matrine pretreatment group was very highly significantly upregulated when measured against those of the other three groups at 24 h (P-value < 0.0001). Interestingly, the levels of IFN-α and IFN-β in the matrine pretreatment group were very highly significantly above those in the other three groups at 48 h after BVDV infection (P-value < 0.0001). Compared with the control group, the mRNA of IFN-α in the BVDV group was very highly significantly downregulated at 24 h (P-value < 0.0001), and highly significantly upregulated at 72 h (P-value < 0.001) (Fig. 4B). The level of IFN-β in the matrine-treated group was very highly significantly higher than those in the control group and the BVDV group at 48 h (P-value < 0.0001), while there were no differences in the mRNA of IFN-α among the matrine-treated group, the control group and the BVDV group (P-value > 0.05). Similarly, the levels of IFN-α and IFN-β in the matrine pretreatment group very highly significantly exceeded those of the other three groups at 72 h after BVDV infection (P-value < 0.0001). The level of IFN-α in the matrine-treated group was raised very highly significantly compared to this level in the control group (P-value < 0.0001), and was also significantly higher than the level in BVDV group at 72 h (P-value < 0.01). The mRNA of IFN-β in the matrine-treated group was upregulated very highly significantly over that of the control group (P-value < 0.0001), while there was no difference between the levels in the matrine-treated group and the BVDV group (P-value > 0.05).

Compared with the BVDV group, the expression levels of IFN-α and IFN-β in the icariin treated group were very highly significantly upregulated at 24 h (P-value < 0.0001) (Figs 4D and 4E). Very highly significant upregulation was noted of the levels of IFN-α and IFN-β in the icariin pretreatment group compared to those of the other three groups at 24 h and 72 h after BVDV infection (P-value < 0.0001). IFN-α was secreted significantly more in the icariin pretreatment group as compared to in the control group, and also was as compared to its secretion in the icariin-treated group and the BVDV group at 48 h (P-value < 0.01). Compared with the control group, the mRNA of IFN-α in the BVDV group was very highly significantly downregulated at 24 h (P-value < 0.0001), and significantly and very highly significantly upregulated at 48 h and 72 h (respective P-values < 0.01 and < 0.0001) (Fig. 4D).

The mRNA levels of IFN-β in the icariin pretreatment group were very highly significantly upregulated over those in the other three groups at 48 h (P-value < 0.0001). IFN-β in the icariin treated group displayed very highly significantly upregulated secretion compared to secretion in the control group and the BVDV group at 48 h (P-value < 0.0001) (Fig. 4E). These results suggested that both matrine and icariin could enhance the expression of IFN-α/β to produce an inhibitory effect on BVDV infections.

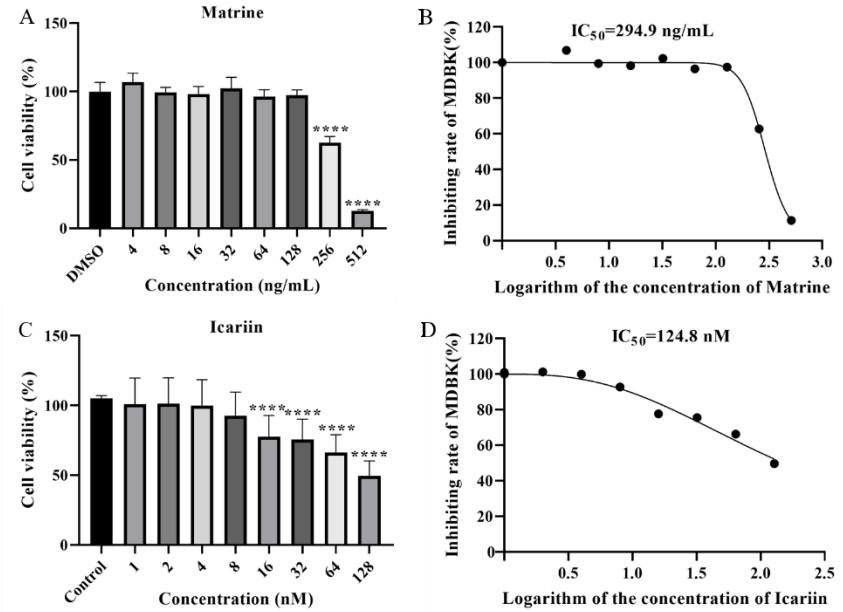

Fig. 1. Cytotoxic effects of matrine and icariin on Madin–Darby bovine kidney (MDBK) cells. A – Matrine's effect on MDBK cell viability; B – Half-maximal inhibitory concentration (IC_{50}) of matrine on MDBK cells; C – Icariin's effect on MDBK cell viability; D – IC_{50} of icariin on MDBK cells. Data are presented as mean ± standard deviation of three independent experiments. DMSO – dimethyl sulphoxide solvent control; Control – cell maintenance solution control group; **** – P-value < 0.0001

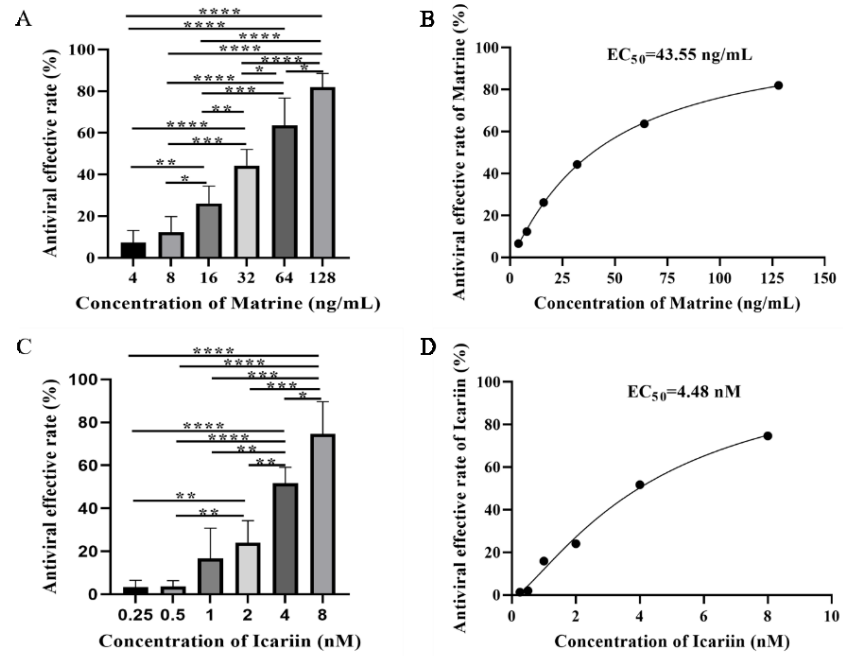

Fig. 2. Effects of the matrine and icariin against bovine viral diarrhoea (BVDV) infection in Madin–Darby bovine kidney (MDBK) cells. A – anti-BVDV effect of matrine in MDBK cells; B – Half-maximal effective antiviral concentration (EC_{50}) of matrine on MDBK cells; C – anti-BVDV effect of icariin in MDBK cells; D – EC_{50} of icariin on MDBK cells; * – P-value < 0.05; ** – P-value < 0.01; *** – P-value < 0.001; **** – P-value < 0.0001

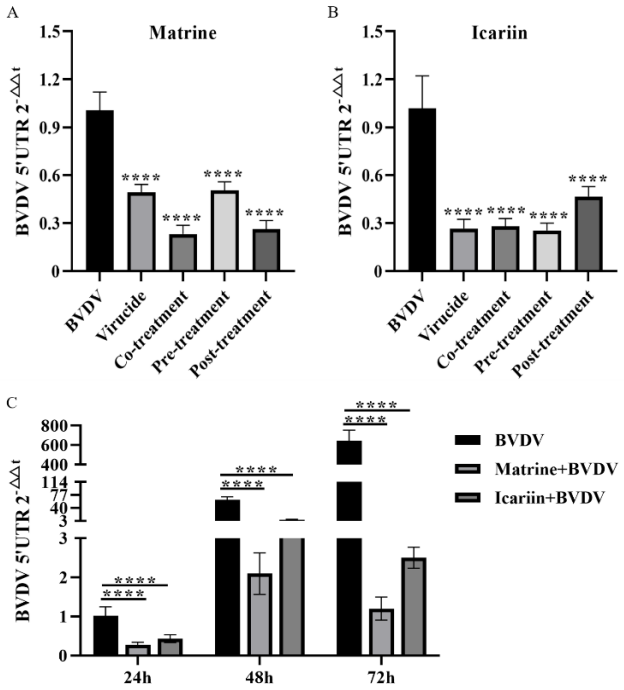

Fig. 3. Evaluation of different administration methods of matrine and icariin on bovine viral diarrhoea virus (BVDV) infection in Madin–Darby bovine kidney (MDBK) cells. A – Effects of different administration methods of matrine on the BVDV 5′ untranslated region (UTR); B – Effects of different administration methods of icariin on the BVDV 5′UTR; C – Evaluation of prophylactic effects of matrine and icariin on the BVDV 5′UTR at different time points; **** – P-value < 0.0001; BVDV group – BVDV and MDBK; Virucide group – BVDV and extract incubated at 4°C for 1 h, MDBK added subsequently; Co-treatment group – BVDV, extract and MDBK incubated at 4°C for 1.5 h; Pre-treatment group – MDBK and extract incubated at 37°C for 3 h, challenged with BVDV subsequently; Post-treatment group – MDBK and BVDV incubated at 37°C for 1 h, extract added subsequently

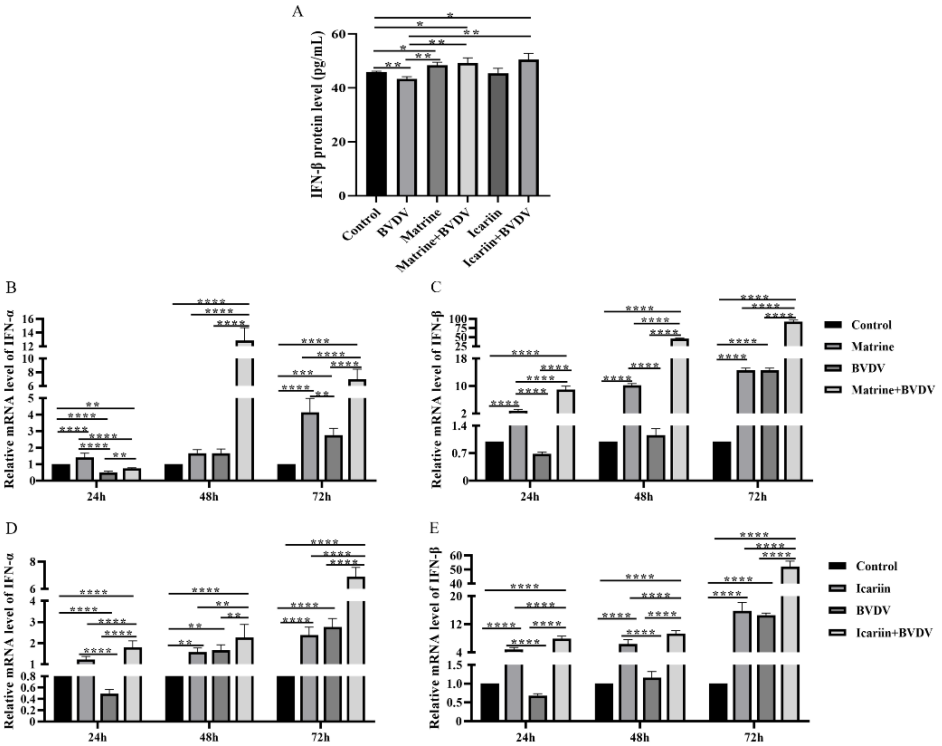

Fig. 4. Effects of matrine and icariin on the expression levels of interferon alpha and beta (IFN-α/β) in Madin–Darby bovine kidney (MDBK) cells infected with bovine viral diarrhoea virus (BVDV). A – Effects of matrine and icariin on the secretion levels of IFN-β in MDBK cells; B – Effects of matrine on messenger RNA (mRNA) expression of IFN-α in MDBK cells; C – Effects of matrine on mRNA expression of IFN-β in MDBK cells; D – Effects of icariin on mRNA expression of IFN-α in MDBK cells; E – Effects of icariin on mRNA expression of IFN-β in MDBK cells; Control – negative group (without BVDV and drug); Matrine – group treated with matrine herb extract at 128 ng/mL; Icariin – group treated with icariin herb extract at 8 nM; + BVDV – group administered an extract and BVDV (100 TCID50); * – P-value < 0.05; ** – P-value < 0.01; *** – P-value < 0.001; **** – P-value < 0.0001

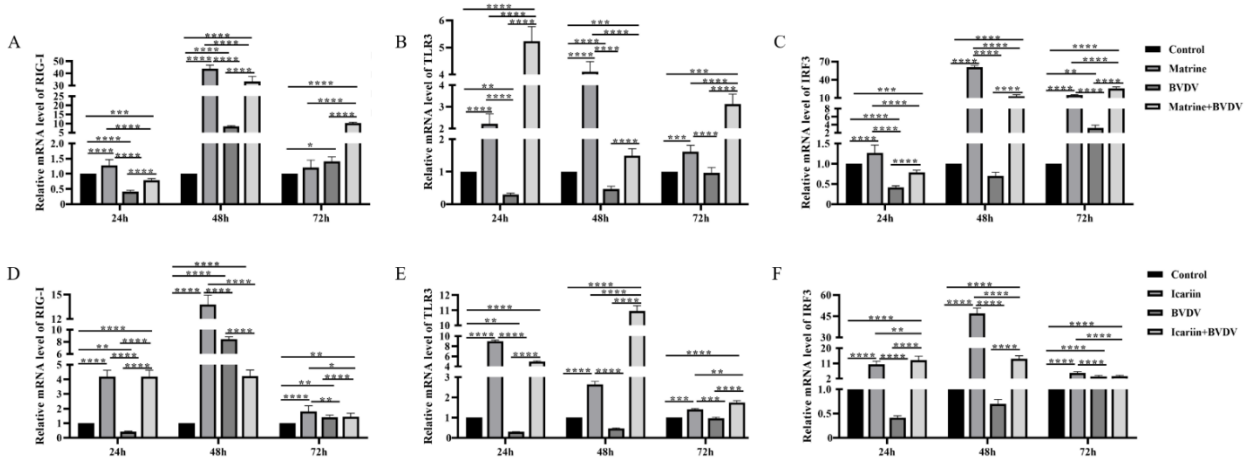

Fig. 5. Effects of matrine and icariin on the messenger RNA (mRNA) expression levels of innate immune response genes in MDBK cells at different time points. A – Effects of matrine on the expression levels of retinoic acid–inducible gene I (RIG-I); B – Effects of matrine on the expression levels of toll-like receptor 3 (TLR3); C – Effects of matrine on the expression levels of interferon regulatory factor 3 (IRF3); D – Effects of icariin on the expression levels of RIG-I; E – Effects of icariin on the expression levels of TLR3; F – Effects of icariin on the expression levels of IRF3; * – P-value < 0.05; ** – P-value < 0.01; *** – P-value < 0.001; **** – P-value < 0.0001

Effects of matrine and icariin on the expression of RIG-I, TLR3 and IRF3 in MDBK cells infected with BVDV. To investigate the pathway through which matrine and icariin improved IFN-α/β expression, the mRNA expression levels of RIG-I, TLR3 and IRF3 were examined. Noteworthily, the expression of RIG-I, TLR3 and IRF3 was significantly downregulated by BVDV infection at 24 h in the BVDV-only group (P-value < 0.01) (Figs 5A–F), indicating BVDV could suppress the innate immune response of host cells. However, the expression of RIG-I, TLR3, and IRF3 gradually rose from 48 to 72 h after BVDV infection. As Fig. 5A shows, compared to the control group, the expression level of RIG-I was very highly significantly downregulated in the BVDV group at 24 h after BVDV infection (P-value < 0.0001), but very highly significantly upregulated at 48 h (P-value < 0.0001) and significantly upregulated at 72 h (P-value < 0.05). Importantly, the mRNA level of RIG-I in the matrine-only group increased dramatically over those in the other three groups at 24 h, 48 h and 72 h (P-value < 0.0001). The mRNA level of RIG-I in the matrine pretreatment group (matrine + BVDV) was also upregulated significantly when measured against the levels in the BVDV group and the control group at 24 h and 48 h (P-value < 0.0001). Notably, the mRNA level of RIG-I in the matrine pretreatment group increased significantly than the other three groups at 72 h (P-value < 0.0001). The mRNA expression of TLR3 in the BVDV group underwent significant and very highly significant downregulation, as comparison with the control group expression level showed respectively at 24 h (P-value < 0.01) and 48 h (P-value < 0.0001) (Fig. 5B). The levels of TLR3 in the matrine pretreatment group were highly significantly above those in the other three groups at 24 h and 72 h (P-value < 0.001). Similarly, the mRNA of TLR3 in the matrine-treated group was markedly higher than those in the BVDV group and the control group at each time point (P-value < 0.001). The mRNA expression of IRF3 in the matrine-treated group very highly significantly exceeded its expression in the other three groups at 24 h and 48 h (P-value < 0.0001) (Fig. 5C). The mRNA of IRF3 in the matrine pretreatment group was highly significantly more abundant than in the BVDV group and the control group at 24 h and 48 h (P-value < 0.001). At 72 h after BVDV infection, the mRNA level of IRF3 in the matrine pretreatment group was very highly significantly upregulated compared to those of the other three groups (P-value < 0.0001).

Compared to the mRNA expression of RIG-I, TLR3 and IRF3 in the control group and the BVDV group, this expression was upregulated very highly significantly in the icariin-treated group at 24 h and 48 h after BVDV infection (P-value < 0.0001) (Figs 5D–F). Compared to the mRNA expression of RIG-I and TLR3 in the BVDV group, mRNA expression of these receptors in the icariin pretreatment group were upregulated very highly significantly at 24 h and 72 h (P-value < 0.0001). Additionally, the mRNA level of TLR3 in the icariin pretreatment group rose very highly significantly above those of the other three groups at 48 h after BVDV infection (P-value < 0.0001) (Fig. 5E). Notably, the mRNA level of IRF3 in the icariin-treated group was enhanced very highly significantly over those of the other three groups at 48 h (P-value < 0.0001) (Fig. 5F). These results suggested that matrine and icariin upregulated IFN-α/β expression levels by potentiating the expression of RIG-I, TLR3 and IRF3, illustrating that the pathways of RIG-I and TLR3 may be involved in the action of matrine and icariin against BVDV.

Discussion

Bovine viral diarrhoea virus can cause disease in a variety of animals such as bovines, goats, deer, alpacas and other livestock and wild animals, causing severe

economic losses to the livestock industry (14, 17, 26). Effective strategies to control the disease which it causes have not been found because of the complexity of BVDV pathogenic types and the variety of clinical symptoms. It is important to identify effective drugs for prevention and treatment of BVD.

The antiviral activity of matrine and its derivatives was reported against hepatitis C virus (11) and porcine reproductive and respiratory syndrome virus (PRRSV) (23). Hepatitis C virus and BVDV are both *Flaviviridae* viruses and have similar structures and physiological functions, suggesting that matrine may have the potential to combat BVDV. The antiviral activity of icariin was reported against feline calicivirus (4) and duck hepatitis virus A (29). These results suggested that icariin may be a prospective broad-spectrum antiviral candidate. To evaluate the efficacy of matrine and icariin against BVDV infection, the cytotoxicity of matrine and icariin to MDBK cells as well as their antiviral effect on BVDV was investigated. The IC_{50} values of matrine and icariin were found to be 294.9 ng/mL and 124.8 nM, respectively, while their EC_{50} values were 43.55 ng/mL and 4.48 nM. Most studies on the antiviral effects of traditional Chinese medicine have shown that the effect of preparations on a virus is dose-dependent within the safe range, which means that the higher the drug concentration, the greater the inhibition of the virus. Consistently with this principle, our results indicated that matrine and icariin inhibited BVDV replication in a dose-dependent manner. The TI is an indicator of drug safety (15), whereby the higher the TI, the safer the drug. It is generally believed that a new drug with a TI greater than 5 can be considered for further research. The TIs of matrine and icariin were calculated to be 6.772 and 27.857, respectively. Therefore, both matrine and icariin may be regarded as safe and effective anti-BVDV drugs, which deserve further research.

The different ways in which matrine and icariin function against BVDV were explored. The virucidal group was set up to make it possible to observe whether matrine and icariin could inactivate the virion. The co-treatment group was established in order that matrine and icariin's exertion of any effects on the process of virus adsorption could be seen. The purpose of the pre-treatment group was to be a means to evaluate whether matrine and icariin could protect the cells from BVDV. The post-treatment group facilitated evaluation of matrine and icariin's suppression of viral replication. The results proved that matrine and icariin can exert antiviral effects through direct virus killing, inhibition of virus adsorption, inhibition of virus replication and prophylactic protection. One report demonstrated that matrine could block the adsorption of hepatitis virus and inhibit the expression of viral proteins (31). Matrine also could inhibit PRRSV infection in Marc-145 cells by directly inactivating PRRSV and reducing its protein expression (23). Icariin was proved to inhibit the early stage of feline calicivirus infection, including the absorption stage (4). The anti–duck hepatitis virus A mechanism of icariin *in vitro* probably involved suppression of virus replication (29).

The innate immunity of the host is the first line of defence against virus invasion. Type I interferon is a core component of innate immunity and plays an important role in antiviral responses (22), and in the IFN-I family, IFN-α and IFN-β are the most important factors. Infection with BVDV is closely related to the suppression of the host's type-I-interferon–mediated innate immunity by the virus (13). The results of this study showed that the secretion level of IFN-β and mRNA expression levels of IFN-α/β were significantly downregulated in the BVDV group compared to the negative control group (without BVDV or extracts) at 24 h after BVDV infection. Previous studies have shown that the inhibition of IFN-α/β by BVDV is due to two mechanisms. One is that of the structural protein E^{rns}, which acts as a nuclease capable of degrading the double-stranded RNA (dsRNA) produced during viral replication in the early stage, making RIG-I and TLR3 unable to recognise the dsRNA in cells and resulting in a downregulation of RIG-I and TLR3 expression levels (12). Retinoic-acid-inducible gene I and TLR are two important pattern-recognition receptors for viral RNA, which are initiated by recognition of dsRNA produced during viral replication (1, 30). The subsequent signalling cascade induces phosphorylation and nuclear translocation of IRF3 and IRF7 to drive type I interferon and limit viral infection (19). Many studies have reported that activation of the RIG-I and TLR3 pathways is a critical point for the expression of IFN against RNA virus infection (2, 8). Compared with the control group, the expression level of RIG-I in the BVDV group was significantly downregulated at 24 h, while TLR3 was significantly downregulated at 24 and 48 h.

The second mechanism by which BVDV inhibits IFN-α/β is that of the non-structural protein N^{pro}, which acts as a protease capable of degrading IRF3 and preventing its binding to DNA (16). Recent research demonstrated that the interaction of the BVDV NS4B non-structural protein with the two-caspase activation and recruitment domains region in the melanoma differentiation-associated protein 5 domain of the RIG-I-like receptor signalling pathway negatively regulates IFN-β and significantly inhibits the phosphorylation of IRF3 (20). The significantly lower IRF3 expression in the untreated BVDV-infected cell group than in the control group (without BVDV and drug) at 24 h in our study is consistent with reported findings (20). Both matrine and icariin significantly increase the expression level of IFN-α/β, implying that they could stimulate the expressions of IFN-α/β by activating the upstream pathway of IFN-I production. Retinoic-acid-inducible gene I (RIG-I) and toll-like receptor (TLR) are two important pattern-recognition receptors (PRRs) for viral RNA, which are initiated by recognition of double-stranded RNA (dsRNA) produced during viral replication (1, 30). The subsequent signalling cascade induces phosphorylation and nuclear translocation of the

key natural immune transcription factors IRF3 and interferon regulatory factor 7 (IRF7) to drive type I interferon and limit viral infection (19). Many studies have reported that activation of the RIG-I and TLR3 pathways is a critical point for the expression of IFN against RNA virus infection (2, 8). Therefore, the effects of matrine and icariin on the expression of those two pattern recognition receptors, RIG-I and TLR3, were further investigated in this study. Compared with the control group, the expression level of RIG-I in the BVDV group was significantly downregulated at 24 h, while TLR3 was significantly downregulated at 24 and 48 h. This may be due to the fact that the E^{rns} protein of BVDV can degrade the dsRNA produced during viral replication in the early stage, making RIG-I and TLR3 unable to recognize the dsRNA in cells, resulting in a downregulation of RIG-I and TLR3 expression levels (12). In this study, they also significantly augmented the expression levels of RIG-I and TLR3 at various time points, indicating that matrine and icariin can increase the downstream expression of IFN-α/β by activating RIG-I and TLR3 pathway to neutralise BVDV or reverse the interferon inhibition effect by BVDV.

Conclusion

The present study demonstrates that matrine and icariin could inhibit the replication of BVDV *in vitro*. However, more assays still need to be applied to further prove the antiviral activity of matrine and icariin, and *in vivo* experimentation needs to be performed, which will be addressed in future work. This study reports for the first time the anti-BVDV activity of matrine and icariin, which is of great value for the prevention and treatment of BVDV.

* These authors contributed equally to this work.

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