Cepharanthine inhibits tick-borne encephalitis virus infection through modulation of the stress and inflammation response

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Abstract. Cepharanthine is a potential candidate for developing antiviral agents against tick-borne encephalitis virus (TBEV), which is a major cause of arboviral neuroinvasive diseases in humans. Cepharanthine is the only bisbenzylisoquinoline alkaloid for treating human diseases due to its unique pharmacological properties. Management of endoplasmic reticulum stress and inflammation response is implicated in therapeutic strategies for TBEV infection. The present study aimed to explore the antiviral efficacy and underlying mechanisms of cepharanthine against TBEV in human lung adenocarcinoma A549 cells and neuroblastoma SH-SY5Y cells. In co-treatment and pre-treatment schemes, cepharanthine exhibited a potent inhibitory effect on TBEV propagation. The antiviral effect of cepharanthine was evidenced by a reduction in TBEV RNA replication, viral protein expression and infectious virus release. The levels of inflammatory cytokines, including tumor necrosis factor-α, interleukin (IL-) 1β and IL-11, induced by TBEV infection were markedly decreased in A549 cells treated with cepharanthine. The induction of IL-11 was impaired in infected SH-SY5Y cells treated with cepharanthine. TBEV infection upregulated the expression of C/EBP homologous protein, which was downregulated by cepharanthine treatment. Phosphorylation of eukaryotic initiation factor 2α was enhanced upon cepharanthine treatment during TBEV infection. These *in vitro* results demonstrated that cepharanthine possesses anti-TBEV efficacy and that modulation of the stress and inflammation response by cepharanthine may be involved in antiviral mechanisms. The results of the present study support further investigation of cepharanthine as an antiviral agent against TBEV.

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Introduction

Flaviviruses are reported to infect up to 400 million individuals annually and can cause a wide spectrum of diseases, representing an ongoing threat to global public health (1). Tick-borne encephalitis virus (TBEV), a member of the flaviviruses, is an emerging health concern with an increasing geographical distribution worldwide (2). TBEV is prevalent in forested areas of the Eurasian continent and northeastern Asia. Tick bites are the primary route of TBEV transmission along with infected milk consumption, aerosols and breast milk from infected mothers to infants (3,4). As a neurotropic arbovirus, TBEV infection attacks the central nervous system (CNS) in humans and may lead to severe neurological disorders such as TBE, long-lasting neurological sequelae and death. In total, 5 TBEV subtypes including the European, Siberian, Far Eastern, Baikalian and Himalayan subtypes have been characterized, which are closely associated with the severity and mortality of TBEV infection (5). The Far Eastern subtype induces the most severe form of TBE with a high mortality rate of up to 30%. In China, TBE is endemic in the northeast, the northwest and the southeast and nearly all the TBEV isolates belong to the Far Eastern subtype (6). There is no specific treatment for the diseases associated with TBEV. Due to an increase in TBEV prevalence and transmission, efforts to combat the growing threat must be made.

Cepharanthine, a biscoclaurine alkaloid mainly extracted from plant *Stephania cepharantha* Hayata, was named in 1934 and rapidly approved for clinical applications in Japan due to the traditional use of the medicinal plant (7). Cepharanthine possesses a variety of pharmacological properties including anti-oxidative, anti-inflammatory, anti-proliferative, anti-metastatic, anti-atherosclerosis, anticancer, anti-parasitic, antiviral and immunomodulatory properties (7,8). These properties of cepharanthine are responsible for the treatment of cancer, shock and inflammatory diseases (9). Moreover, cepharanthine is the only bisbenzylisoquinoline alkaloid in clinical use to treat human diseases and has unique pharmacological properties, excellent safety and limited side effects (10). After its initial use for the treatment of tuberculosis, cepharanthine has been approved to treat a diverse range of acute and chronic diseases such as venomous snakebites, radiation-induced leukopenia, alopecia, malaria, xerostomia, sarcoidosis and refractory anemia (11). Cepharanthine has also been applied for the treatment of T cell acute lymphoblastic leukemia, autoimmune

diseases and immune thrombocytopenic purpura (12,13). Therefore, cepharanthine is a fascinating drug with various clinical benefits and complex action modes.

In addition to the aforementioned clinical applications, the antiviral properties of cepharanthine have been implicated in the management of certain viral diseases. Cepharanthine was shown to be a potent inhibitor of human immunodeficiency virus type 1 replication in a chronically infected monocytic cell line, U1 (14). Additionally, cepharanthine has an inhibitory efficacy towards herpes simplex virus type 1 (15). Cepharanthine can also inhibit the replication and hepatitis B e antigen production of wild-type or lamivudine-resistant hepatitis B virus isolates (16). Cepharanthine induces the apoptosis of the SIT leukemia cell line infected with human T-lymphotropic virus type 1 (17). The antiviral efficacy of cepharanthine has been proven in PK-15 cells infected with porcine circovirus type 2 (18). Cepharanthine has been identified as one of the most potent inhibitors of porcine reproductive and respiratory syndrome virus (19). Inhibition of porcine epidemic diarrhea virus propagation by cepharanthine is observable *in vitro* and *in vivo* (20). Cepharanthine blocks entry of swine acute diarrhea syndrome coronavirus (21). Cepharanthine also inhibits dengue virus infection at the initial viral replication state and decreases secretion of interleukin (IL-) 6 (22). Furthermore, cepharanthine markedly suppresses the replication of human coronavirus OC43 and expression of viral proteins (23). Additionally, cepharanthine was considered a promising repurposing drug for coronavirus disease 2019 therapy (24). Cepharanthine has been identified as one of the most potent inhibitors of severe acute respiratory syndrome coronavirus type 2, with predominant inhibition of entry and replication, suggesting therapeutic potential as an antiviral agent (25).

TBEV is a major pathogen of arboviral encephalitis with increasing expansion and potential outbreaks (2,3). The development of effective anti-TBEV components is needed to control this emerging disease. Cepharanthine displays distinct antiviral activities that are crucial to modulating viral propagation and the inflammatory response. Viruses are obligate intracellular parasites and viral proteins are synthesized by the endoplasmic reticulum (ER) of host cells. ER stress is closely regulated to maintain cellular homeostasis. Viruses may comply with ER regulatory processes or subvert them to support viral propagation. TBEV infection induces neuron damage as well as immunopathology due to extreme inflammation (4,5). As ER stress and inflammatory response are involved in TBEV pathogenesis, the antiviral efficacy of cepharanthine against TBEV should be investigated. Therefore, the present study aimed to elucidate whether cepharanthine possesses anti-TBEV efficacy, which step(s) of the TBEV life cycle might be affected and how the stress and inflammatory response are modulated.

Materials and methods

Cells and TBEV. Human lung adenocarcinoma A549 cells (cat. no. CCL-185), human neuroblastoma SH-SY5Y cells (cat. no. CRL-2266), African green monkey kidney Vero cells (cat. no. CCL-81) and porcine kidney PK-15 cells (cat. no. CCL-33) were kindly provided by Professor Rong Ye (School of Basic Medical Sciences, Fudan University, Shanghai, China) and were originally sourced from ATCC. All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.), 1% L-glutamine and 1% non-essential amino acid solution, 100 μg/ml streptomycin and 100 U/ml penicillin at 37˚C in a humidified atmosphere of 5% CO_2. Unless otherwise stated, the products for cell culture were from Invitrogen (Thermo Fisher Scientific, Inc.).

TBEV (provided by Professor Rui-Wen Ren; Center for Disease Control and Prevention of Southern Theater Command, Guangzhou, China) propagation in Vero cells was previously described (26). Vero cells seeded in a T-75 culture flask were cultured and reached 90% confluency. The medium was then replaced with a mixture of 1 ml TBEV stock and 5 ml culture medium and allowed to proceed for 2-h adsorption at 37˚C. After the adsorption, fresh culture medium was added to the cells, followed by an additional 72-h incubation at 37˚C. The culture supernatants were collected at 72 h post incubation and centrifuged at 1,000 x g for 10 min to remove cell debris at room temperature. TBEV stock obtained was frozen in aliquots at -80˚C until use. Titers of TBEV were determined by plaque assay with PK-15 cells before use. Infection experiments with TBEV were conducted in the Biological Safety Level 3 Laboratory in accordance with the guidelines by the Committee on Safety of Biomedicine at Naval Medical University (Shanghai, China).

Plaque assay. Plaque assay was performed to determine TBEV titers in the culture supernatants collected from TBEV-infected cells with and without cepharanthine treatment. PK-15 cells were cultured overnight in 12-well culture plates to form a confluent monolayer. Serial ten-fold dilutions of the culture supernatants were prepared and added to the confluent monolayer of PK-15 cells (three wells per dilution). After 3 h of adsorption at 37˚C, the viral inoculum was removed. The cells were washed once with phosphate-buffered saline (PBS) and incubated with 2% carboxymethylcellulose sodium (cat. no. SLBN8656V; Sigma-Aldrich; Merck KGaA) overlay medium. Following an incubation period of 6 days, the cells were washed once with PBS and fixed for 15 min with 4% paraformaldehyde at room temperature. The cells were then stained with 1% crystal violet for 15 min at room temperature. Plaques formed were counted and the plaque-forming unit (PFU) was calculated. The virus titers are expressed as PFU/ml.

Cytotoxicity assay. Cepharanthine (cat. no. S4238; Selleck Chemicals) was dissolved in dimethyl sulfoxide (DMSO; cat. no. D2650; Sigma-Aldrich; Merck KGaA) to yield a stock solution of 10 mM. Cytotoxicity of cepharanthine in A549, SH-SY5Y and Vero cells was assessed. Cells seeded into 96-well culture plates were cultured overnight to form a confluent monolayer. After removal of the culture medium, the cells were treated for 48 h at 37˚C with fresh culture medium containing cepharanthine at increasing concentrations ranging from 10 to 60 μM (experiment group). The cells maintained in fresh culture medium were used as the control group. DMSO was added to the cell monolayer at a final concentration of 0.1% as the dissolvent group. The culture medium was directly added to cell-free wells as a blank group. All the

groups were examined in three separate wells. Cell viability was determined using CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit containing MTS (cat. no. G3582; Promega Corporation) according to the manufacturer's protocol. The optical density (OD) was measured at 490 nm on a Synergy 2 Multi-Mode Microplate Reader (BioTek; Agilent Technologies, Inc.). Cell viability was determined using the following formula: ($OD_{experiment\ group/dissolvent\ group}$-$OD_{blank\ group}$/$OD_{control\ group}$-$OD_{blank\ group}$) x100%.

TBEV infection. Cells were seeded into 12-well culture plates and cultured overnight to form a confluent monolayer. The cells were then infected with TBEV at a multiplicity of infection (MOI) of 0.1 and allowed to proceed for 2 h adsorption at 37˚C. Following the adsorption, unbound virus was removed by washing with PBS. The cells were subsequently maintained in fresh culture medium for 48 h. The time point for cepharanthine addition experiments was measured from the end of the 2 h adsorption. Cells were infected with TBEV at an MOI of 0.1 throughout the present study.

Cepharanthine addition experiments. In total, three cepharanthine addition experiments were adopted to treat A549, SH-SY5Y and Vero cells. Confluent monolayer cells in 12-well culture plates were treated with cepharanthine at increasing concentrations (5, 10 and 20 μM; three wells per concentration). For co-treatment of cepharanthine, the cells were maintained in fresh culture medium containing TBEV and cepharanthine for 48 h. For pre-treatment of cepharanthine, the cells were pre-treated with cepharanthine in fresh culture medium for 12 h, washed once with PBS and incubated for 2 h with TBEV. Following adsorption for 2 h, the cells were washed once with PBS and cultured with fresh culture medium for another 48 h. For post-treatment with cepharanthine, the cells were incubated for 2 h with TBEV. After removal of the viral inoculum, the cells were washed once with PBS and cultured in fresh culture medium containing cepharanthine for 48 h. Cells without TBEV infection served as a mock control (Mock; three wells). Cells infected with TBEV served as an untreated control (0 μM; three wells). TBEV-infected cells were treated with DMSO at a final concentration of 0.1% as a dissolvent control (DMSO; three wells).

Immunofluorescence staining. A549 cells cultured in 96-well culture plates were treated with cepharanthine at increasing concentrations (10 and 20 μM; three wells per concentration) and TBEV. Cells just infected with TBEV served as an untreated control (0 μM cepharanthine; three wells). TBEV-infected A549 cells treated with 0.1% DMSO served as the dissolvent control (DMSO; three wells). Following a 48 h incubation, the cells were subjected to immunofluorescence staining for TBEV-positive cells as described previously (26). Briefly, the cells were fixed in 4% paraformaldehyde for 15 min at room temperature, washed with PBS and permeabilized with methanol for 20 min at -20˚C. After washing with PBS, blocking was achieved with 3% bovine serum albumin (BSA; lot. no. 4255715; Affymetrix Inc.) for 2 h at room temperature. The cells were incubated with formaldehyde-inactivated TBEV immunized mouse ascites recognizing the viral surface antigen (1:500; provided by Professor Rui-Wen Ren) overnight at 4˚C, washed with PBS and incubated with Alexa Fluor 488-conjugated goat anti-mouse antibody (1:1,000; cat. no. ab150113; Abcam) for 1 h at room temperature. The cells were washed with PBS and subsequently treated with mounting medium with 4',6'-diamidino-2-phenylindole (cat. no. ab104139; Abcam) for 5 min at room temperature to visualize cell nuclei. The cells were counted and analyzed with Gen5 3.10 software (BioTek; Agilent Technologies, Inc.) by a Cytation 5 imaging reader (BioTek; Agilent Technologies, Inc.). Finally, the cells were imaged under a fluorescence microscope (Olympus Corporation).

ELISA. Culture supernatants from TBEV-infected A549 and SH-SY5Y cells with and without cepharanthine treatment were collected and tested for cytokine production. ELISA was conducted with Quantikine ELISA kits (R&D Systems China Co., Ltd.) for human tumor necrosis factor-α (TNF-α; cat. no. VAL105), human IL-1β (cat. no. VAL101), human C reactive protein (CRP; cat. no. VAL120) or human IL-11 (cat. no. D1100) following the manufacturer's protocol. The OD was measured at 450 nm on the Synergy 2 Multi-Mode Microplate Reader. Cytokine concentrations were calculated based on standard curves generated for each assay with Sigma Plot version 10.0 software (Systat; Grafiti LLC) and are shown as pg/ml.

Reverse transcription-quantitative PCR (RT-qPCR). For extraction of total RNA, cells were washed once with ice-cold PBS and RNA was extracted using TRIzol® Reagent (cat. no. 15596026; Thermo Fisher Scientific, Inc.). The concentration and purity of the extracted RNA were examined using the Synergy 2 Multi-Mode Microplate Reader. Subsequently, cDNA was synthesized from the RNA with moloney murine leukemia virus reverse transcriptase (cat. no. M1705; Promega Corporation) and dNTP mix (cat. no. C1141S; Promega Corporation) following the manufacturer's protocol. qPCR was carried out to detect gene expression of TBEV and C/EBP homologous protein (CHOP). The reaction was performed in a Rotor-Gene 3000 Thermal Cycler (Corbett Life Science) in volumes of 20 μl containing 10 μl SYBR Green 2X PCR Master Mix (cat. no. LS2062; Promega Corporation), 2 μl cDNA template, 0.4 μl of each primer (10 μM) and 7.2 μl nuclease-free water. The thermocycler conditions for qPCR were as follows: An initial denaturation at 2 min at 95˚C and 40 cycles of 10 sec at 95˚C, 10 sec at 55˚C and 25 sec at 72˚C. Expression of genes was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The levels of TBEV RNA and CHOP mRNA were quantified compared with the relative control by the threshold cycle ($2^{-\Delta\Delta Cq}$) method (27). The primer sequences for CHOP were as follows: 5'-AGCTGGAACCTGAGGAGAGA-3' (forward) and 5'-TGGATCAGTCTGGAAAAGCA-3' (reverse). The primer sequences for TBEV and GAPDH were described previously (26).

Western blotting. For total protein extracts, cells were washed once with ice-cold PBS and lysed in blue loading buffer containing dithiothreitol (cat. no. 7722; Cell Signaling Technology, Inc.) according to the manufacturer's protocol. Protein samples were prepared and total protein concentration was determined by BCA protein assay. Equal amounts of

protein samples (30 μg/lane) were separated by SDS-PAGE on 12% gels and transferred onto a polyvinylidene difluoride membrane (cat. no. 1620177; Bio-Rad Laboratories, Inc.). The membranes were blocked for 2 h at room temperature in Tris-buffered saline containing 0.1% Tween 20 (TBST) with 5% non-fat milk powder. Mouse monoclonal antibody for CHOP (cat. no. 2895) and rabbit antibodies specific for eukaryotic initiation factor 2α (eIF2α; cat. no. 5324), phospho-eIF2α (cat. no. 3398), β-actin (cat. no. 4967) or GAPDH (cat. no. 5174) were used for immunoblotting overnight at 4˚C. All primary antibodies (1:1,000; Cell Signaling Technology, Inc.) were diluted with 5% BSA in TBST. After three washes with TBST, the membranes were incubated for 2 h with horseradish peroxidase-conjugated goat anti-mouse (1:2,000; cat. no. 1706516; Bio-Rad Laboratories, Inc.) or goat anti-rabbit secondary antibodies (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) at room temperature. The membranes were washed three times with TBST and the bound antibodies were detected with Clarity Western ECL Substrate (cat. no. 1705060; Bio-Rad Laboratories, Inc.) on a GeneGnome HR image capture. The bands were quantified by densitometric analysis with GeneTools version 4.01.02 software (SynGene; Synoptics Ltd.).

Statistical analysis. All data are presented as the mean ± standard deviation of three independent experiments. Group comparisons were conducted using one-way ANOVA followed by Tukey's post hoc test with GraphPad Prism software (version 8.0; Dotmatics). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Influence of cepharanthine on the viability of A549 and SH-SY5Y cells. Human lung adenocarcinoma A549 cells are highly susceptible to TBEV and are therefore used to characterize strains of TBEV (28). Some compounds have been evaluated for their antiviral activities against TBEV in A549 cells (29). Human neuroblastoma SH-SY5Y cells have been used to explore the replication and pathogenesis of the Zika virus, which is a major pathogen of human neurological disorders (30). Food Drug Administration-approved bioactive small molecules have been screened for their abilities to inhibit the death of SH-SY5Y cells infected with La Crosse virus, which is a mosquito-borne Orthobunyavirus that leads to viral encephalitis (31). In the present study, the effects of cepharanthine on TBEV propagation and pathogenicity were assessed using both A549 and SH-SY5Y cells.

Time-of-drug addition experiments were adopted to explore which step(s) of the viral life cycle may be disturbed by cepharanthine (32). Anti-TBEV activity of polyphenol complex isolated from *Zosteraceae* seagrass (main components containing rosmarinic acid, luteolin disulfate and bioflavonoids) has been characterized using several versions of the addition experiments (33). In the present study, three schemes including co-treatment, pre-treatment and post-treatment with cepharanthine were adopted for evaluating anti-TBEV efficacy. A schematic presentation of the co-treatment and pre-treatment cepharanthine experiments is shown in Fig. 1A. First, the co-treatment and pre-treatment experiments were carried out to investigate the anti-TBEV efficacy of cepharanthine. TBEV RNA replication, viral protein expression and virus propagation were synchronously detected in cells following cepharanthine treatment.

It was necessary to analyze the cytotoxicity of cepharanthine prior to determining its antiviral efficacy. For this, A549 and SH-SY5Y cells were treated with increasing concentrations of cepharanthine. MTS assay showed that cepharanthine was not cytotoxic to A549 cells at concentrations of up to 30 μM (Fig. 1B). A significant reduction in A549 cell viability was observed upon treatment with cepharanthine at concentrations of 40, 50 and 60 μM ($P<0.05$ or $P<0.001$). As for SH-SY5Y cells, cepharanthine did not exert a cytotoxic effect at concentrations of up to 20 μM. Cepharanthine at concentrations ranging from 30 to 60 μM significantly decreased SH-SY5Y cell viability ($P<0.01$ or $P<0.001$). There was no significant difference in the cell viability between the DMSO dissolvent and the untreated control groups. The maximal concentration of 20 μM of cepharanthine was thereby selected for evaluation of its antiviral efficacy.

Inhibition of TBEV RNA replication and protein expression by cepharanthine. Whether co-treatment or pre-treatment with cepharanthine displays antiviral efficacy against TBEV was next assessed. The cellular RNA obtained from the infected cells treated with cepharanthine at the increasing concentrations was analyzed for TBEV RNA expression. RT-qPCR analysis showed that TBEV RNA replication was inhibited by cepharanthine in a concentration-dependent manner (Fig. 1C). The TBEV RNA levels were significantly reduced in the infected A549 cells with co-treatment or pre-treatment with 20 μM cepharanthine ($P<0.05$ or $P<0.01$). There was no notable reduction in TBEV RNA levels in the infected A549 cells treated with the DMSO dissolvent. Co-treatment or pre-treatment with cepharanthine also inhibited TBEV RNA replication in SH-SY5Y cells (Fig. 1D). For co-treatment, the TBEV RNA levels were significantly reduced in SH-SY5Y cells treated with cepharanthine at 5, 10 or 20 μM ($P<0.05$ or $P<0.001$). For pre-treatment, a significant reduction in TBEV RNA levels was observable in SH-SY5Y cells treated with the cepharanthine at 5, 10 and 20 μM ($P<0.001$ or $P<0.0001$, respectively). While the TBEV RNA levels were significantly decreased in SH-SY5Y cells treated with the DMSO dissolvent ($P<0.05$; Fig. 1D, pre-treatment) compared with the untreated group, the cepharanthine at 5, 10 and 20 μM significantly inhibited the viral RNA replication compared with the DMSO dissolvent ($P<0.01$ or $P<0.001$). In summary, co-treatment or pre-treatment with cepharanthine displayed a potent inhibitory effect on TBEV RNA replication.

Next, immunofluorescence staining was performed to detect TBEV protein expression in infected A549 cells with cepharanthine co-treatment. As shown in Fig. 2, expression of TBEV protein was detectable in TBEV-infected A549 cells and that treatment with cepharanthine at 20 μM effectively inhibited such expression. TBEV protein expression was undetectable in the mock group. The DMSO group showed a minor influence on viral protein expression. In parallel with the marked inhibition of TBEV RNA replication by cepharanthine at 20 μM (Fig. 1C, co-treatment), the percentage of TBEV-positive cells was significantly decreased upon

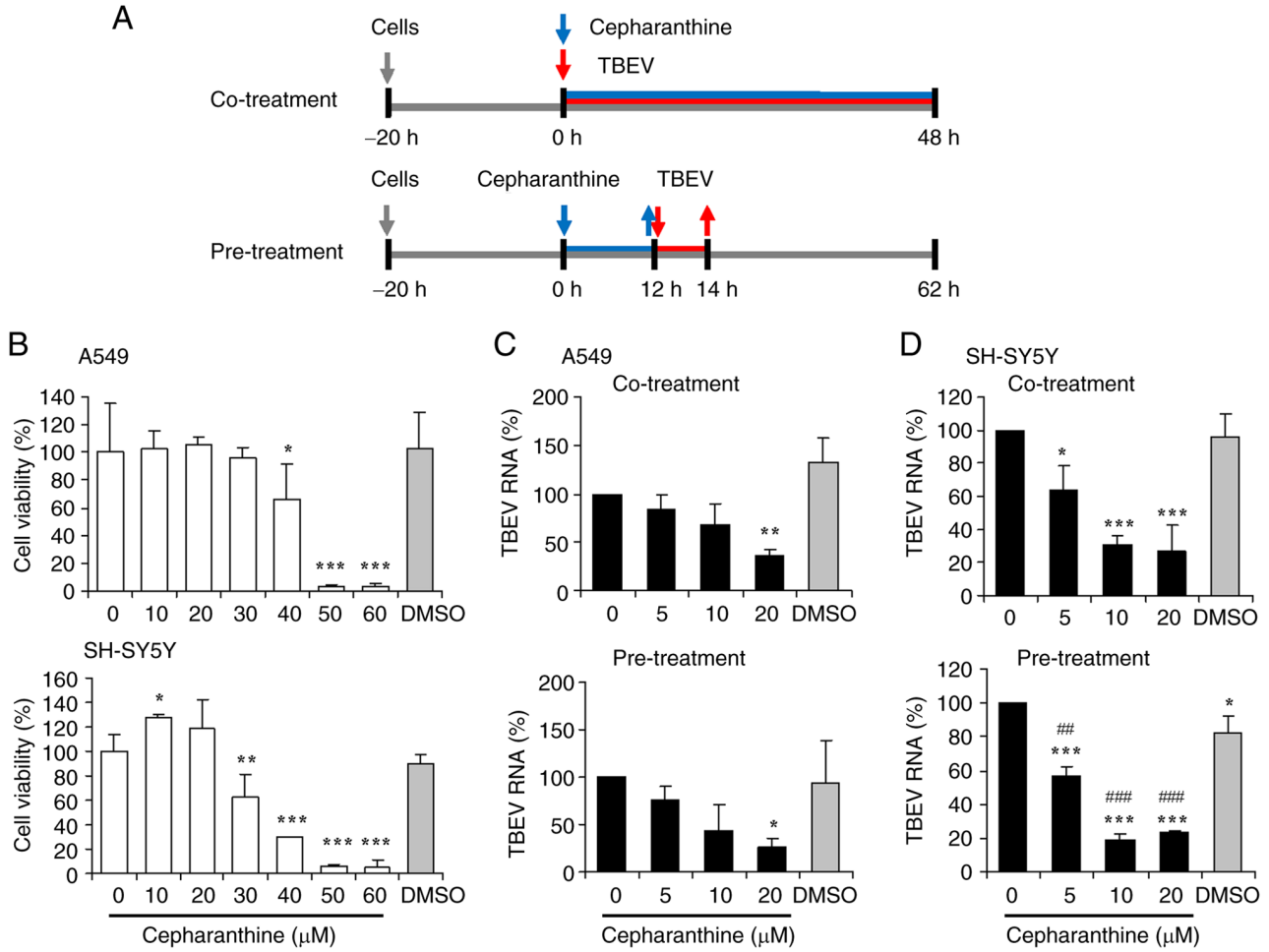

Figure 1. Cepharanthine displays a marked inhibitory effect on TBEV RNA replication in A549 and SH-SY5Y cells. (A) Schematic representation of cepharanthine addition experiments. (B) Influence of cepharanthine on cell viability. A549 and SH-SY5Y cells were treated with cepharanthine at the indicated concentrations and cell viability was determined by MTS assay. Inhibition of TBEV RNA replication by cepharanthine in (C) A549 and (D) SH-SY5Y cells. Reverse transcription-quantitative PCR was carried out to quantify TBEV RNA levels in the infected cells treated with the increasing concentrations of cepharanthine via co-treatment or pre-treatment. Data are shown as relative percentages of the untreated control (0 μM); n=3. *P<0.05, **P<0.01, ***P<0.001 vs. the untreated control; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. DMSO. TBEV, tick-borne encephalitis virus; DMSO, dimethyl sulfoxide.

cepharanthine treatment (P<0.001). These results demonstrated that TBEV RNA replication and protein expression were inhibited by cepharanthine.

Effects of different cepharanthine treatment conditions on TBEV propagation. The antiviral efficacy of cepharanthine was further confirmed by plaque assay to detect TBEV titers in the culture supernatants obtained from cells treated with cepharanthine via different schemes. The virus titers were significantly decreased in infected A549 cells co-treated with cepharanthine at 20 μM (P<0.05) or pre-treated with cepharanthine at 10 and 20 μM (P<0.05 or P<0.01; Fig. 3A). In SH-SY5Y cells, the TBEV titers were also significantly decreased upon co-treatment with cepharanthine at 10 and 20 μM (P<0.001) or pre-treatment with cepharanthine at concentrations ranging from 5 to 20 μM (P<0.01) (Fig. 3B). The DMSO group showed no or a minor inhibitory effect on the virus titers, suggesting that the inhibition of TBEV propagation was attributed to the cepharanthine treatment. These results were consistent with the significant inhibition of TBEV RNA replication in A549 and SH-SY5Y cells co-treated or pre-treated with cepharanthine (Fig. 1C and D).

In addition to the co-treatment and pre-treatment schemes, post-treatment of cepharanthine was also adopted for evaluating antiviral efficacy. A schematic presentation of cepharanthine post-treatment is shown in Fig. 4A. TBEV titers were not influenced in the A549 cells with cepharanthine post-treatment (Fig. 4C). Furthermore, whether cepharanthine exhibited antiviral efficacy against TBEV was analyzed using Vero cells. Cepharanthine at concentrations ranging from 20 to 60 μM exerted a marked cytotoxic effect on Vero cells, implying variable cytotoxicity of cepharanthine among different cell types (Fig. 4B). Vero cells were thus treated with cepharanthine at 5 and 10 μM for subsequent assays. Plaque assays showed that TBEV titers were markedly decreased upon co-treatment with cepharanthine at 10 μM (P<0.05) and that a significant reduction in the virus titers was observed in Vero cells with cepharanthine pre-treatment at 5 and 10 μM (P<0.01 or P<0.001) (Fig. 4D). Conversely, post-treatment with cepharanthine did not lead to a notable reduction in TBEV titers.

In A549, SH-SY5Y and Vero cells, TBEV propagation was inhibited by cepharanthine co-treatment and pre-treatment. This characterization allowed for further examination of the

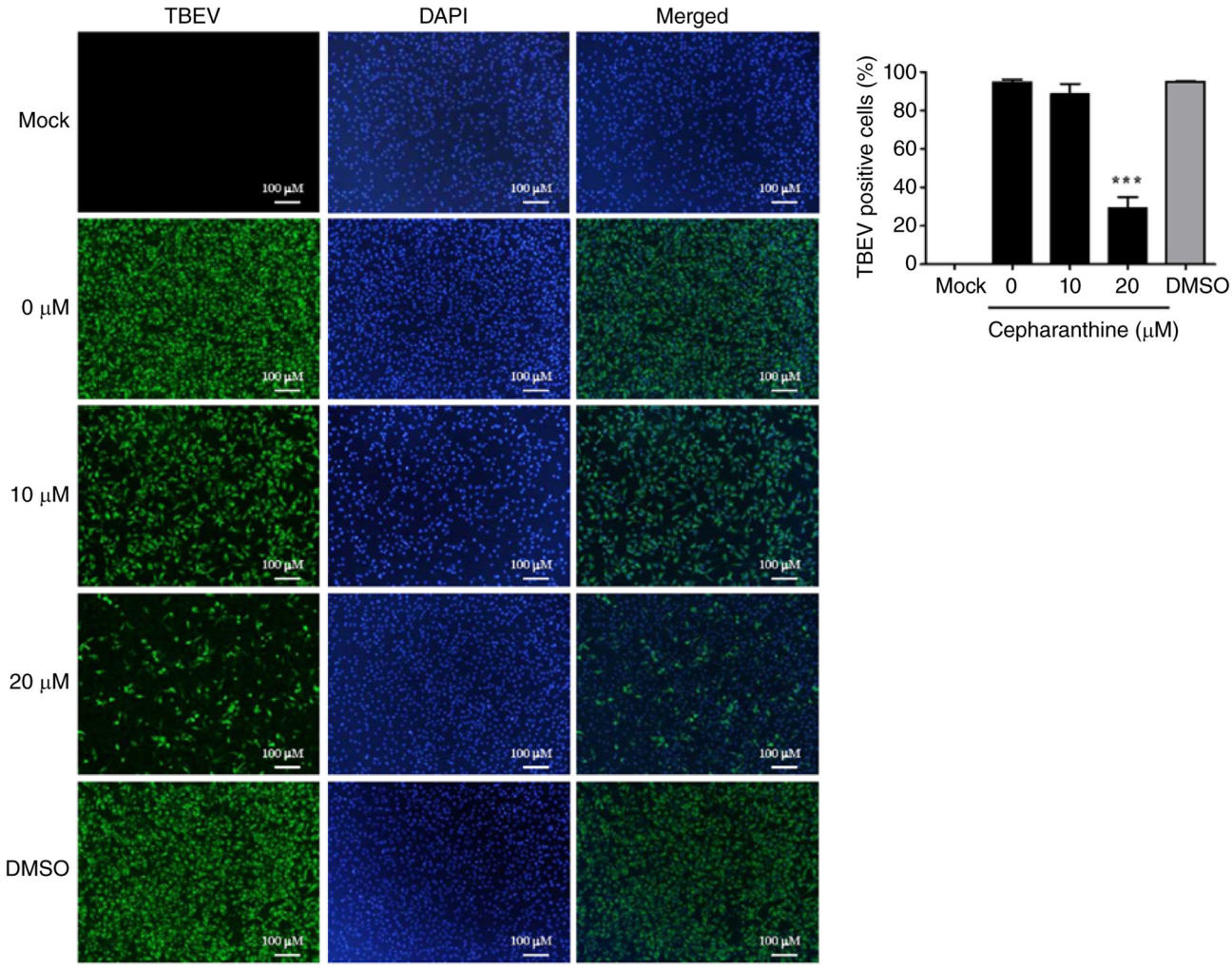

Figure 2. Cepharanthine effectively inhibits TBEV protein expression in A549 cells. The cells were incubated with TBEV and the indicated concentrations of cepharanthine. Immunofluorescence staining was performed and monitored by fluorescence microscopy (TBEV in green; nuclei in blue). The representative images of three experiments are shown (scale bar, 100 μm). The number of TBEV-positive cells in each group is shown as the relative percentage of the untreated control (0 μM); n=3. ***P<0.001 vs. the untreated control. TBEV, tick-borne encephalitis virus; DAPI, 4',6'-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide.

mechanisms underlying the effects of cepharanthine on TBEV propagation and pathogenicity based on the co-treatment and pre-treatment schemes.

Management of TBEV-induced inflammatory cytokine release by cepharanthine. Inflammatory cytokines are closely linked to severe inflammatory symptoms during TBEV infection. In mouse brain and human neural cells, TBEV is responsible for the elevated production of a variety of cytokines and chemokines that cause pathological neuroinflammation (34). In the present study, the influence of cepharanthine on the TBEV-induced inflammatory response was evaluated. ELISA was carried out to detect the production of inflammatory cytokines in the culture supernatants obtained from infected cells treated with the cepharanthine. The levels of TNF-α, IL-1β and IL-11 were notably increased in TBEV-infected A549 cells compared with the mock group (P<0.01 or P<0.001; Fig. 5A and B). The co-treatment with cepharanthine at 10 and 20 μM led to a significant reduction in the production of TNF-α, IL-1β and IL-11 induced by TBEV (P<0.05, P<0.01 or P<0.001; Fig. 5A). The amounts of TNF-α, IL-1β and IL-11 induced by TBEV were significantly decreased in A549 cells pre-treated with cepharanthine at 10 and 20 μM (P<0.001; Fig. 5B). DMSO dissolvent showed no notable inhibitory effects on the production of these cytokines. The production of TNF-α, IL-1β and IL-11 induced by TBEV was reduced by co-treatment or pre-treatment with cepharanthine.

CRP is an acute inflammatory protein that plays important roles in inflammation processes and the host response to pathogen infection (35). However, little is known about the contribution of TBEV infection to CRP release. Therefore, whether CRP release is associated with TBEV infection was assessed. As shown in Fig. 6A, TBEV infection showed little influence on the levels of CRP in A549 and SH-SY5Y cells and co-treatment with cepharanthine had a minor effect on the CRP levels, indicating that CRP was not likely involved in the inflammatory response to TBEV infection. Similarly, the release of TNF-α and IL-1β was not notably affected in the TBEV-infected SH-SY5Y cells (Fig. 6B), suggesting differential regulation of TNF-α and IL-1β by TBEV in a cell type-dependent manner. There were no notable changes in the production of TNF-α and IL-1β in infected SH-SY5Y cells co-treated with cepharanthine. Based on these data, the influence of cepharanthine pre-treatment on the release of CRP,

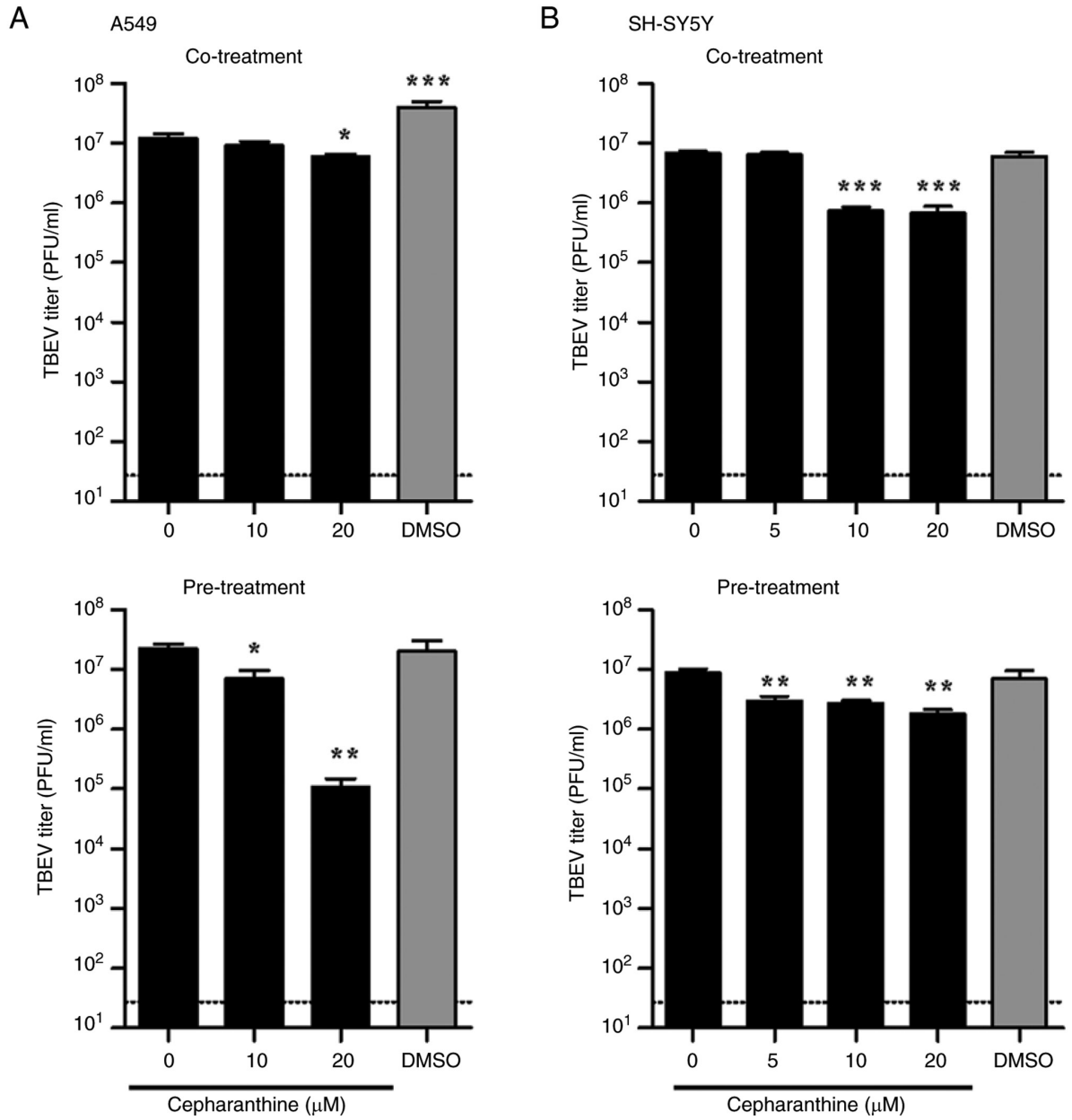

Figure 3. Pre- or co-treatment with cepharanthine decreases TBEV titers in A549 and SH-SY5Y cells. The cells were infected with TBEV and pre- or co-treated with the indicated concentrations of cepharanthine. TBEV titers were determined in the culture supernatants from the (A) A549 and (B) SH-SY5Y cells by plaque assay. The horizontal dashed line indicates the minimum detectable threshold of 1.44 $\log_{10}$PFU/ml; n=3. *P<0.05, **P<0.01, ***P<0.001 vs. the untreated control (0 μM). TBEV, tick-borne encephalitis virus; PFU, plaque-forming unit; DMSO, dimethyl sulfoxide.

TNF-α and IL-1β was not further analyzed in TBEV-infected SH-SY5Y cells. However, TBEV infection markedly enhanced IL-11 release from SH-SY5Y cells and a significant reduction in the IL-11 production induced by TBEV was observed upon co-treatment with cepharanthine at 10 and 20 μM (P<0.01 or P<0.001; Fig. 6C). Pre-treatment with cepharanthine at 20 μM significantly reduced IL-11 production in infected SH-SY5Y cells (P<0.05; Fig. 6D). Notably, 10 μM cepharanthine resulted in a significant increase in IL-11 production (P<0.05), implying that IL-11 management may be related to the cell type and cepharanthine concentration. Inhibitory effects of the DMSO dissolvent on IL-11 release were ruled out. These results suggested that co-treatment and pre-treatment with cepharanthine exhibited differential inhibitory effects on the TNF-α, IL-1β and IL-11 production induced by TBEV.

Downregulation of TBEV-induced CHOP expression by cepharanthine. CHOP, a major component of ER stress-induced apoptosis, is implicated in the progression of human diseases as well as in microbial infection processes (36). Host cells maintain ER homeostasis by triggering the defense response to viral infection, which in turn impacts viral survival and replication. Fig. 7A showed that CHOP mRNA expression was significantly upregulated in TBEV-infected A549 cells (P<0.001). Similarly, significant upregulation of CHOP mRNA expression was observed in infected SH-SY5Y cells (P<0.01; Fig. 7B). In infected A549 cells, the CHOP mRNA levels were significantly decreased upon co-treatment (20 μM; P<0.01) or pre-treatment (10 and 20 μM; P<0.05) with cepharanthine (Fig. 7A). In infected SH-SY5Y cells, co-treatment as well as pre-treatment with cepharanthine also led to a significant

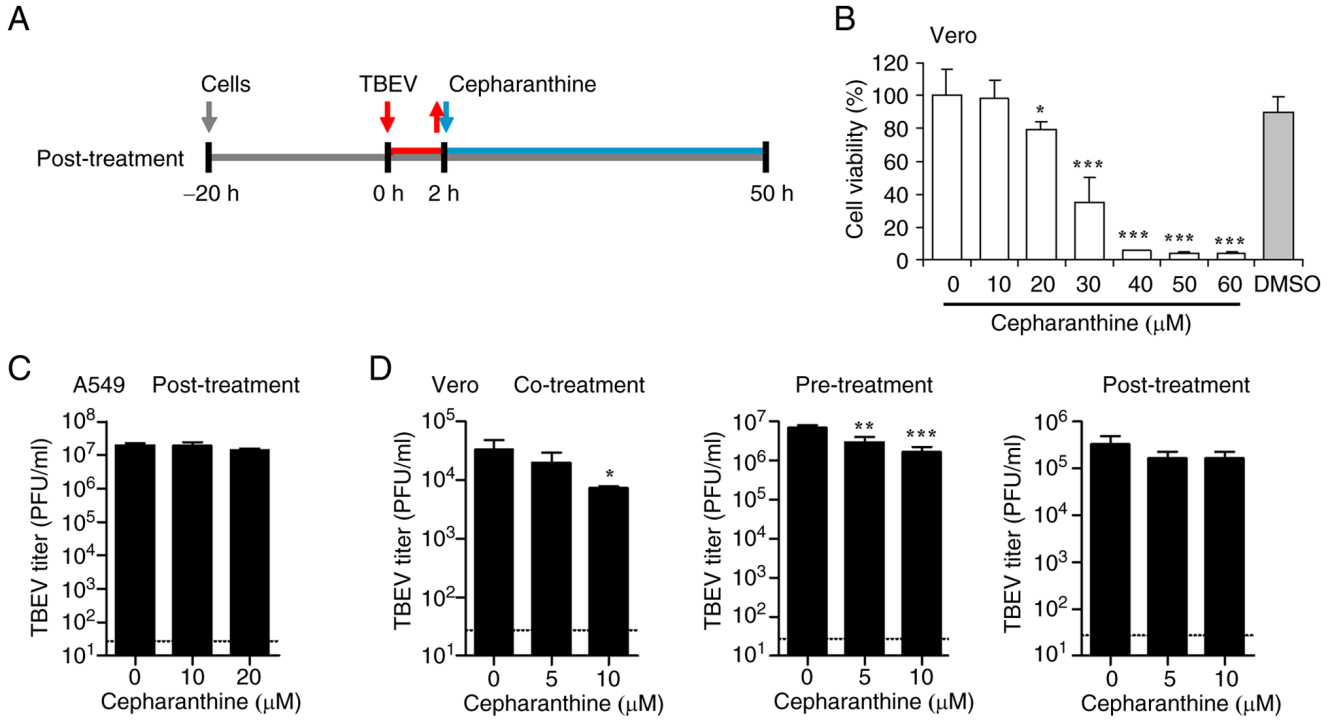

Figure 4. Management of TBEV propagation by cepharanthine. (A) Cepharanthine post-treatment scheme. (B) Influence of cepharanthine on cell viability. Vero cells were treated with cepharanthine at the indicated concentrations and cell viability was determined by MTS assay. Data are shown as relative percentages of the untreated control (0 μM). (C) Influence of cepharanthine post-treatment on TBEV propagation in A549 cells. TBEV titers were determined in the culture supernatants from the infected cells with and without (0 μM) the cepharanthine treatment by plaque assay. (D) Effects of co-treatment, pre-treatment or post-treatment with cepharanthine on TBEV propagation in Vero cells. Plaque assay was carried out to determine TBEV titers in the culture supernatants from the infected cells with and without (0 μM) the cepharanthine treatment. The horizontal dashed line indicates the minimum detectable threshold of 1.44 $\log_{10}$PFU/ml; n=3. *P<0.05, **P<0.01, ***P<0.001 vs. the relative control (0 μM). TBEV, tick-borne encephalitis virus; PFU, plaque-forming unit; DMSO, dimethyl sulfoxide.

decrease in the CHOP mRNA levels (P<0.05; Fig. 7B). The DMSO dissolvent showed a minor inhibitory effect on the CHOP mRNA expression.

Moreover, the expression of CHOP was verified at the protein level. Western blot analysis showed that TBEV infection markedly induced CHOP expression in A549 cells and that co-treatment (20 μM) or pre-treatment (10 μM) with cepharanthine significantly reduced such an induction (P<0.05; Fig. 7C). The CHOP protein levels were increased in infected SH-SY5Y cells and these levels were decreased upon treatment with cepharanthine (Fig. 7D), but these results were not statistically significant. Pre-treatment with cepharanthine displayed an inhibitory effect on CHOP expression in A549 and SH-SY5Y cells. Thus, co-treatment and pre-treatment with cepharanthine effectively downregulated TBEV-induced CHOP expression.

Enhancement of eIF2α phosphorylation by cepharanthine during TBEV infection. eIF2α plays pivotal roles in stress response and eIF2α/ATF4/CHOP signaling is involved in viral infection (37,38). Since CHOP expression was upregulated in TBEV infected cells, a potential association between TBEV infection and eIF2α phosphorylation was investigated, together with an evaluation of the effects of cepharanthine on eIF2α phosphorylation. Western blotting was performed to measure the total and phosphorylated levels of eIF2α in the cell lysates. In parallel with the upregulation of CHOP expression (Fig. 7), the levels of phosphorylated eIF2α were increased in TBEV-infected A549 and SH-SY5Y cells. Compared with levels of phosphorylated eIF2α in the mock group, TBEV infection resulted in a notable (co-treatment) or minor (pre-treatment) elevation in eIF2α phosphorylation in the A549 cells and a marked elevation in the SH-SY5Y cells (Fig. 8A and B), suggesting the phosphorylation and activation of eIF2α induced by TBEV. The levels of phosphorylated eIF2α were significantly increased in the A549 and SH-SY5Y cells co-treated or pre-treated with cepharanthine at 20 μM (P<0.05 or P<0.01; Fig. 8A and B). DMSO dissolvent did not exert enhancement of eIF2α phosphorylation in the infected cells. Treatment with cepharanthine via the two schemes enhanced eIF2α phosphorylation during TBEV infection.

Taken together, co-treatment or pre-treatment with cepharanthine displayed an anti-TBEV effect in A549 and SH-SY5Y cells. The management of eIF2α phosphorylation by cepharanthine may contribute to the antiviral effect against TBEV.

Discussion

TBEV infection leads to severe neurological manifestations and does not have a specific antiviral therapy. Using a cell-based infection model, we previously performed high-throughput screening of the Food Drug Administration-approved drug library consisting of 2,580 compounds for potential anti-TBEV agents and identified ribavirin and cepharanthine as potent inhibitors of TBEV replication by immunofluorescence assay (Tang and Zhao, unpublished data). The anti-TBEV efficacy

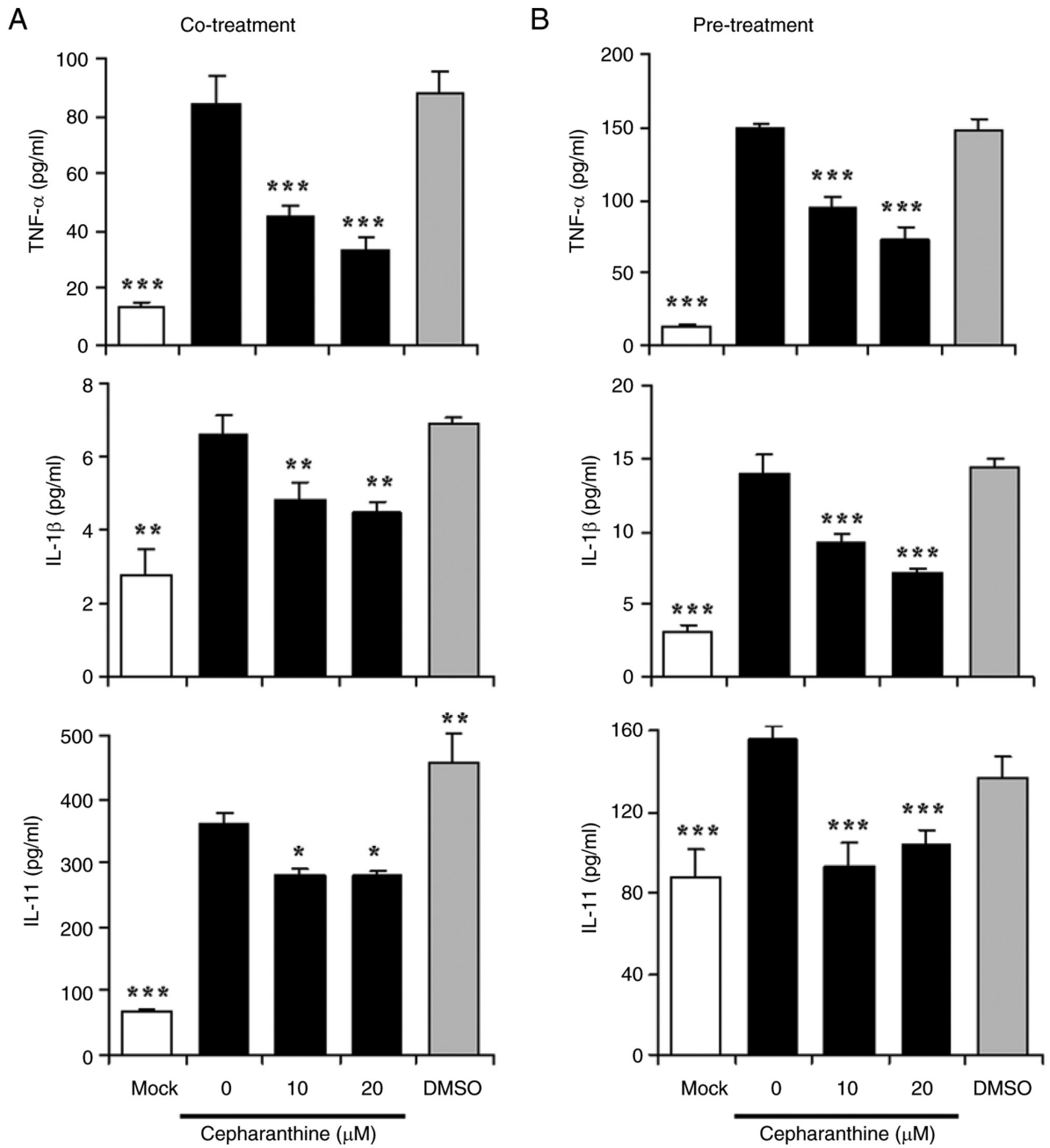

Figure 5. Co- or pre-treatment with cepharanthine reduces inflammatory cytokine induction by TBEV in A549 cells. The cells were infected with TBEV and treated with the indicated concentrations of cepharanthine via (A) co-treatment or (B) pre-treatment. The levels of TNF-α, IL-1β and IL-11 were determined in the culture supernatants by ELISA; n=3. *P<0.05, **P<0.01, ***P<0.001 vs. the untreated control (0 μM). TNF-α, tumor necrosis factor α; IL, interleukin; DMSO, dimethyl sulfoxide.

and mechanisms of ribavirin and cepharanthine required further investigation using various experimental systems. We previously reported that co-treatment or post-treatment with ribavirin exhibited a marked anti-TBEV effect in A549 and SH-SY5Y cells by upregulating the expression of myxovirus resistance A and promoting the phosphorylation of signal transducer and activator of transcription 3, implying that ribavirin primarily inhibits TBEV replication through the upregulation of antiviral response (26). The present *in vitro* study demonstrated that cepharanthine exerted the anti-TBEV and anti-inflammatory properties by modulating ER stress and inflammatory response, providing new insights into TBEV-host interaction and guiding future *in vivo* studies.

Recent studies have provided evidence for controlling virus infection using cepharanthine. For instance, cepharanthine displays anti-porcine deltacoronavirus activity through prevention of viral entry by competing with porcine aminopeptidase N protein for viral binding as well as inhibition of viral replication by downregulating the immune response and inducing autophagy (39). Cepharanthine may also impair Japanese encephalitis virus infection (40). Cepharanthine has proven to be the top inhibitor for Nipah virus glycoprotein, which plays a critical role in viral transmission (41). In particular, cepharanthine has been proposed as a prophylactic and therapeutic agent in preventing and treating human immunodeficiency virus-associated CNS disorders, upon

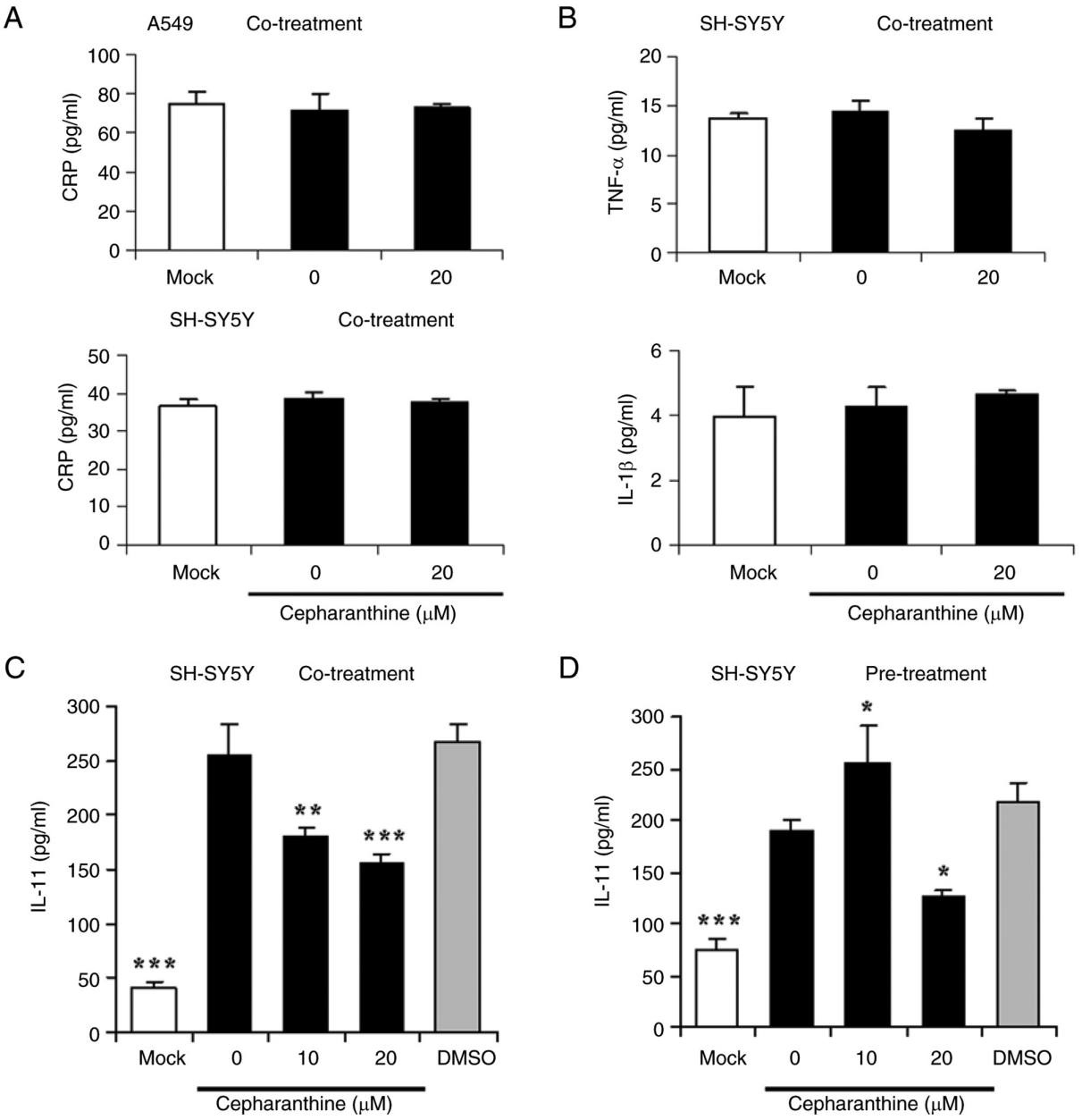

Figure 6. Cepharanthine regulates the release of CRP and inflammatory cytokines by SH-SY5Y cells infected with TBEV. (A) Influence of co-treatment with cepharanthine on CRP induction by TBEV in A549 and SH-SY5Y cells. CRP levels in the culture supernatants were measured by ELISA. (B) Effects of co-treatment with cepharanthine on TNF-α and IL-1β induction by TBEV in SH-SY5Y cells. The levels of TNF-α and IL-1β in the culture supernatants were measured by ELISA. Influence of (C) co-treatment or (D) pre-treatment with cepharanthine on IL-11 induction by TBEV in SH-SY5Y cells. IL-11 levels in the culture supernatants were measured by ELISA. n=3; *P<0.05, **P<0.01, ***P<0.001 vs. the untreated control (0 μM). CRP, C-reactive protein; TNF-α, tumor necrosis factor α; IL, interleukin; DMSO, dimethyl sulfoxide.

its penetration of the CNS and inhibiting release of inflammatory cytokines that are responsible for CNS damage (14). Similarly, TBEV infection disseminates to the CNS and leads to viral encephalitis. These properties led to the evaluation of the potential anti-TBEV efficacy of cepharanthine in the present study.

The cytotoxicity of cepharanthine is variable among cell lines, which may be associated with its impairment of cell growth by targeting relevant signaling pathways (42). Therefore, in the present study, the concentrations of cepharanthine ranging from low to the maximal without cytotoxicity were chosen for the evaluation of antiviral efficacy. Co-treatment and pre-treatment, but not the post-treatment, with cepharanthine displayed an anti-TBEV effect. Therefore, the antiviral effect and underlying mechanism of cepharanthine were investigated using the co-treatment and pre-treatment schemes. Compared with co-treatment, pre-treatment with cepharanthine exhibited a more notable inhibition of TBEV RNA replication and virus propagation, implying that the early steps of the viral life cycle, such as attachment, entry and replication, may be impacted by cepharanthine. The results of the present study showed that cepharanthine exhibited notable anti-TBEV efficacy, supporting its broad-spectrum antiviral activities and highlighting its potential as an antiviral agent against TBEV. This *in vitro* finding deserves further research based on various experimental models.

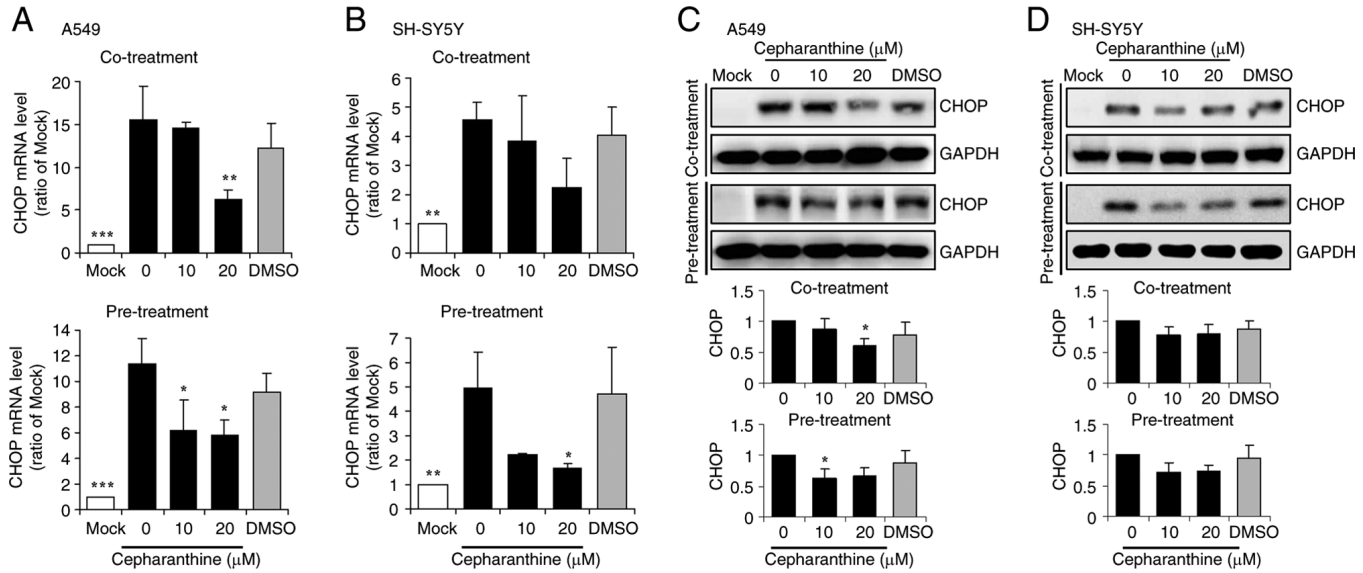

Figure 7. Co- or pre-treatment with cepharanthine decreases CHOP expression induced by TBEV in A549 and SH-SY5Y cells. The cells were infected with TBEV and co- or pre-treated with the indicated concentrations of cepharanthine. Reverse transcription-quantitative PCR was carried out to quantify the CHOP mRNA levels in (A) A549 and (B) SH-SY5Y. Data are shown as the change in CHOP mRNA levels as a ratio of the Mock. Western blot analysis of CHOP levels in (C) A549 and (D) SH-SY5Y cells. The upper panels show representative images and the lower graphs show semi-quantification of the CHOP protein levels. Data are shown as the amount of CHOP normalized to GAPDH as a ratio of the untreated control (0 μM); n=3. *P<0.05, **P<0.01, ***P<0.001 vs. the untreated control. CHOP, C/EBP homologous protein; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

TBE is a neuropathological disorder characterized by pathological neuroinflammation. The host inflammatory response plays a pivotal role in the development and progression of TBE. TBEV non-structural protein 1 elevates the production of early pro-inflammatory cytokines such as TNF-α, IL-6 and IL-10 in mouse splenocytes or mice (43). The levels of early inflammatory cytokines are differentially increased in different samples from patients with TBE (44). Consistent with the elicitation of the inflammatory response by TBEV, the present study showed that the release of TNF-α, IL-1β and IL-11 was markedly upregulated in A549 cells while only IL-11 release was upregulated in SH-SY5Y cells following TBEV infection. However, there were no notable changes in CRP release in the infected cells. These results indicated that the release of inflammatory cytokines upon TBEV infection was dependent on the cytokine and cell type. Since cepharanthine possesses anti-inflammatory properties, the present study evaluated the effect of cepharanthine on the inflammatory response to TBEV infection. Notably, the release of TNF-α, IL-1β and IL-11 induced by TBEV was significantly downregulated in A549 cells treated with cepharanthine. The marked downregulation of IL-11 release was detectable in infected SH-SY5Y cells treated with cepharanthine. Such downregulation of TBEV-induced inflammatory cytokines by cepharanthine not only represent its anti-inflammatory property but also run in parallel with its inhibition of TBEV propagation. Cepharanthine is a therapeutic agent for certain inflammatory diseases. Cepharanthine inhibits the release of TNF-α, IL-6 and IL-1β in RAW264.7 cells and downregulates the levels of cytokines in a mouse acute lung injury model (45). Cepharanthine suppresses replication of human coronavirus OC43 and reduces the virus-induced inflammatory response in MRC-5 cells (23). In an acute lung injury rat model, cepharanthine reduces pulmonary edema and hemorrhage, accompanied by a reduction in TNF-α and IL-6 levels (46). Additionally, cepharanthine ameliorates synovial inflammation and reduces TNF-α levels in the serum and joints of arthritic mice (47). In keeping with these reports, the findings of the present study indicated that co-treatment or pre-treatment with cepharanthine differentially downregulated the release of inflammatory cytokines induced by TBEV, providing *in vitro* experimental evidence for the antiviral efficacy of cepharanthine. The reduction in inflammatory cytokine release by cepharanthine requires further validation in animal models.

Perturbation of ER homeostasis causes ER stress, which is involved in a variety of diseases such as diabetes, ischemia and neurodegenerative disorders (48). CHOP, an important downstream transcription factor of multiple stress signaling pathways, plays multifunctional roles in ER stress. Dissection of CHOP function and regulation during microbial infection has implications for the development of antimicrobial therapy (36). West Nile virus infection activates the unfolded protein response and leads to CHOP induction (49). CHOP expression is upregulated at the late phase of human astrovirus infection (50). TBEV infection induces the gene expression of CHOP in a mouse macrophage cell line (51). The reports regarding ER stress in TBEV infection are limited. In the present study, a notable induction of CHOP expression in infected A549 and SH-SY5Y cells was observed. The data also established that co-treatment and pre-treatment with cepharanthine inhibited the TBEV-induced CHOP expression at the mRNA and protein levels. The management of TBEV-induced CHOP expression by cepharanthine may contribute to its antiviral effect against TBEV. Consistent with these findings, suppression of hepatitis B virus replication by nucleos(t)ide analogs has been linked to its suppression of CHOP-mediated apoptosis; AMG487 inhibits Japanese encephalitis virus infection in conjunction with downregulation of the unfolded

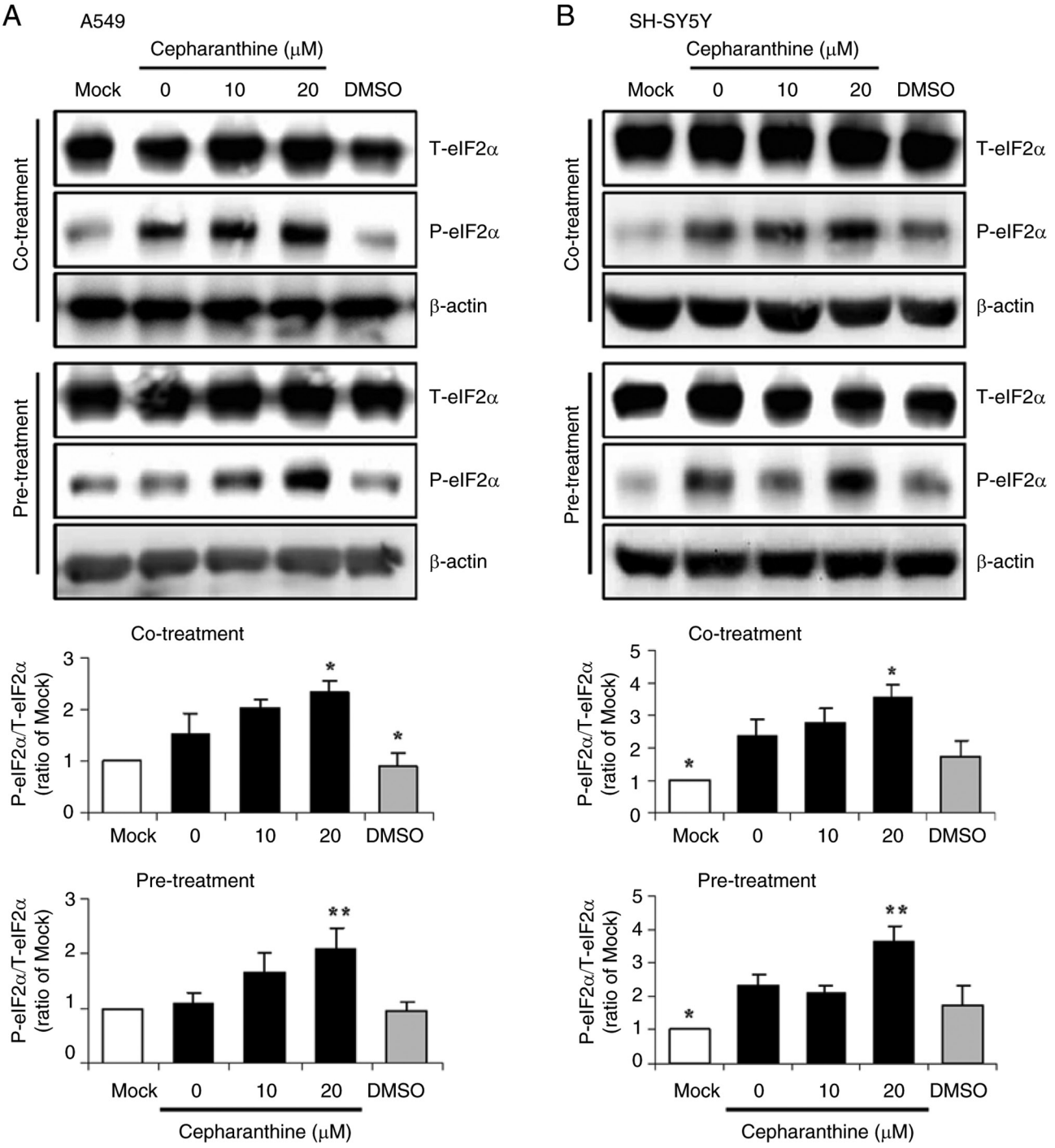

Figure 8. Co- or pre-treatment with cepharanthine enhances eIF2α phosphorylation in A549 and SH-SY5Y cells infected with TBEV. The cells were infected with TBEV and co- or pre-treated with the indicated concentrations of cepharanthine. Western blotting was performed to detect T- and P-eIF2α in the lysates obtained from (A) A549 and (B) SH-SY5Y cells. The upper panels show representative images and the lower graphs show semi-quantification of eIF2α levels. Data are present as the level of P-eIF2α to T-eIF2α after normalization to β-actin, as a ratio of the Mock; n=3. *P<0.05, **P<0.01 vs. the untreated control (0 μM). eIF2α, eukaryotic initiation factor 2α; DMSO, dimethyl sulfoxide; T-, total; P-, phosphorylated.

protein response via CHOP (52,53). The improved understanding of the interplay between TBEV infection and CHOP expression may assist with the development of antiviral agents.

Cellular signaling events triggered by viruses are the subject of intense research due to their importance in the pathogenesis of viruses. Phosphorylation of eIF2α results in translation suppression or decline in protein synthesis, and viral infection triggers cellular stress response, leading to translation shut-off via eIF2α phosphorylation and viral protein synthesis arrest (54). A number of viruses have evolved gene products targeting the protein kinase R (PKR)/eIF2α pathway, suggesting involvement of this pathway in antiviral defense (55,56). The results of the present study showed that TBEV infection increased eIF2α phosphorylation, which was in line with the upregulation of CHOP expression. Although the role of eIF2α activation in maintaining ER stress remains to be explored, the present findings suggest that eIF2α activation may play a critical role in TBEV pathogenesis.

Supporting this, Newcastle disease virus-induced translation shut-off is attributed to sustaining the phosphorylation of eIF2α (57). Peste des petits ruminants virus nucleocapsid protein induces the formation of stress granules through activation of the PKR/eIF2α pathway (58). The molecular mechanisms underlying the pharmacological properties of cepharanthine include the modulation of signaling pathways such as nuclear factor-κB, apoptosis, mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (8). Cepharanthine has been identified as an inhibitor of porcine reproductive and respiratory syndrome virus by suppressing the integrins/integrin-linked kinase/receptor for activated C kinase 1/protein kinase C α/nuclear factor-κB signaling axis (19). The upregulation of the STING/TBK1/P62 pathway and the inhibition of the PI3K/Akt and p38 MAPK pathways by cepharanthine are responsible for its inhibitory effects on herpes simplex virus type 1 (59,60). The treatment of coronavirus disease 2019 with cepharanthine is associated with its modulation of PI3K/Akt, relaxin, vascular endothelial growth factor and hypoxia-induced factor 1 pathways (61). However, the molecular mechanisms of cepharanthine against TBEV remain unclear. Considering the involvement of eIF2α activation in ER stress and the inhibition of TBEV propagation by cepharanthine, the present study examined the influence of cepharanthine on eIF2α signaling in infected cells. Consistent with its inhibition of TBEV propagation, co-treatment and pre-treatment with cepharanthine was sufficient to enhance the levels of phosphorylated eIF2α, implying that cepharanthine may exert an anti-TBEV effect via the activation of eIF2α. Although a direct association between eIF2α activation and decline in TBEV protein synthesis remains to be elucidated, these results have implications for antiviral strategies adopted by cepharanthine through modulating the ER stress to limit TBEV propagation.

However, the present study has some limitations that need to be addressed in future research. First, evaluation of the antiviral efficacy of cepharanthine primarily relied on data generated from human cancer cell lines without validation with normal human cell lines and *in vivo* experimental models. Although the two cell lines are widely used in TBEV research, the use of normal human cell lines may mimic natural viral infection, enable efficacy assessment in a cellular environment close to physiological status and provide a reliable platform for development of antiviral agents. Future research based on normal human cell lines remains to be pursued for cross-validation of the antiviral efficacy of cepharanthine against TBEV. Second, the lack of representative images for the stained plaque assays is a concern. Although all the plaque assays were strictly conducted and the data were statistically analyzed, the visual documentation in supporting quantitative data is important. Thus, future research will provide PFU calculation combined with stained cell images for presentation of TBEV titers to demonstrate reliability and integrity of the results. Third, the effect of cepharanthine via the post-treatment scheme was only presented as the changes in the virus titers, which would be strengthened with analysis of inflammatory cytokines and the eIF2α/CHOP pathway. Finally, assessment of other ER stress-related genes and cell apoptosis may provide important functional insights into the antiviral mechanisms of cepharanthine. To more comprehensively explore the anti-TBEV effect and underlying mechanisms of cepharanthine, further studies are needed to clarify these issues.

Due to the various clinical applications and the distinct antiviral activities of cepharanthine, the present study focused on the antiviral effect and underlying mechanisms of cepharanthine against TBEV *in vitro*. The findings demonstrated the antiviral and anti-inflammatory effects of cepharanthine, together with the management of ER stress response during TBEV infection and should be further explored as an antiviral agent against TBEV using animal experiments.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

WDT carried out the experiments and contributed to the analysis and interpretation of the data. LJZ was responsible for the study design, validation, supervision, funding acquisition, project administration, conceptualization and writing, reviewing and editing the manuscript. WDT and LJZ confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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