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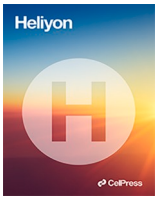

Research article

Resveratrol inhibits rabies virus infection in N2a cells by activating the SIRT1/Nrf2/HO-1 pathway

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A B S T R A C T

Rabies is a highly lethal infectious disease with no existing treatment available, thus investigating effective antiviral compounds to control rabies virus (RABV) infection is of utmost importance. Resveratrol is a natural phenolic compound that, as a phytoalexin, exhibits several biological activities, including antiviral activity. In this study, we evaluated the inhibitory effect of resveratrol on RABV infection and investigated its molecular antiviral mechanism. We found that resveratrol significantly inhibited RABV infection, including the phases of adsorption, replication, and release, and also directly inactivated RABV and inhibited its infectivity. However, resveratrol had no significant effect on RABV internalization. Resveratrol also reduced RABV-induced oxidative stress, specifically reactive oxygen species and malondialdehyde levels. Western blotting analysis revealed that resveratrol enhanced antioxidant signaling via the SIRT1/Nrf2/HO-1 pathway and inhibited viral replication. Viral infection was enhanced after SIRT1 knockdown, which inhibited the SIRT1/Nrf2/HO-1 antioxidant signaling pathway, suggesting that this pathway plays an important role in RABV replication. Overall, resveratrol prevented the adsorption, replication, and release of RABV and directly inactivated RABV, but failed to inhibit RABV internalization. Furthermore, resveratrol activated the SIRT1/Nrf2/HO-1 pathway to inhibit RABV replication and suppressed RABV-induced oxidative stress. These findings highlight the therapeutic potential of resveratrol for fighting RABV infections.

1. Introduction

Rabies is a zoonotic disease caused by the rabies virus (RABV), and has a mortality rate of up to 100 %. Globally, the majority of all infections are transmitted through animal bites (especially free roaming dogs) and are prevalent mainly in Asia and Africa [1]. RABV is a neurotropic, negative-stranded RNA virus that spreads primarily by entering the nervous system through the skin from an animal bite and eventually travels to the brain for replication, ultimately leading to multiple organ failure and death [2]. As a highly fatal infectious disease, rabies has been a major public health concern [3].

The RABV genome comprises a single-stranded negative-sense RNA approximately 12 kb in length [4]. It encodes five structural

Abbreviations: RABV, Rabies Virus; SIRT1, silent information regulator sirtuin 1; Nrf2, Nuclear factor erythroid 2-related factor 2; HO-1, Heme oxygenase-1; CAT, Catalase; SOD, superoxide dismutase; GPx-1, Glutathione peroxidase 1; GSH-Px, glutathione peroxidase; DFA, Direct fluorescent antibody; FFU, Fluorescent focus unit; MDA, malondialdehyde.

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proteins, including nucleocapsid (N), phosphoprotein (P), matrix (M), glycoprotein (G), and RNA-dependent RNA polymerase protein (L), each of which is involved in a different stage of the life cycle. First, the G proteins on the surface of RABV bind to possible host cell receptors (including p75NTR, NCAM, and mGluR2), triggering endocytosis of the viral particle [5]. The low pH of the endosomes catalyzes G protein-mediated fusion of the viral envelope with the endosomal membrane, during which the viral N, P, and L proteins (which together form the ribonucleoprotein (RNP) with RNA polymerase activity) are released into the cytoplasm. The viral replication and transcription processes are then followed in Negri's body, which have been used as histologic evidence of RABV infection as a typical manifestation of rabies infection in the brain [6]. The L-P polymerase complex is transcribed from the 3′ end, and the expression level of viral mRNA and encoded proteins decreases from the 3′ to the 5′ end of the genome in the order 3′-N > P > M > G > L -5′. When adequate amounts of N and P proteins are synthesized in the cytoplasm, L-P polymerase activity changes from transcription to replication, synthesizing full-length positive-stranded replicative RNA that becomes the template for the negative-stranded RNA genome, where the primary transcription is completed [7]. The newly synthesized viral RNA is packaged into RNP along with the N-P-L complex; this is followed by M binding to the RNP complex, which condenses the RNP and localizes it to the cell membrane in the presence of G. Finally, the mature viral particle crosses the host cell membrane, releasing the mature rabies virus particle [8,9]. Rabies virus has evolved a mechanism to alter the steepness of the transcript gradient by downregulating the expression of distal genes thereby attenuating the increase in cytosolic case effect and prolonging host cell survival [6,10,11].

Resveratrol is a polyphenol stilbene extracted primarily from plants such as grapes and mulberries and is known for its antioxidant properties [12,13]. Numerous studies have reported that resveratrol also has anti-inflammatory, anti-aging, anti-cancer, and anti-viral properties [14–16]. In antiviral studies, resveratrol has been found to inhibit the activity of viruses such as influenza virus, hepatitis C virus, respiratory syncytial virus (RSV), and human immunodeficiency virus (HIV), and its antiviral mechanism has been widely studied [17–20]. Furthermore, it was found that resveratrol could inhibit the replication and inflammatory response of enterovirus 71 (EV71) by inhibiting the oxidative stress-mediated autophagy signaling pathway [21]. In addition, resveratrol activates the antioxidant signaling pathway, which inhibits COVID-19 replication [22,23]. Other viruses, such as the hepatitis C virus and HIV, induce oxidative stress to promote intracellular replication. In animal models, reactive oxygen species (ROS) inhibitors reduce pathogenesis and viral replication [24]. Resveratrol, an inhibitor of ROS, can attenuate oxidative stress and inflammatory injury by activating the expression of silent information regulator 2 homologue 1 (SIRT1), which regulates downstream transcription factors such as Nrf2 and NF-κB [25,26]. Among them, SIRT1 is a protein deacetylase that controls the acetylation of specific transcription factors and proteins, with multiple functions such as energy metabolism, stress response, inflammation, and redox homeostasis [27,28]. Nrf2 has anti-inflammatory and anti-oxidative stress properties and is the main transcription factor responsible for regulating the antioxidant response, which can activate downstream antioxidant signaling pathways, as well as the expression of downstream stress-inducible proteins such as heme oxygenase-1 (HO-1) [29,30]. Furthermore, Nrf2 reduces lipid peroxidation, increases the antioxidant activities of superoxide dismutase (SOD) and glutathione peroxidase 1 (GPx-1), and enhances catalase (CAT) expression [31–33].

Currently, the only mode of rabies virus prevention and control is via vaccination due to the specificity of the pathogenicity of the rabies virus, therefore more effective antiviral drugs need to be developed to respond to the viral infection process [34–36]. More attention has been paid to screening for anti-RABV drugs among broad-spectrum antivirals [37–39], and only a few studies have been conducted to find antiviral drugs from the perspective of rabies pathogenesis. It has been demonstrated that RABV infection can use ROS for the enhancement of pathogenesis [40,41]. Therefore, evaluating anti-RABV drugs through the perspective of oxidative stress reduction could be potentially fruitful. Here, we evaluated the antiviral effects of resveratrol on RABV using the N2a cell model and elucidated the molecular mechanisms underlying its anti-RABV activity from the perspectives of blocking the virus-associated life cycle and inhibiting oxidative stress.

2. Materials and methods

2.1. Compounds and antibodies

Dimethyl sulfoxide (DMSO), resveratrol, and acetylcysteine (N-acetylcysteine, NAC) were purchased from Selleck (St. Louis, MO, USA). FITC-labeled RABV antibody was purchased from Fujirebio (Tokyo, Japan) and Evans Blue from Sigma Aldrich (St. Louis, MO, USA)

Primary antibodies used for Western blot were: anti-β-actin (Cell Signaling Technology (CST), Danvers, MA, USA), anti-RABV N protein (generated in our laboratory), anti-p-AMPK (CST), anti-HO-1 (Abcam, Cambridge, UK), anti-SIRT1 (CST), and anti-Nrf2 (CST).

2.2. Cell culture and virus

Mouse neuroblastoma cells (N2a cells) were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 10 % fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin G, and 100 g/ml streptomycin (Gibco, Waltham, MA, USA).

The RABV strains challenge virus standard 11 (CVS-11), SC-16, and CTN-1 were propagated in N2a cells and stored at −80 °C.

2.3. Fluorescent focus unit (FFU) assay

After diluting the virus stock solution with serum-free DMEM in a 96-well plate at a 5-fold multiplicity, 50 μL of diluted virus was added to a new 96-well plate, followed by the addition of 50 μL of cells, and incubated in a 37 °C incubator for 24 h. Viral fluorescent

foci were detected using Direct Fluorescent Antibody (DFA) assay and the number of green fluorescent foci <20 per well was recorded. The formula used for calculating the viral titer was [42]:

Viral titer (fluorescent foci units [FFU]/mL) = (average number of fluorescent foci in the last four wells × dilution factor × 1000)/50.

2.4. Direct fluorescent antibody (DFA) test

The cell supernatant was discarded from the 96-well plate to be tested and the cells were washed thrice with phosphate buffered solution (PBS). Acetone was added, and the cells were fixed at −20 °C for 15 min. Acetone was discarded, and the cells were stained with anti-RABV fluorescent antibody for 1 h. The viral fluorescent foci were observed under a fluorescence microscope (Olympus 1X51, Olympus, Tokyo, Japan). The fluorescent antibodies included FITC-labeled and Evans blue diluted in PBS, where FITC-labeled anti-RABV antibody:PBS was 1:50 and Evans blue:PBS was 1:10000.

2.5. Reverse transcription (RT)-quantitative PCR (qPCR)

RNA was extracted from the cell samples using the TRIzol method, and 1–2 μg of RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA), oligo (dT) (6-mer) primer, and 10 mM dNTPs (TaKaRa, Dalian, China). The cDNA was then subjected to PCR using the GoTaq Qpcr (Promega). The related genes were detected using CFX96 Touch Real-Time PCR system (Bio-Rad, Hercules, CA, USA). The primers used in this study were: β-actin-F, GTGCTATGTTGCTCTAGACTTCG; β-actin-R, ATGCCACAGGATTCCATA; rabies virus N gene-F, AATCTCACCGCAAGGGAAGC; rabies virus N gene-R, ATGCAGCAATAACCGTCGCA; RABV Genome F, AGAAGAAGCAGACATCGTCAGTTG; RABV Genome R, GGAGACCACCTGATTATTGACTTTGA.

2.6. Western blotting analysis

Cells were lysed using RIPA buffer (Beyotime, Shanghai, China) at 4 °C for 20 min; then, 1:50 protease inhibitor and 1:100 phosphatase inhibitor were added (Beyotime). Cell lysates were collected and centrifuged at 12,000×*g* for 10 min at4 °C. The supernatant was collected and protein concentration was determined using a BCA kit (Beyotime). The collected protein samples were boiled in a buffer containing 5 × β-mercaptoethanol for 10 min. The samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were transferred to a polyvinylidene fluoride membrane, followed by incubation with 5 % (w/v) skimmed milk in PBS containing 1 % (v/v) Tween-20 for 1 h at26 °C. Then, the proteins were incubated 16 h at 4 °C with primary antibody diluted in primary antibody diluent and a 1:5000 dilution of horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibody (CST) for 1 h. The membrane was then incubated for 1 h with an enhanced chemiluminescence (ECL) reagent (Beyotime) as a substrate to detect the proteins blotted on the membrane.

2.7. Antiviral assay

N2a cells were seeded into 24-well plates at a density of 1×10^6 cells per well. When the cell concentration reached 70–80 % confluence, the cells were infected with CVS-11, SC-16, and CTN-1 (multiplicity of infection [MOI] = 0.1) for 1 h at 37 °C, and then treated with different concentrations of resveratrol for 24 h. The cell supernatants were then collected to determine the viral titer, and the cell lysates were collected for qPCR or Western blot assay.

2.8. Time-of-drug-addition assay

CVS-11 was incubated with N2a cells at an MOI of 0.01 at −1 to 0 h. Resveratrol (40 μM) was added at four time points: −3 to 0 h (pre-infection), −1 to 0 h (co-infection), 0–24 h (post-infection) and −3 to 24 h (complete infection). Cell lysates and cell supernatants from each treatment were harvested after 24 h for RT-qPCR analysis and viral titer determination.

2.9. Translation inhibition

Cycloheximide inhibits protein translation and prevents the synthesis of new viral proteins, so that viral RNA synthesis is performed through primary transcription by the RABV-associated polymerase. After the cells were infected with virus at MOI = 3 for 1 h and the supernatant was removed, the cells were treated with cycloheximide (150 μg/ml) or resveratrol (20 μM and 40 μM) and cycloheximide (150 μg/mL) for 24 h, and the cells were lysed to extract RNA for transcriptional analysis [7].

2.10. Viral binding, entry, and release assays

For the binding assay, N2a cells were cooled at 4 °C for 1 h and incubated with different concentrations (20 μM and 40 μM) of resveratrol and CVS-11 with MOI = 10 for 1 h at 4 °C. Cells were lysed after washing twice with PBS and RNA was extracted for RT-qPCR analysis. For the entry assay, the N2a cells were first exposed to 4 °C with CVS-11 at MOI = 10 for 1 h, after the supernatant was discarded, the cells were transferred to resveratrol of different concentrations (20 and 40 μM) for 1 h at 37 °C; the cells were washed twice with PBS and treated with proteinase K (1 mg/mL) for 45 min at 4 °C to remove adsorbed but not internalized virus. Cells were

lysed and RNA was extracted for further analysis by RT-qPCR. For release assay, cells were incubated with CVS-11 at MOI = 1 for 24 h at 37 °C, then washed with PBS and incubated with different concentrations of resveratrol (5, 10, 20, and 40 μM) for 4 h at 37 °C. The cells were then incubated for 1 h at 37 °C, washed twice with PBS, and incubated for 4 h at 37 °C. After the different treatments, the supernatants were collected and titrated with FFU assay.

2.11. Viral particle inactivation assay

CVS-11 was diluted to 1×10^5 FFU/mL with serum-free medium, then different concentrations of resveratrol (0, 5, 10, 20, and 40 μM) were added to the viruses at this titer and incubated in an incubator at 37 °C for 1 h, 2 h, and 4 h, respectively, and the incubated mixture was collected for determination of the viral titer.

2.12. Cell viability assay

N2a cells were inoculated into 96-well plates at a density of 1×10^5 cells per well. When the cell confluence reached over 80 %, the cells were treated with different concentrations of resveratrol (0, 2.5, 5, 10, 20, and 40 μM) for 24 h. After the cells were washed thrice with PBS, the viability of the cells was determined by the CCK-8 assay (Yeasen, Shanghai, China). The absorbance was then measured at an OD of 450 nm.

2.13. Small interfering RNA (siRNA) knockdown of SIRT1

The siRNA targeting SIRT1 was obtained from GenePharma (Shanghai, China). The target sequences of the three siRNAs were siSIRT1-1: sense 5′-GCACCGAUCCUCUCGAACAAUTT-3′, siSIRT1-2: sense 5′-CCCUCAAGCCAUGUUUGAUTT-3′, siSIRT1-3: sense 5′-UUCUCCGAACGUGUCACGUTT-3′. For the knockdown assay, 100 nM siRNA was transfected into N2a cells with Lipofectamine 3000 (Invitrogen) following the manufacturer's protocol for 24 h. The cells were then incubated with CVS-11 for 1 h, and then replaced with fresh DMEM for 24 h. Western blotting was performed on the collected cells.

2.14. Reactive oxygen species (ROS) detection

After CVS-11 infected N2a cells with MOI = 0.1 for 1h, the cells were treated with different concentrations of resveratrol (0,10, 20, and 40 μM) for 24 h. After removing the supernatant, the cells were washed thrice with PBS, and the accumulation of ROS was detected using a ROS detection kit (Shanghai Yeison Biotechnology Co., Ltd., Shanghai, China) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

2.15. Lipid peroxidation assay

CVS-11 was used to infect N2a cells with MOI = 0.1 for 1 h. After treating the cells with resveratrol at different concentrations (0, 10, 20, and 40 μM) for 24 h, the cells were lysed and centrifuged at 10,000×*g* for 10 min at 4 °C. Total protein concentration was measured using the BCA Protein Assay kit (Beyotime) and the total amount of malondialdehyde (MDA) was measured using the Lipid Peroxidation MDA Detection Kit (Beyotime) following the manufacturer's protocol.

2.16. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) activity assays

CVS-11 infected N2a cells with MOI = 0.1 for 1h were treated with different concentrations of resveratrol (0,10, 20, and 40 μM) for 24 h. The cells were washed twice in ice-cold PBS and lysed. The homogenate was centrifuged at 10,000×*g* for 10 min at 4 °C, and the SOD, CAT, and GSH-Px activities in the supernatants were determined using SOD, CAT, and GSH-Px Activities Test kits (Beyotime), according to the manufacturers' instructions. The total protein content was determined using the BCA Protein Assay Kit (Beyotime).

2.17. Statistical analysis

Data was analyzed using SPSS version 20.0 (IBM Corp., Armonk, NY, USA). Graphs were plotted using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Imagine Lab (Imagine Lab, USA) was used to calculate the density of the bands. All data are expressed as the mean ± standard deviation (SD). For multiple groups, statistical significance was evaluated by a non-parametric test. $^*p < 0.05$, $^{**}p < 0.01$.

3. Results

3.1. Resveratrol inhibits RABV infection in N2a cells

To evaluate the potential antiviral effects of resveratrol on RABV, green fluorescent foci of RABV N protein in N2a cells were observed with DFA. The results showed that increasing the concentration of resveratrol reduced the quantity of the fluorescent foci (Fig. 1A). The cytotoxic effect of resveratrol on N2a cells was evaluated using the CCK-8 assay. The results showed that resveratrol at 5,

10, 20, or 40 μM had no significant effect on the viability of N2a cells after 24 h treatment. (Fig. 1B). The titer of RABV was detected with an FFU assay and decreased significantly as the resveratrol concentration increased, with reduction rates of 79–97.6 % (Fig. 1C). To verify the inhibitory effect of resveratrol on RABV replication, the level of RABV *N* mRNA was assessed by RT-qPCR. As expected, we observed that increasing the resveratrol concentration in the treated RABV-infected cells significantly decreased the RABV *N* mRNA level (Fig. 1D). In addition, western blotting analysis showed that resveratrol dose-dependently decreased the expression level of RABV N protein (Fig. 1E). The effect of resveratrol on primary transcription of RABV was also evaluated. The inhibition of protein translation by cycloheximide (CHX) prevented the synthesis of new viral proteins, and the results showed that 40 μM resveratrol reduced the RABV genome number and the level of primary transcription of the N protein of RABV. These results indicate that resveratrol has anti-RABV activity in N2a cells.

3.2. Resveratrol affects the different infection cycles of RABV

An experimental scheme was designed to further evaluate the effect of resveratrol on the intracellular infection cycle of RABV (Fig. 2A) [43,44]. To characterize the effect of resveratrol on the RABV infection cycle, RABV *N* mRNA levels were assessed by RT-qPCR, as shown in Fig. 2B. The inhibitory effect of resveratrol on the viral replication phase (0–24 h p.i.) was significant (>90 %) compared with that in the control in which no resveratrol was added. During the −3 to 24 h post-infection period, resveratrol exhibited an inhibitory effect of over 80 %. In experiments conducted during the early stage of RABV infection (−1 to 0 h p.i.), resveratrol inhibited early infection by approximately 35 %. However, when N2a cells were pre-incubated with resveratrol (−3 to 0 h p.i.), the inhibitory effect of resveratrol on RABV was reversed, indicating that resveratrol facilitated virus entry after pre-incubation. The DFA results were consistent with the RT-qPCR results (Fig. 2C). Given the inhibitory effect of resveratrol on the early infection phase (−1 to 0 h p.i.) of RABV, we further explored the effect of resveratrol on RABV adsorption to cells and entry into cells. In the simulated virus adsorption assay, resveratrol reduced the RABV genome number, and 40 μM resveratrol inhibited the adsorption of viruses by approximately 70 %, indicating that resveratrol inhibited RABV adsorption to cells. However, in the simulated viral entry assay, resveratrol had no significant effect on RABV genome number, implying that resveratrol does not affect RABV entry into N2a cells (Fig. 2D and E). Together, these results suggest that resveratrol mainly targets the replication phase of the virus and that it inhibits viral adsorption.

3.3. Resveratrol inhibits the release of RABV and directly inactivates the virus

In the viral release assay, an increase in resveratrol concentration resulted in a dose-dependent decrease in the RABV titer. At 40 μM resveratrol, the titer was reduced by over 50 %. The results indicate that resveratrol inhibits the release of RABV (Fig. 3C). Virus inactivation experiments were performed to determine whether resveratrol directly targets RABV virus particles. The results demonstrated that increasing the concentration of resveratrol incubated with RABV significantly reduced the titer of RABV. Prolonging the incubation time of resveratrol with RABV significantly reduced the viral titer, exhibiting a virucidal effect. In particular, RABV particles with 40 μM resveratrol inactivated about 94 % of the virus after 1 h of co-incubation and over 98 % of the virus after 2 h of co-incubation. After treatment for 4 h, the virucidal effect of resveratrol was >99 % (Fig. 3D). Thus, resveratrol kills RABV particles and inhibits the infectivity of RABV. These results suggest that resveratrol can both inhibit virus release and directly inactivate RABV virion.

3.4. Resveratrol inhibits the virus activity of strains SC-16 and CTN-1

In order to determine the inhibitory effect of resveratrol on other RABV strains, various methods determined its impact on the street virus SC-16 and the vaccine CTN-1 strains. As shown in Fig. 4A, the findings exhibited a gradual reduction in the number of green fluorescent foci of SC-16 and CTN-1 strains with increasing concentration of resveratrol, alongside a decrease in their titers. Additionally, the inhibitory effect of resveratrol on vaccine strain CTN-1 was more pronounced compared with strain SC-16. Specifically, the inhibition rate of 40 μM resveratrol was approximately 80 % against CTN-1, but only approximately 60 % against SC-16 (Fig. 4B). Further analysis of the mRNA expression levels of the *N* genes of the two strains revealed that 20 μM resveratrol had a more noticeable inhibitory effect on the RABV *N* mRNA levels of the CTN-1 strain compared with the SC-16 strain (Fig. 4C). In conclusion, these findings indicate that resveratrol can potentially inhibit the viral activity of both street and vaccine strains of RABV.

3.5. Resveratrol alleviates RABV-induced ROS and malondialdehyde (MDA) levels in N2a cells

Some studies have shown that oxidative stress plays an important role in RABV infections [40]. Oxidative stress induces elevated ROS levels and lipid peroxidation [45]. Therefore, we investigated ROS and MDA levels in N2A cells after CVS-11 infection. As shown in Fig. 5A, infection by CVS-11 increased ROS levels, suggesting that RABV infection increases ROS and causes oxidative stress. Notably, the ROS levels decreased dose-dependently after the addition of different concentrations of resveratrol. Resveratrol at 40 μM reduced the ROS levels by approximately 30 %, whereas the levels of ROS induced by CVS-11 decreased by about 19 % after treatment with 20 μM NAC, a ROS scavenger. We then examined MDA levels before and after CVS-11 infection, and found that MDA increased after CVS-11 infection. After treatment with resveratrol, MDA levels decreased dose-dependently, suggesting that resveratrol attenuates the lipid peroxidation induced by CVS-11. These results indicate that resveratrol inhibits RABV-induced ROS and MDA levels.

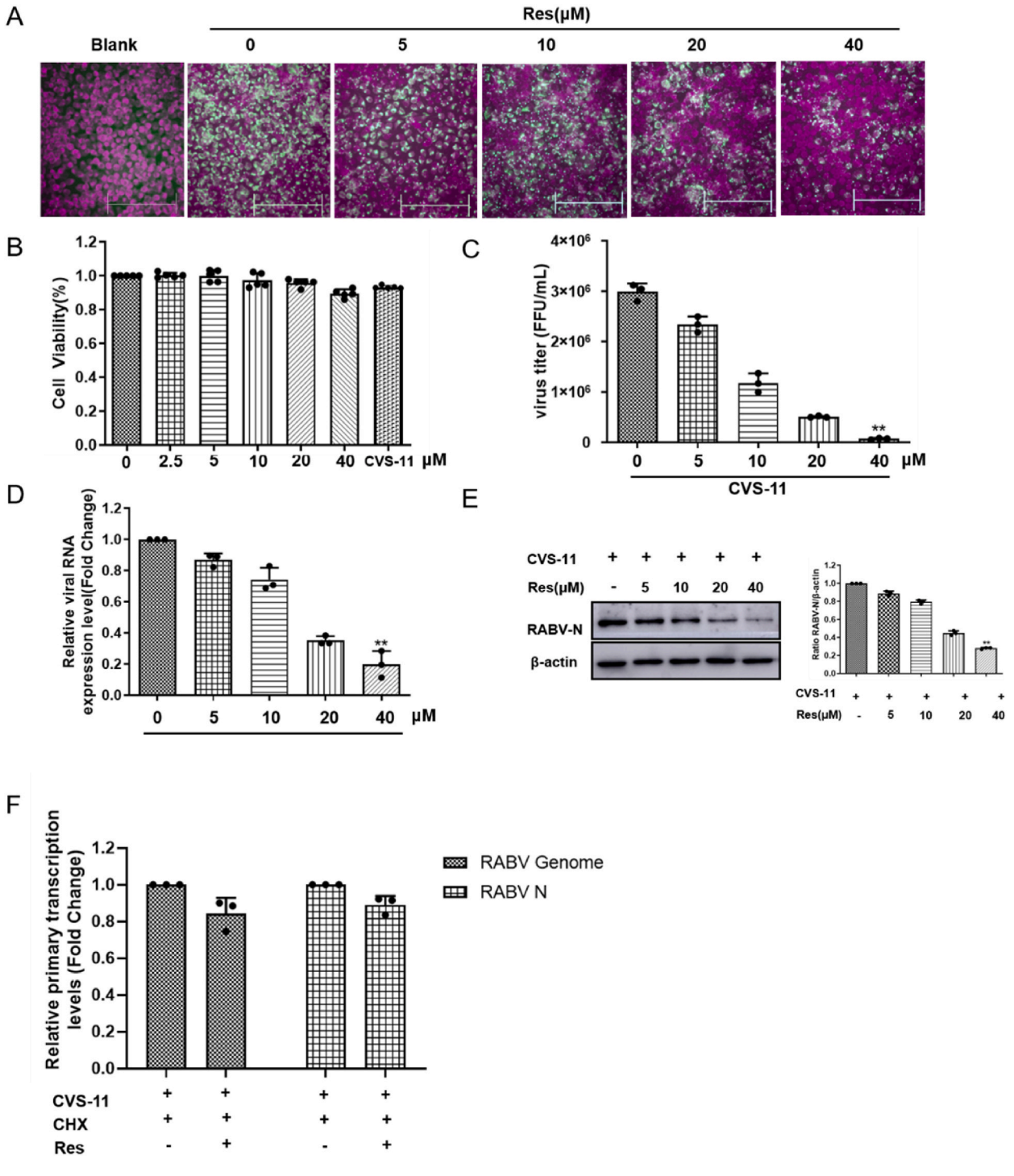

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Fig. 1. Effect of resveratrol on RABV activity. (A) Treatment with different concentrations of resveratrol (0, 5, 10, 20, and 40 μM) for 24 h on RABV infection examined in N2a cells using DFA. Scale bar, 50 μm (B) The impact of resveratrol and CVS-11 on cell viability was determined using CCK-8 assay for 24 h. Results are presented as a percentage of control values. (C) The impact of resveratrol on the CVS-11 titer measured using FFU for 24 h. (D) The effect of resveratrol on CVS-11 *N* gene mRNA levels assessed using RT-qPCR for 24 h. The ratios were normalized to the RNA levels of the viral control (arbitrarily set to 1). (E) Analysis of RABV N protein expression using western blotting for 24 h (Left figure). Ratio of the intensity of rabies virus N protein bands to the intensity of β-actin bands (Right figure). (F) Effect of resveratrol on primary transcription of RABV. Virus-infected N2a treated with 150 μg/mL cycloheximide (CHX) and/or 40 μM resveratrol for 24 h. RNA was extracted, and the mRNA of the RABV genome and N protein quantified by qRT-PCR. The figure shows that after normalizing the transcripts from RABV 24 h infected alone, the mRNA transcribed from cells infected by CHX treatment was set to 1. All results shown in the figure represent at least three experiments. Statistical significance was calculated with a non-parametric test and is defined as $^{**}p < 0.01$.

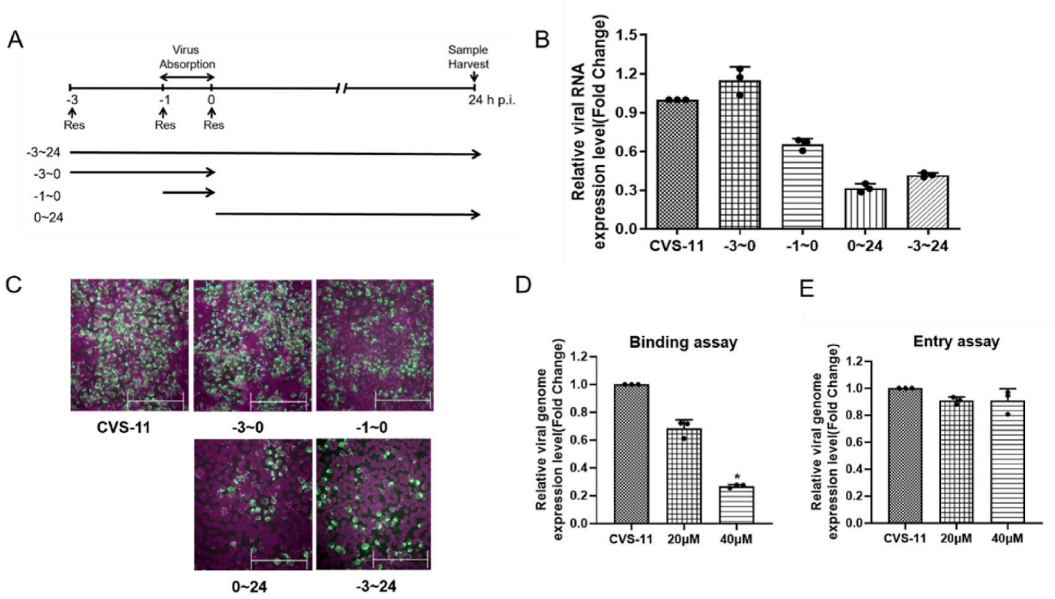

Fig. 2. Effect of resveratrol on CVS-11 infection of N2a cells with different infection cycles (A) Illustration of the addition time approach. (B) FFU investigation of the impact of 40 μM resveratrol on viral titers throughout pre-infection (between −3 and 0 h p.i.(post incubate)), co-infection (between −1 and 0 h p.i.), post-infection (0–24 h p.i.), and complete infection phases (−3 to 24 h p.i.). (C) The effect of CVS-11 with resveratrol (40 μM) at different stages of infection was analyzed using DFA. Scale bar, 50 μm. (D) Binding assay. The effect of resveratrol (20 μM and 40 μM) on the number of RABV genomes in the CVS-11 binding phase was evaluated by RT-qPCR. (E) The effect of resveratrol (20 μM and 40 μM) on the number of RABV genomes in the CVS-11 entry phase was evaluated by RT-qPCR. Data in the bar graphs are the mean ± standard deviation (SD) of at least three independent experiments. $^{*}p < 0.05$.

3.6. Resveratrol inhibits RABV replication by activating the SIRT1/Nrf2/HO-1 antioxidant pathway

To further determine how resveratrol mitigates oxidative stress caused by RABV, we evaluated SIRT1/Nrf2 antioxidant-signaling-pathway-related proteins in RABV-infected N2a cells treated with resveratrol using western blotting analysis. Our findings indicate that CVS-11 infection caused an increase in SIRT1, Nrf2, HO-1, and p-AMPK expression; however, the addition of resveratrol further increased the expression of these proteins, while only the expression of RABV N protein decreased. (Fig. 6A and B). We investigated the association between resveratrol-induced inhibition of RABV replication and activation of the intracellular antioxidant pathways. Accordingly, we used three SIRT1-targeted siRNAs to knock down SIRT1 in N2a cells. The results indicated that SIRT1-knockdown results in decreased expression of Nrf2 and HO-1, and increased expression of RABV N protein (Fig. 6C and D). We assessed the activity of several significant antioxidant enzymes within the antioxidant pathways, specifically SOD, CAT, and GSH-Px, which were observed to be lower in virus-infected cells when compared with that in uninfected cells. When resveratrol was administered to virus-infected cells, there was an increase in intracellular antioxidant enzymes (SOD, CAT, and GSH-Px) activities corresponding to the concentration of resveratrol used. At 40 μM, resveratrol significantly increased the activities of these antioxidant enzymes, although the increase was moderate. The results indicate that resveratrol inhibits the replication of RABV by activating the SIRT1/Nrf2/HO-1 signaling pathway, boosting antioxidant enzyme activity, and reducing RABV-induced oxidative stress.

4. Discussion

Rabies is a highly fatal infectious disease, and it exhibits no obvious symptoms in the early stages. During disease progression, the

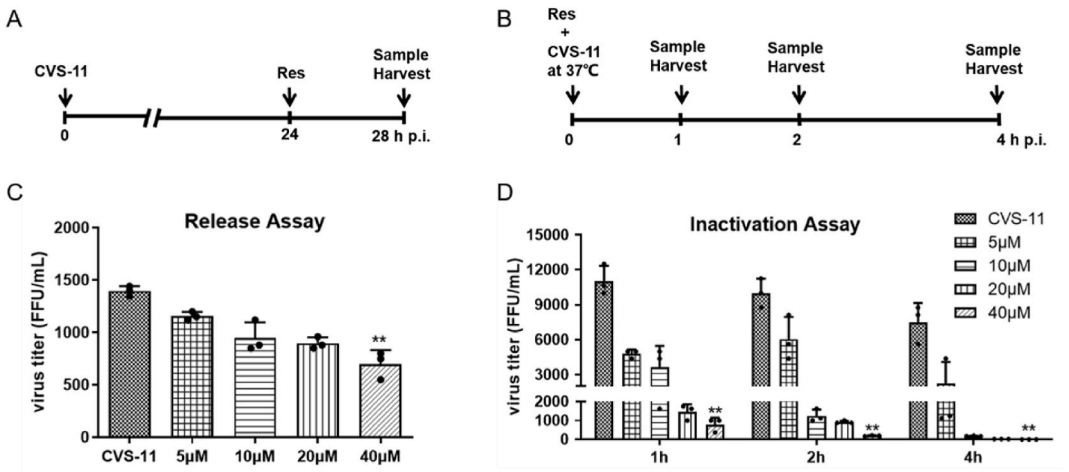

Fig. 3. Effect of resveratrol on CVS-11 release and inactivation. (A) Experimental design for release assay. (B) Experimental design for inactivation assay. (C) Effect of resveratrol on the titer of the CVS-11 release phase detected by FFU assay. (D) FFU assay for the effect of resveratrol on the viral titer after direct inactivation of CVS-11 virus particles. Statistical significance was calculated with a non-parametric test, $^{**}p < 0.01$ compared with the CVS-11 control.

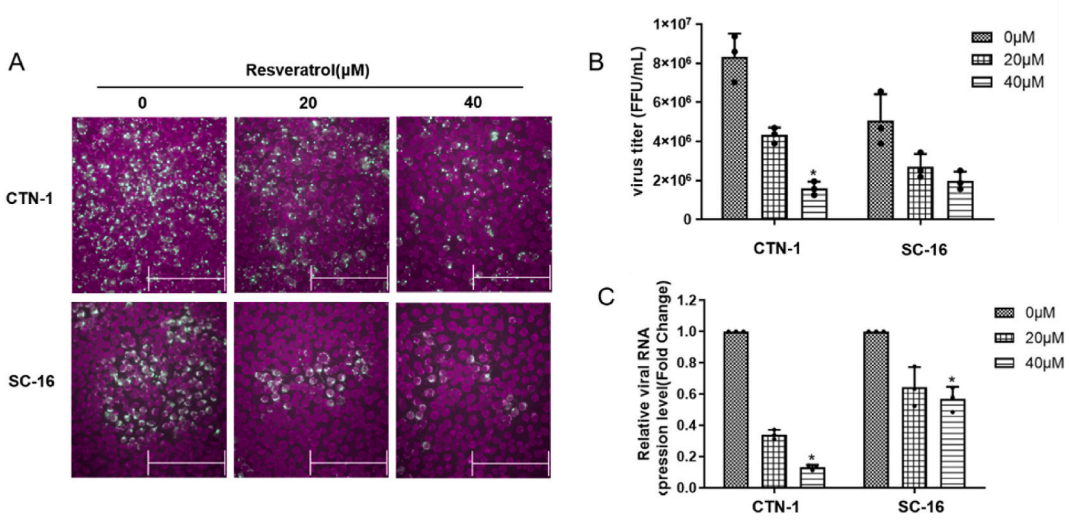

Fig. 4. Effect of resveratrol on the replication of SC-16 and CTN-1 strains. (A) DFA assay examined the replication effect of resveratrol on both strains. Scale bar, 50 μm. (B) FFU assay detected the effect of resveratrol on the viral titer of two strains. (C) RT-qPCR assay detected the effect of resveratrol on the *N* gene mRNA levels of both strains. Statistical significance was calculated with a non-parametric test, $^{*}p < 0.05$ compared with the uninfected virus.

virus enters the immune-privileged central nervous system [46]. Analysis of the molecular pathogenesis revealed no apparent cytopathic effects after RABV infection of cells. While RABV utilizes the host to complete its infection cycle and spread, the host cells themselves were not significantly damaged by the virus [47]. This suggests that there is a delicate balance between RABV and the host that selectively drives different levels of mRNA and protein expression in the cell, mainly involving key metabolic pathways, signaling, immune and inflammatory responses [44]. However, the lack of information in the pathogenic mechanisms underlying rabies infection makes the treatment of rabies extremely difficult. Therefore, identifying effective anti-rabies virus drugs is an important step in treating rabies until an effective rabies treatment can be established [48–50]. Previous studies have reported that RABV infection causes mitochondrial dysfunction, leading to oxidative stress and degeneration of neuronal processes [40]. However, the relationship between oxidative stress and RABV replication remains poorly understood. Therefore, we conducted an *in vitro* investigation of the antiviral activity of resveratrol against RABV and its molecular mechanism. Our results provide insight into the pathogenesis of rabies and provide a basis for further therapeutic research.

In the time-of-addition assay, it was observed that resveratrol promoted viral replication after pre-infecting the cells, and this promotion may also be responsible for the more pronounced inhibitory effect of resveratrol on the replicative phase (0–24 h p.i.)

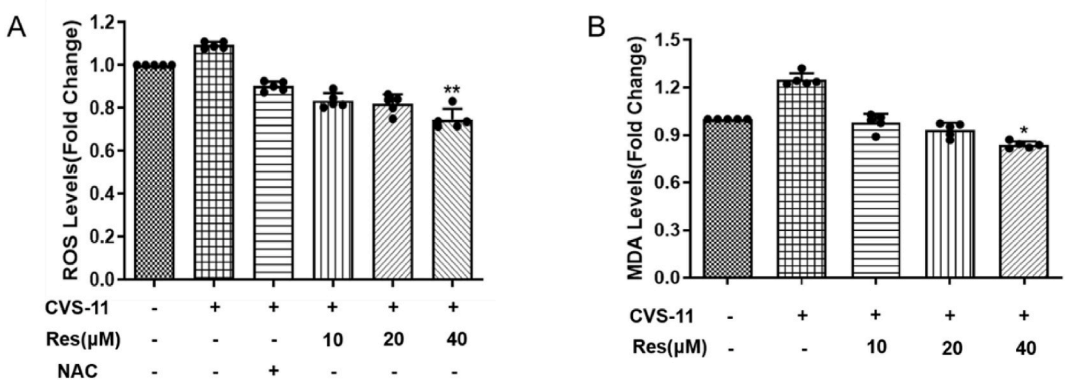

Fig. 5. Effect of resveratrol on ROS and MDA levels in CVS-11-infected N2a cells. (A) Detection of ROS levels in N2a cells after resveratrol treatment of cells infected with CVS-11. The vertical coordinate ROS level represents fluorescence intensity. (B) Detection of MDA levels intracellularly in N2a cells after resveratrol treatment of cells infected with CVS-11. The vertical coordinate MDA levels are measured in μmol/mg. The value of control was set to 1. Statistical significance was calculated with a non-parametric test, $*p < 0.05$, $**p < 0.01$ compared with control.

compared with the −3 to 24 h p.i., which may be related to the fact that resveratrol enhances cellular metabolism and protects cells [51]. However, whether resveratrol can be added earlier in the process of isolating RABV to promote virus amplification needs to be confirmed in future studies. Co-incubation experiments have shown that resveratrol inhibits the adsorption phase of RABV, and subsequently, during the early infection phase targeting RABV; moreover, RABV binding to cells can be interfered by increasing the concentration of resveratrol. In addition, earlier studies have found that resveratrol interferes with the binding of human cytomegalovirus to cells, thereby preventing early infection of host cells by RABV [52]. Moreover, in viral entry phase trials, resveratrol showed no inhibitory effect on viruses already bound to cells, similar to the inhibition of Zika virus replication by resveratrol [53].

Resveratrol inhibits the release of RABV in a dose-dependent manner, suggesting that the continuous addition of resveratrol in the late stage of viral infection can limit the spread of RABV. In the inactivation test, the inactivation effect of RABV was positively correlated with the concentration of resveratrol; therefore, whether resveratrol could be used as a RABV inactivator needs to be further verified. Moreover, a higher inactivation effect may be correlated to the reduction of the RABV titer with an extended incubation period of resveratrol and RABV particles. The effect of resveratrol on the replication of RABV street and vaccine strains differed from the inhibitory effect on deoxynivalenol toxin, where resveratrol inhibited the vaccine strain more than the street strain. Against the virus itself, the RABV vaccine strain was weaker than the street strain and, therefore, was more likely to be affected by apoptosis and inducing an immune response. However, resveratrol, as a cellular rescuer, could inhibit infection by the RABV vaccine strain through its anti-apoptotic and anti-inflammatory properties [54]. Therefore, we hypothesized that the inhibitory effect of resveratrol on the CTN-1 vaccine strain might be achieved by inhibiting virus-induced apoptosis by resveratrol. Notably, resveratrol inhibits porcine hemagglutinating encephalomyelitis virus (PHEV) replication *in vitro* and *in vivo*, and also attenuates neuronal damage from PHEV infection [17]. Thus, resveratrol is a potential anti-RABV drug with a multilevel efficacy that warrants further investigation.

Fig. 6a shows that RABV infection increases SIRT1/Nrf2/HO-1 expression and further experiments showed that silencing the SIRT1 protein resulted in the suppression of the antioxidant pathway SIRT1/Nrf2/HO-1. Since SIRT1 is a nuclear protein with several roles in metabolic control and mitochondrial biogenesis, which has a positive effect on cell survival, we hypothesized that RABV may regulate mitochondrial function through the SIRT1/Nrf2 pathway, thereby affecting viral replication [55,56]. Resveratrol treatment of RABV-infected cells enhanced the activation of the antioxidant signaling pathway SIRT1/Nrf2 by increasing SIRT1 expression. This could potentially be attributed to the additional effects of resveratrol on the function of SIRT1/Nrf2, thereby inhibiting viral replication. Previous studies have demonstrated increased oxidative stress during RABV infection. However, this study demonstrates that treatment with resveratrol diminishes oxidative stress, as evidenced by lower ROS and MDA levels in cells [24,40]. Resveratrol attenuates the level of oxidative stress, which has been achieved by reducing ROS [57]. Resveratrol is a ROS scavenger and the scavenging effect of is mainly achieved by regulating gene expression in cells, including increasing the expression of various antioxidant enzymes [58,59]. The primary mechanism behind these effects of resveratrol is to enhance the antioxidant signaling pathway by activating SIRT1 or Nrf2 to target the expression of antioxidant enzymes, thus reducing the level of oxidative stress [31,60,61]. Additionally, the RABV P protein possesses a structural domain that can interact with complex I within mitochondria, resulting in mitochondrial dysfunction, heightened ROS production, and oxidative stress. However, further experimental evidence is needed to determine whether resveratrol affects ROS production through RABV P protein [62]. Our results indicate that resveratrol treatment activated three antioxidant enzymes, including SOD, CAT, and GSH-Px (Fig. 6E). Furthermore, Nrf2 target genes encode various antioxidant enzymes, demonstrating that resveratrol can influence antioxidant enzyme activity through SIRT/Nrf2 [32,63]. In addition, we found that resveratrol inhibited RABV infection in BHK-21 cells by activating the SIRT1/Nrf2/HO-1 antioxidant signaling pathway (results not shown). Therefore, resveratrol may be a potential anti-RABV drug.

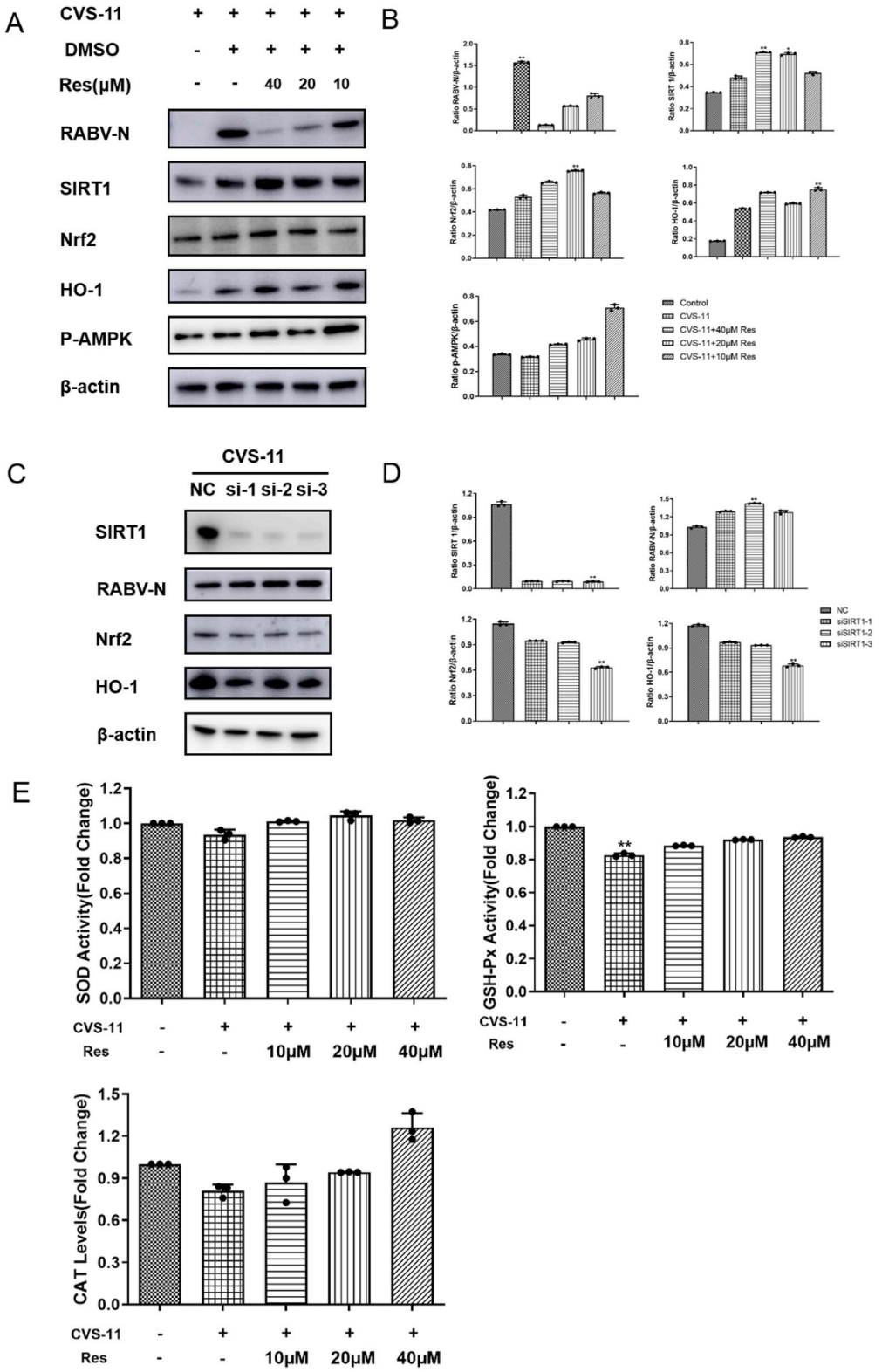

Fig. 6. Effects of resveratrol on the expression and activity of antioxidant-pathway-related proteins in RABV-induced N2a cells. (A) Cells were pretreated with RABV strain CVS-11 for 1 h, followed by exposure to varying concentrations of resveratrol for 24 h. Western blotting analyzed the expression levels of RABV N protein, SIRT1, Nrf2, HO-1, p-AMPK, and β-actin. (B) Ratios of the target protein band intensities to that of β-actin. (C) After transfection with siRNA to knock down SIRT1, protein samples were collected, and western blotting analyzed the expression of RABV N

protein, SIRT1, Nrf2, HO-1, and β-actin in the RABV-infected cells. (D) Ratios of the target protein band intensities to that of β-actin. (E) N2a cells were pretreated with CVS-11 for 1 h, followed by exposure to varying concentrations of resveratrol for 24 h. SOD (OD value), CAT (units/mL), and GSH-Px (mU/mg) levels were measured with a spectrophotometer. Statistical significance was calculated with a non-parametric test, $*p < 0.05$, $**p < 0.01$.

5. Conclusion

In summary, resveratrol can inhibit multiple stages of the RABV life cycle, including adsorption, replication, and release, and can directly inactivate RABV particles. Moreover, resveratrol reduced the level of RABV-induced oxidative stress and inhibited RABV replication by activating the SIRT1/Nrf2/HO-1 antioxidant signaling pathway. These findings further elucidates RABV infection mechanisms and establish a theoretical foundation for designing efficient anti-RABV medications.

Data availability statement

The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Ethics statement

Ethics are not involved in this study.

CRediT authorship contribution statement

Qian Liu: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Qing He:** Investigation, Data curation. **Xiaoyan Tao:** Conceptualization. **Pengcheng Yu:** Data curation. **Shuqing Liu:** Data curation. **Yuan Xie:** Data curation. **Wuyang Zhu:** Writing – review & editing, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36494.

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