

Autophagy Inhibitors Treatment Alleviates Degree of Infection and Cerebral Inflammatory Responses in Mouse Model of Japanese Encephalitis

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Abstract

Background: Japanese Encephalitis (JE) is a zoonotic natural epidemic disease caused by Japanese Encephalitis Virus (JEV) infection. Currently, there is no specific medicine for Japanese encephalitis. At present, autophagy regulating drugs have played an important role in the treatment of tumors, heart diseases and other diseases. We hope that by studying the effects of autophagy-regulating drugs on JEV infection and host response in mice, will provide effective clinical trials for autophagy-regulating drugs in the treatment of Japanese encephalitis and other viral infectious diseases.

Methods: After establishing appropriate animal model. We observed the neurological symptoms of the mice and counted their survival rate. We compared the degree of viral infection in the brain of mice infected with JE virus. We compared the extent of neuroinflammatory responses in the brain of mice and explored the signaling processes involved in neuroinflammation.

Results: We found autophagy inhibitors wortmannin (Wort) and chloroquine (CQ) alleviate degree of viral infection in the brain of JEV-infected mice. Autophagy inhibitors reduced the neuroinflammation in Mouse Model of Japanese encephalitis. We speculated that autophagy inhibitors may attenuate the activation of the PI3K/AKT/NF- κ B pathway, thereby reducing the brain inflammation in mice, thereby protecting mice from JEV-induced death. This result is not significant enough, the specific mechanism still needs further study.

Conclusions: Our study suggests that autophagy inhibitors wortmannin and chloroquine could reduce the degree of viral infection and inflammatory response in the brain of JEV infected mice, providing a clinical basis for the treatment of Japanese encephalitis.

Background

Autophagy is a highly conserved homeostatic process through which cytoplasmic macromolecules, excess or damaged organelles, and some pathogens are delivered to lysosomes for degradation [1–2]. Autophagy usually occurs at a basal level in all cells, but is upregulated in response to extracellular or intracellular stress and pathogen infection [3]. Autophagy activates antigen-specific T cells for removal of pathogens or their proteins by degrading or enhancing type I interferons or by processing major histocompatibility complex (MHC) antigens and presenting them to T cells [4]. A growing body of data indicates that microbes can eliminate or use autophagy processes to enhance their replication or transmission [5–10].

Autophagy is triggered by UNC-51-like kinase 1/2 complex (ULK1/2 complex, the mammalian orthologs of autophagy-related 1, Atg1), and the ULK1/2 complex is regulated by rapamycin complex 1 (MTORC1) [11–12]. Rapamycin was investigated initially for its antifungal properties [13]. Since the first description of its immunosuppressive activity in 1977 [14], much has been learned about the complex mechanisms of action of this macrolide and its site of action, the mammalian target of rapamycin (mTOR) [15]. Rapamycin (Rapa) is a commonly used autophagy inducer that can promote the occurrence of

autophagy by inhibiting the MTOR pathway [16–17]. Wortmannin, a hydrophobic fungal metabolite of the fungus *Talaromyces wortmanni*, has been widely used as a powerful tool to examine the role of PI3K in cellular signaling [18]. Wortmannin as an early autophagy inhibitor, can prevent the formation of the autophagosome, primarily by inhibiting the activity of class III Phosphatidylinositol 3 kinases [19–20]. Chloroquine (CQ), an aminoquinoline used in malaria treatment [21], also played a promising role in the management of the Zika virus and SARS-CoV outbreaks [22]. Chloroquine (CQ) can inhibit autophagosome and lysosome fusion and/or prevent degradation of autophagic lysosomal material downstream (in the late stage) of autophagosome formation [23–24].

Japanese encephalitis (JE) is a mosquito-borne zoonotic infection caused by Japanese encephalitis virus (JEV). Currently, JEV is mainly prevalent in the Asia-Pacific region, with about 68,000 cases of JEV infection each year and a mortality of 25–30%, and 50% of the survivors are affected with neuropsychiatric sequelae [25–27]. In the future, JEV is likely to become an emerging global pathogen [28]. JEV is a neurotropic virus whose clinical manifestations range from hyperthermia syndrome to multifocal central nervous system disease to death [29]. The pathological changes of brain tissue caused by JEV infection are characterized by varying degrees of haemorrhage, hyperaemia, perivascular cuff, lymphocytic infiltration, neuronal degeneration, necrosis, glial cell proliferation, and glial nodule formation [30]. JE is an inflammatory disease of the central nervous system. JEV infection can cause excessive activation of microglia in the brain and, in turn, release of a large number of pro-inflammatory cytokines such as IL-6, TNF- α , and RANTES, which promote the migration and penetration of numerous white blood cells into the brain, resulting in an inflammatory storm that causes severe damage to the brain tissues [31–32].

It is reported that the autophagy pathway or other pathways under the action of autophagy-regulating drugs is related to the process of virus replication, translation and even entry into host cells [33]. This study employed the autophagy inducer Rapamycin, early autophagy inhibitor wortmannin and late autophagy inhibitor chloroquine to investigate the effect of autophagy-regulating drugs on JEV infection in the mouse brain.

Methods

JEV strain

JEV (P3 strain) was donated by Professor Cao Shengbo, the State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University. The JEV P3 strain was amplified in the brains of neonatal mice and virulence was determined by a plaque test in BHK-21 cells [34].

Establishment of the JE mouse model and treatment

Six-week-old female BALB/c mice were used in this study and the Regulations on the Administration of Laboratory Animals in Hubei Province were strictly followed. Number of the using of Laboratory Animal was **HZAUMO-2018-059** approved by The Scientific Ethic Committee of Huazhong Agricultural University.

The 305 mice were divided into 8 groups, 272 in the infection groups including JEV (10^5 PFU, 0.1 mL) infection group, JEV (10^5 PFU, 0.1 mL) + rapamycin (Rapa, 5 mg/kg (0.5mg in 1mL DMEM per mouse), 0.2 mL) group, JEV (10^5 PFU, 0.1 mL) + wortmannin (Wort, 1 mg/kg (0.2mg in 1mL DMEM per mouse), 0.2 mL) group, JEV (10^5 PFU, 0.1 mL) + chloroquine (CQ, 50 mg/kg(5mg in 1mL DMEM per mouse), 0.2 mL) group. The remaining mice are divided into the control groups including DMEM (0.1 mL) control group, Rapa (5 mg/kg, 0.2 mL) group, Wort (1 mg/kg, 0.2 mL) group, CQ (50 mg/kg, 0.2 mL) group. In particular, Rapa, Wort, and CQ were administered 2 h prior to JEV challenge, and then administered daily for 10 consecutive days. The mice were continuously fed for 20 d, with daily observation and recording of their clinical symptoms. Mouse symptoms were scored based on a reported clinical symptom scale for JEV-infected mice [34]. All experiments were conducted using the protocol recommended by the Research Ethics Committee of the College of Veterinary Medicine, Huazhong Agricultural University, Hubei Province, China.

Collection of mouse brain samples

The mice were sacrificed at 5d, 7d, 10 d, 15d and 20 d after JEV infection, with 5 mice each group at 5d, 7d, 10d, 15d after JEV infection. The remaining mice all were sacrificed at 20d after JEV infection. The mice were sacrificed by cervical dislocation prior to collection of brain tissue samples. The left brain was frozen. Portions of the right cerebral cortex were taken (about the size of a sesame seed) and fixed in 2.5% glutaraldehyde, with the remaining portions fixed in 4% formaldehyde.

Transmission electron microscopy

After the small pieces of tissue were completely fixed in 2.5% glutaraldehyde, the tissues were embedded using a pure embedding medium (anhydrous acetone mixed with an embedding agent in a volume ratio of 1:1). After the tissue boundaries were trimmed, the tissues were sliced into ultra-thin sections (80-100 nm), which were stained sequentially with 4% uranyl acetate and lead citrate. The samples were observed and photographed under a transmission electron microscope (TECNA110, Philips, Netherlands).

Paraffin sectioning

After the brain tissues were fixed with 4% formaldehyde for 48 h, they were dehydrated using an ethanol gradient, embedded in paraffin with the cut surface down, and serially sectioned at 5 μ m, with the sections subjected to different staining methods.

Haematoxylin-eosin (HE) staining

The standard haematoxylin-eosin (HE) staining method was adopted to stain selected tissues: the nuclei were stained by haematoxylin and the cytosol and extracellular matrix (ECM) were stained by eosin, followed by mounting with neutral gum.

Immunohistochemical staining

Paraffin sections were dewaxed and placed in 3% H₂O₂ for 30 min to quench endogenous peroxidase. The sections were incubated in 96 °C citrate buffer for 30 minutes to complete antigen retrieval. After washing, the sections were blocked in 5% BSA for 1 h, and then incubated with a mouse anti-JEV primary antibody (1:100, provided by the State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University) overnight at 4 °C. After washing, a secondary antibody (HRP-labelled goat anti-mouse/rabbit IgG, Gene Tech Co., Ltd., Shanghai, China) was added dropwise, and then the sections were incubated for 45 min. Finally, colour development was performed with DAB, and haematoxylin was counterstained. All immunohistochemical stained sections were scanned using the Leica Aperio CS2 section scanning system.

RNAScope staining

RNAScope staining was performed according to the instructions of the RNAScope staining kit as follows. The sections were dewaxed and completely dried, after which RNAScope hydrogen peroxide was added dropwise. After incubation at room temperature, the slides were placed in a boiling RNAScope target retrieval reagent for antigen retrieval. RNAScope® Protease Plus was added dropwise, and the mixture was incubated at 40 °C in a hybridization oven, followed by the addition of an appropriate probe for continual incubation at 40 °C. Next, the mixture was washed in turn by Amp1-Amp6, coloured with a RED working solution, counterstained with haematoxylin, and mounted in glycerogelatin. All immunohistochemical stained sections were scanned using a Leica Aperio CS2 scanner.

Tissue immunofluorescence

Paraffin sections were dewaxed to water and placed in 3% H₂O₂ for 30 min to quench endogenous peroxidase. Antigen retrieval was performed by incubating the sections in citrate buffer at 96 °C for 30 min. After washing, the sections were blocked in 5% BSA for 1 h and then incubated with primary antibodies (mouse anti-JEV-E antibody, 1:100, State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, rabbit anti-LC3A/B antibody, 1:100, Seville Biotech Co., Ltd.) overnight at 4 °C. After washing, secondary antibodies (FITC Goat Anti-Mouse IgG, Cy3 Goat Anti-Rabbit IgG) were added dropwise and were incubated for 2 h, followed by washing, DAPI staining, and mounting with an anti-fluorescence quencher. Fluorescence signals were detected using a fluorescence confocal microscope.

Quantitative Real-time PCR (Q-PCR)

The total RNA of brain tissue was extracted with Trizol by following the manufacturer's instructions and then reverse transcribed into cDNA using a TAKARA reverse transcription kit. Then, the Q-PCR reaction was carried out using a TAKARA Q-PCR kit. The primer sequences for the Q-PCR reaction are shown in Table 1. The reaction conditions of Q-PCR were as follows: pre-denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s, and melting curve analysis at 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. The relative expression levels of target genes in each sample were calculated using the $2^{-\Delta\Delta C_t}$ analysis method.

Table 1 Q-PCR primer sequences

Genename	Forward primer(5'-3')	Reverse primer(5'-3')
β -actin	cactgccgcacatcctcttctctccc	caatagtgatgacctggccgt
IL-6	agacttccatccagttgcct	tctcctctccggacttgtaa
IL-1 β	atgaaagacggcacacccac	gcttgctctgcttgtag
TNF- α	tggcctccctctcatcagtt	ttgagatccatgccgtggc

Western blotting

Total tissue protein was extracted using RIPA lysate, and protein quantitation was performed using the BCA method, with each sample adjusted to have the same protein content. After a prepared gel of a suitable concentration was fixed in an electrophoresis tank, protein samples and a marker were added to the sample wells using a micropipette for electrophoresis. Each excised protein band (gel slice) was transferred to a PVDF membrane, and blocked with 5% skim milk at 37 °C, followed by incubation with TBST-diluted primary antibodies overnight at 4 °C. The primary antibodies were: PI3 Kinase P85 alpha (ABclonal), Phospho-AKT (BOSTER), Phospho-JNK1/2 (ABclonal), Phospho-ERK1 (ABclonal), NF-kB (ABclonal), and GAPDH (Servicebio). Next, TBST-diluted secondary antibodies were added for incubation at 37 °C, followed by using a colour-developing solution to produce coloured bands, whose grey values were analysed using Image J software. The relative expression levels of PI3K, P-AKT, P-ERK, P-JNK, and P65 proteins in brain tissues were detected using GAPDH as an internal reference. Quantitative statistics of the grey values of related proteins were performed using Image J software.

Statistical analysis

Data are expressed as the mean \pm SD, and inter-group differences were analysed using One-way ANOVA, with $P < 0.05^*$ and $P < 0.01^{**}$ indicating significance and extreme significance, respectively.

Results

Neurological symptoms in mice infected with JE virus

Neurological symptoms in mice infected with JE virus were observed and scored. The duration of symptoms in the JEV+Rapa group was long (at 5-20 d post JEV infection), and there were noticeable phenotypic symptoms of piloerection, arched back and motor dysfunction. The JEV group had a short duration of symptoms at 5-12 d after JEV infection and showed significant neurological symptoms. The JEV+Wort group showed only mild mental depression and piloerection at 7-10 d post JEV infection and then returned to normal. No significant neurological symptoms were observed in the JEV+CQ, DMEM control, and drug control groups (Fig. 1A and Fig. 1B). The morbidity in the JEV+Rapa group was 65.5%, with the lowest survival rate. The JE incidence in the JEV group was 32.7%. The morbidity in the

JEV+Wort group was lower, with a survival rate of 90%. The survival rate in the JEV+CQ group was nearly 100%. No obvious illness in DMEM control group and drug control group (Fig. 1C and Fig. 1D).

Relationship between autophagy and viral infection

The brain tissues of 3 mice in different treatment groups were subjected to transmission electron microscopy to observe the damage of the subcellular structure in the brain, and the co-localization of the autophagy factor LC3 with JEV E protein was observed using tissue immunofluorescence. These results showed that severe mitochondrial damage occurs in the brain tissue of mice in the JEV and JEV+Rapa groups, while the damage in the brain tissue of mice in the JEV+Wort and JEV+CQ groups was mild. (Fig. 2). Meanwhile, Co-localization of LC3 and E protein occurred in more neurons in brain tissue of JEV and JEV+Rapa groups. However, a small number of neurons in brain tissue of JEV+Wort and JEV+CQ groups exhibited co-localization (Fig. 3A, 3B).

Distribution of JEV in the brain tissues of JEV-infected mice

Brain tissues were collected at day 10 post infection. Using RNAScope staining technique to observe the distribution of JEV nucleic acid positive signal and immunohistochemical staining (IHC) to detect the distribution of JEV antigen positive signal. RNAScope staining revealed that the viral nucleic acids in the brain tissues of the JEV group were mainly distributed in the cerebral cortex and thalamus. For the JEV+Rapa group, JEV nucleic acids were mainly distributed in the cerebral cortex, olfactory tubercle, thalamus, mesencephalon, pons, and medulla oblongata. There was no obvious JEV nucleic acids positive signal was seen in the brain tissues of JEV+Wort and JEV+CQ groups. Immunohistochemical staining revealed that at 10 d after JEV infection, the JEV antigen in the brain tissues of the JEV group, as well as the JEV+Wort and JEV+CQ groups, was mainly distributed in the cerebral cortex, while for the JEV+Rapa group the cerebral distribution of JEV antigen was mainly concentrated in the cerebral cortex, thalamus, hypothalamus, mesencephalon, and pons (Fig. 4A). To further confirm that autophagy inhibitors treatment can alleviate JEV infection in JEV-infected mice, Q-PCR was performed to detect the JEV load in mice brain tissues at 10 d and 20 d after JEV infection, revealing that the JEV and JEV + Rapa groups had a significantly higher JEV load than the JEV+Wort and JEV+CQ groups on day 10 post infection, the JEV+Rapa group had a significantly higher JEV load than the JEV, JEV+Wort, and JEV+CQ groups on day 20 post infection (Fig. 4B)

Inflammatory responses

To ensure the effectiveness of autophagy inhibitors in JEV-induced encephalitis, mice brain tissues were collected at 10 d and 20 d post JEV infection, fixed in formaldehyde, embedded in paraffin, and sectioned for HE staining, followed by observation of histological changes of the brain tissues under a microscope. The brain tissues of the JEV and JEV+Rapa groups showed obvious vascular inflammatory responses and microglia proliferation. There were mild vascular inflammatory responses in the brain tissues of the JEV+Wort and JEV+CQ groups, but obvious microglia proliferation was observed. Pathological changes in the brain tissues of the control and single administration groups were not evident (Fig. 5).

Pro-inflammatory cytokines

To further confirm that autophagy inhibitors can alleviate inflammatory responses in mice brain tissues, Q-PCR was performed to detect the expression levels of pro-inflammatory cytokines IL-6, IL-1 β , and TNF- α in the mice brain tissues of different treatment groups. Mice brain tissues were collected at 10 d and 20 d post JEV infection, frozen, and subjected to RNA extraction, followed by Q-PCR analysis. The results showed that the secretion of pro-inflammatory cytokines in the brain tissues of the JEV and JEV+Rapa groups was significantly higher than that in the JEV+Wort and JEV+CQ groups at 10d post JEV infection. (Fig. 6).

Inflammatory response signalling

Studies have shown that the activation of p13k/AKT pathway is related to the inflammatory response caused by JE virus infection [36]. Moreover, wortmannin is an inhibitor of PI3k, so we study the effect of autophagy inhibitors on PI3k/AKT pathway. Mice brain tissues were collected at 10 d after JEV infection and subjected to protein extraction, followed by western blot analysis to evaluate the regulation of downstream signals by the PI3K/AKT pathways and the effects of the pathway on the nuclear translocation of NF- κ B. The levels of PI3K, P-AKT, P-JNK, and P65 proteins in the JEV and JEV+Rapa group mice had an upregulation trend, but not significant (Fig. 7).

Discussion

Japanese encephalitis virus, a neurotropic virus, causes severe inflammatory reactions in the central nervous system [37]. Previous in vitro experiments have shown the effect of autophagy inducers and inhibitors on JE virus-infected cells [3, 38]. Due to the complex mechanism of the body's response, it is important to further study the effect of autophagy-regulating drugs on the infection degree of JEV and the host response in mice infected with JEV. In this study, a JEV mouse model was established and the mice were intraperitoneally injected with an autophagy inducer and inhibitors, aimed at identifying the effect of autophagy-regulating drugs on JEV infection and JE symptoms in the model mice as well as the effect mechanisms.

When infected with JEV, mice develop notable neurological symptoms such as ataxia and dyskinesia [35]. After the mice began to show symptoms, we observed the symptoms of the mice every day and scored the symptoms of the mice based on the score. Take the last symptoms of the mice as the final criterion. The results of this study showed that the JEV-infected mice treated with autophagy inducers showed clinical manifestations of varying severity at 5–20 d after JEV infection, including piloerection, arched back, eye congestion and hind limb paralysis. JEV-infected mice without any other treatment developed clinical symptoms at 5–10 d after JEV infection, but recovered to normal in the late stage of infection. Some of the JEV-infected mice treated with the autophagy inhibitors Wort and CQ developed early mild symptoms. Wort-treated JEV-infected mice had a survival rate of nearly 90%, and the CQ-treated JEV-infected mice had a survival rate of nearly 100%. Cellular invasion of a pathogen depends on its ability to bind to the corresponding cellular receptor [39]. It has been reported that autophagy can be

activated as an innate immune mechanism to control infection after intracellular pathogen invasion [39–42]. This study preliminarily implicated Rapa as a positive regulator of JEV infection. However, autophagy inhibitors Wort and CQ have certain protective effects on JEV infected mice.

TEM technology plays an important role in revealing the morphology of all organelle structures with nanometer-scale resolution [43]. It is worth comparing that we found that the brain tissue of mice in JEV + Wort group and JEV + CQ group had less mitochondrial damage. Combined with the analysis of clinical symptoms in animal experiments in mice, we initially determined that the autophagy inhibitors Wort and CQ can attenuate JEV infection, which has a certain protective effect on cytoplasmic structure of brain tissue of mice.

Among autophagy-related ATG proteins, microtubule-associated proteins (LC3I, LC3II), a homolog of mammalian ATG8, was identified as a marker of autophagosomes [44]. The study found that compared with the mice in the JEV + Wort group and JEV + CQ group, the co-localization of LC3 and JEV-E appeared in more neurons in the brain tissue of mice in JEV and JEV + Rapa groups. In combination with previous experiments, we analyzed that the autophagy inhibitors Wort and CQ can attenuate the interaction between autophagy and Japanese encephalitis virus infection and have a certain protective effect on mice infected JEV. As to whether the early stage or the late stage of autophagy has a stronger influence on viral infection, due to the complexity of the body's response and the complex mechanism of autophagy, better research methods and larger clinical trials are needed to research.

JEV is mainly distributed in the cerebral cortex, basal ganglia, thalamus, mesencephalon, pons, and medulla oblongata [45]. The results of this study showed that JEV was distributed in the cerebral cortex, thalamus or even the whole brain of the mice in JEV + Rapa group, while JEV was mainly distributed in the cerebral cortex and thalamus in the brain tissues of mice in JEV group. And JEV was mainly distributed in the cerebral in the brain tissues of mice in JEV + Wort group and JEV + CQ group. In addition, the results of RNAScope staining method and IHC staining method for detecting positive virus signals in this study are different, which may be different from the section interval and the detection level of the two methods. In addition, we detected that the viral load in the brain tissue of mice in the JEV + Wort group and JEV + CQ group was significantly lower than that in the JEV group and JEV + Rapa group at 10 days after virus infection, which was consistent with the above test results. The viral load in the brain tissue of mice in the JEV + Wort group and JEV + CQ group was still relatively low at 20 days after virus infection. This result further confirmed that the autophagy inhibitors Wort and CQ can attenuate the degree of virus infection in brain tissue of mice infected with JEV.

As a neurotropic virus, JEV has a marked pathogenic effect on the brain tissues of the central nervous system [46]. JEV infection mainly affects brain tissue, showing pathological changes in brain tissue to varying degrees, mainly manifested as degeneration and necrosis of neurons, glial cell proliferation and vascular cuff characteristics. The histopathological results of this study showed that the brain tissue of mice in the JEV group and the JEV + Rapa group had obvious vascular inflammation and late glial cell proliferation, while the mice in the JEV + Wort group and the JEV + CQ group had slight vascular

inflammatory response and obvious glial cell proliferation. Other groups of mice showed normal. After JE virus infection, the number of inflammatory monocytes, dendritic cells, macrophages and granulocytes increases in the spleen and other lymphoid tissue in the mice [48]. During JEV infection, a number of immune cells migrates to the central nervous system. In mice, the major cells behind the brain infiltration are believed to be monocytes and macrophages. When the mice infected with JEV, the brain infiltrating monocytes cells stains positive for IL-6 and TNF- α . The number of reactive astrocytes and activated microglia are reported to increase in mice post infection. Astrocytes which belong to rodents produce cytokines like IL-6, IL-1 and IL-18 and they release only CCL5 chemokine. JEV infection leads to excessive microglia activation and the subsequent release of numerous pro-inflammatory cytokines, resulting in an inflammatory storm [31–32]. In order to further confirm that autophagy inhibitors can alleviate the vascular inflammatory responses of brain tissues in JEV-infected mice, we detected that the expression levels of IL-1, IL-6 and TNF in the brain tissues of mice in the JEV + Wort and JEV + CQ groups were significantly down-regulated compared to the JEV and JEV + Rapa groups. Therefore, this study found that autophagy inhibitors can attenuate the degree of inflammatory response in brain tissues of mice infected with JEV, which may be related to autophagy inhibitors attenuated JEV virus infection in mice infected with JEV. In addition, it was also possible to reduce the release of inflammatory factors by affecting the PI3K/AKT pathway.

The PI3K/AKT pathway plays an important role in regulating various inflammatory responses [47]. Activation of the PI3K/AKT pathway leads to proliferation of B cells and activation of nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B), triggering proinflammatory responses [48]. The endoplasmic reticulum is an organelle for viral replication and maturation, and a growing body of studies has shown that endoplasmic reticulum stress induces autophagy [49]. During viral production, infected cells synthesize large quantities of viral proteins and unfolded or misfolded proteins, which results in endoplasmic reticulum stress [49–50]. The aggregation of viral proteins in the endoplasmic reticulum is known as the unfolded protein response (UPR) [50]. In addition to IRE1–XBP1 activation, JEV also induces activation of the RIDD cleavage pathway [51]. Studies suggest that many UPR-related transcription factors manage Atg expression [52]. Early studies have shown that hepatitis C virus and hepatitis B virus promote autophagosome formation by inducing ER stress, and the UPR signalling pathway is involved in activating autophagy pathways [53]. Therefore, we wondered whether the effect of autophagy regulating drugs on Japanese encephalitis virus infection has a certain relationship with UPR. The specific mechanism still needs to be studied. In this study, the results showed that PI3K/AKT/NF- κ B signaling pathway did not show significant activation, which may be related to the complexity of the body's response.

Based on the results of this experiment, we initially found that the autophagy inhibitors wortmannin and chloroquine slowed the occurrence of neurological symptoms in JEV-infected mice, attenuated the damage of subcellular structure of brain tissues in JEV-infected mice, and reduced the prevalence. In addition, wortmannin and chloroquine reduced the distribution of JEV in the brain tissue of infected mice and weaken the inflammatory response in the brain tissue of infected mice. As well as, the antiviral mechanism of chloroquine drugs on enveloped RNA viruses such as SARS-CoV. We analyzed that the

antiviral effect of chloroquine drugs on JEV may be related to the drug's interference with the terminal glycation of cell receptors to weaken the ability of the virus to bind to the receptor. Due to the complexity of the body's response and the uncertainty of the pathogenic mechanism of Japanese encephalitis virus. The protective mechanism of autophagy inhibitors wortmannin and chloroquine on mice infected with JE virus still needs large-scale experiments for further study.

Conclusions

In summary, this study initially showed that autophagy inhibitors wortmannin and chloroquine attenuate the inflammatory response in the brain of mice infected with JE virus and have a certain protective effect on mice infected with JE virus.

Abbreviations

CNS: Central Nervous System, CQ: Chloroquine, HCQ: Hydroxychloroquine, JEV: Japanese Encephalitis Virus, JE: Japanese Encephalitis, LC3: Microtubule-associated proteins, MHC: Major histocompatibility complex, MTORC1: Rapamycin complex 1, NF- κ B : Nuclear factor- κ B , PI3K III: class III Phosphatidylinositol 3-kinase, Rapa: Rapamycin, ULK1/2 complex: UNC-51-like kinase 1/2 complex, UPR: Unfolded protein response, Wort: Wortmannin.

Declarations

Ethics approval and consent to participate

All experiments were conducted using the protocol recommended by the Research Ethics Committee of the College of Veterinary Medicine, Huazhong Agricultural University, Hubei Province, China. Number of the using of Laboratory Animal was **HZAUMO-2018-059** approved by The Scientific Ethic Committee of Huazhong Agricultural University.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ZJ designed the research, performed the majority of the study, analyzed the data, generated the figures, and wrote the manuscript. CG, LX, ZW, XC and HX provided advice in the design of the study. CS provided virus strain. GC provided advice on the manuscript. HW, GM and WX conducted parts of the animal surgery and performed the experiments. All authors have read and approved the final version of the manuscript.

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Not applicable.

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Figures

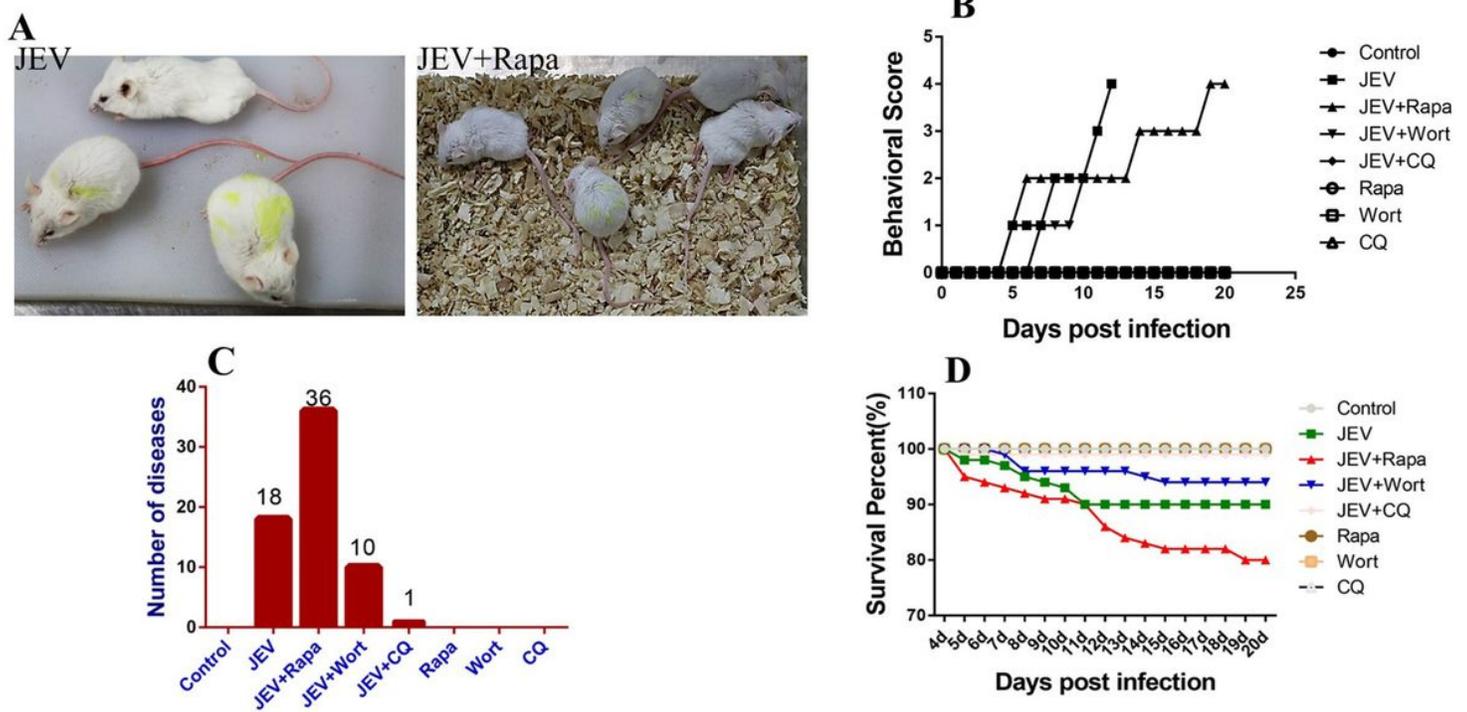


Figure 1

Autophagy inhibitors alleviated neurological symptoms in Japanese encephalitis virus (JEV)-infected mice. A: the JEV and JEV+ Rapa groups developed neurological symptoms at 5-12 d after JEV infection, B: Behavioural scores showed that the neurological symptoms disappeared in the JEV group at 12 d after JEV infection, The mice of JEV+Rapa group had obvious neurological symptoms 5-20 days after virus infection, the JEV+ Wort group developed the symptom of piloerection. The other groups did not show evident neurological symptoms. NOTE: Behaviour Score, 0 = asymptomatic, 1 = mental depression and piloerection, 2 = mental depression, piloerection, and arched back, 3 = mental depression, piloerection, arched back, and motor disorder, 4 = mental depression, piloerection, arched back, motor disorder, and eyelid swelling. C: Statistical analysis of JEV prevalence in mice (The ratio of the number of symptomatic mice to the total number). D: Statistical analysis of survival rate of mice infected with virus after 20 days (The ratio of the number of surviving mice to the total number).

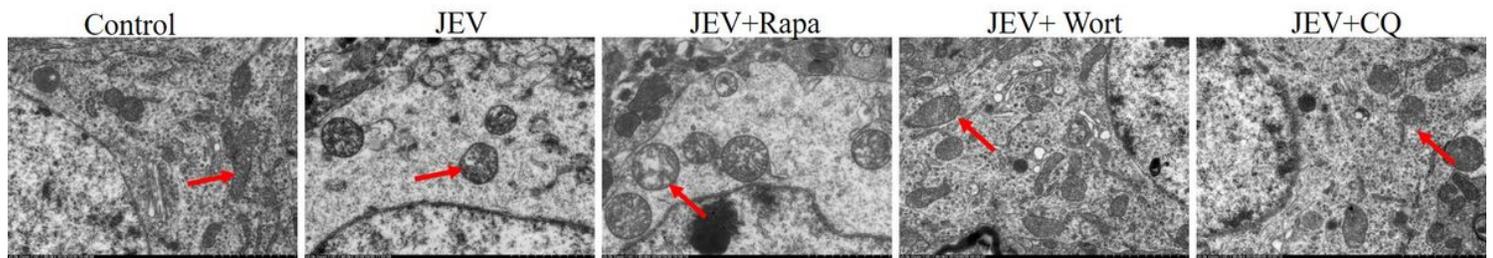


Figure 2

Autophagy inhibitors had a protective effect on the subcellular structure of brain tissue. Transmission electron microscopy (TEM) was used to observe the pathological changes of subcellular structure in mice

brain tissue. Severe mitochondrial damage to brain tissue of mice in JEV+Rapa and JEV groups, and slight damage to mitochondria of brain tissue in mice of JEV+Wort and JEV+CQ groups (Red arrow: mitochondria, Scale bar: 2µm).

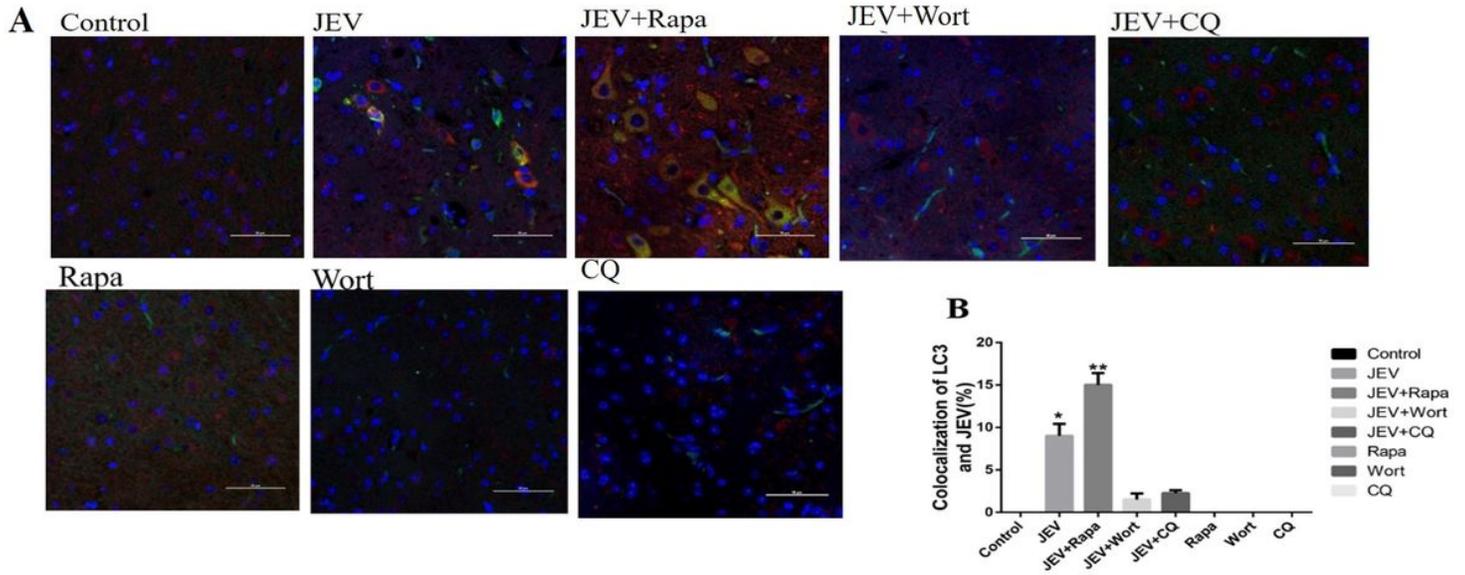


Figure 3

Autophagy inhibitors reduced the colocalization of the autophagy factor LC3 and Japanese encephalitis virus (JEV) E protein in brain tissues of mice infected with JEV. A: Confocal immunofluorescence images of LC3 protein (red) and JEV E protein (green) with a scale bar of 50 µm. Co-localization of a large number of neurons in the brain of mice in the JEV+Rapa and JEV groups, and co-localization of fewer neurons in the brain of the mice in the JEV+Wort and JEV+CQ groups. B: Average number of cells with co-localized LC3 protein and JEV E protein in multiple fields of view. Each error bar represents the standard deviation (SD) of the number of cells in 3 independent experiments, with each experiment involving 10 fields of view and each field containing about 150 cells.

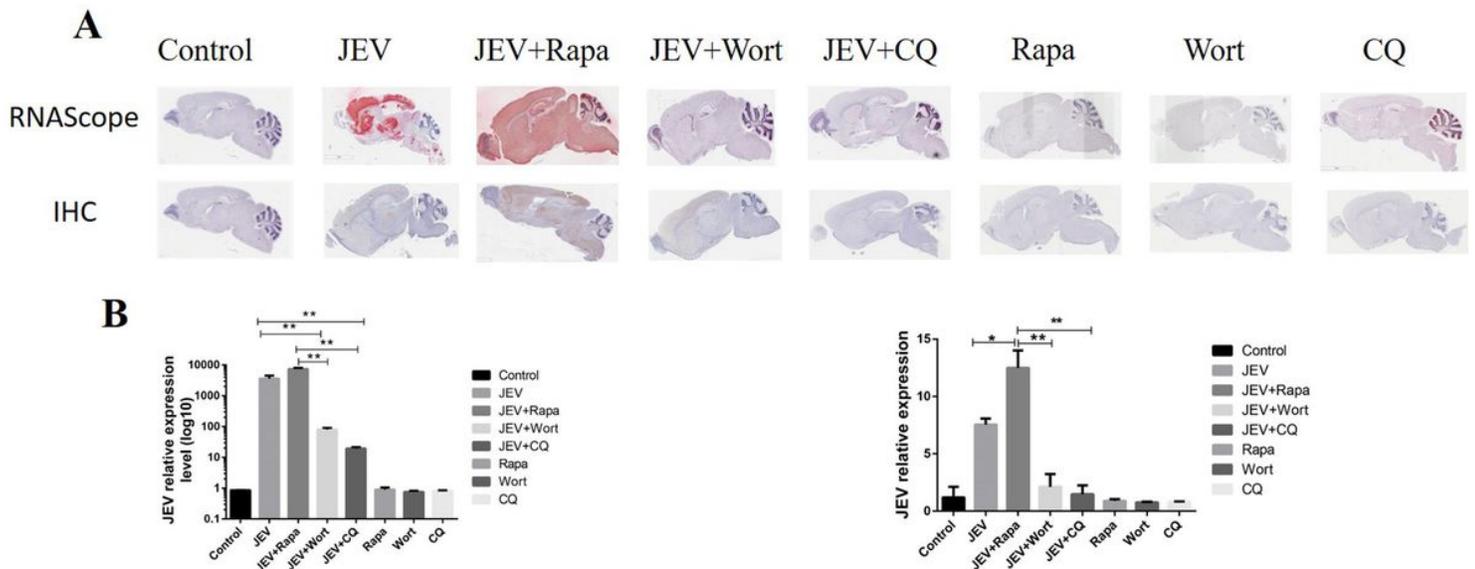


Figure 4

Autophagy inhibitors reduced the distribution of Japanese encephalitis virus (JEV) in brain tissues of mice infected with JEV. A: 10 days after JEV infection, RNAScope staining technique was used to observe the distribution of JEV nucleic acid positive signal (red) and immunohistochemical staining technique (IHC) was used to detect the distribution of JEV antigen positive signal (brown) (400 X). B: The JEV load in mice brain tissues was evaluated using qPCR (On the left was the viral load 10 days after JEV infection, On the right was the viral load 20 days after JEV infection). Total RNA of JEV-infected cells was extracted by Trizol and reverse transcribed into cDNA using a TAKARA PrimeScript™ RT reagent Kit with gDNA Eraser, followed by CT value detection based on the fluorescent dye in the TAKARA TB Green™ Premix Ex Taq™ II kit and appropriate primers. Each error bar represents the standard deviations of 3 independent measurement results for 3 mice in a group. One-way ANOVA test was performed using Graph Pad Prism 6 software, ** $p < 0.01$, * $p < 0.05$, compared with each group.

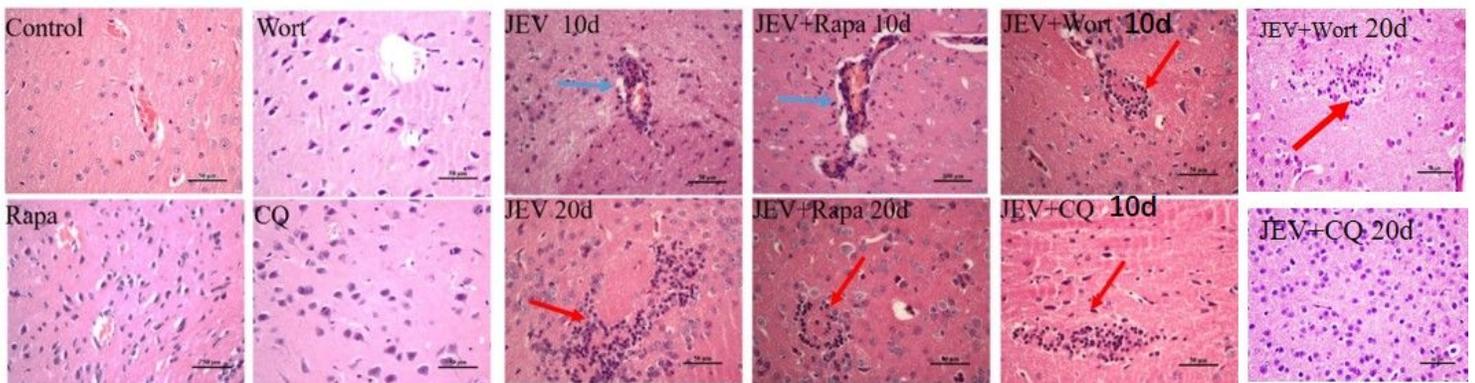


Figure 5

Autophagy inhibitors alleviated histopathological changes in the brain tissues of Japanese encephalitis virus (JEV)-infected mice. Significant perivascular cuffs were observed in the brain tissues of the JEV and JEV+Rapa groups at 10 d after JEV infection (blue arrows). Glial cell proliferation occurred in the brain tissues of the JEV and JEV+Rapa groups at 20 d after JEV infection (red arrows). The JEV+Wort and JEV+CQ groups showed Mild vasculitis and obvious glial cell proliferation after JEV infection. The control and drug control groups showed normal brain tissue morphology (400X).

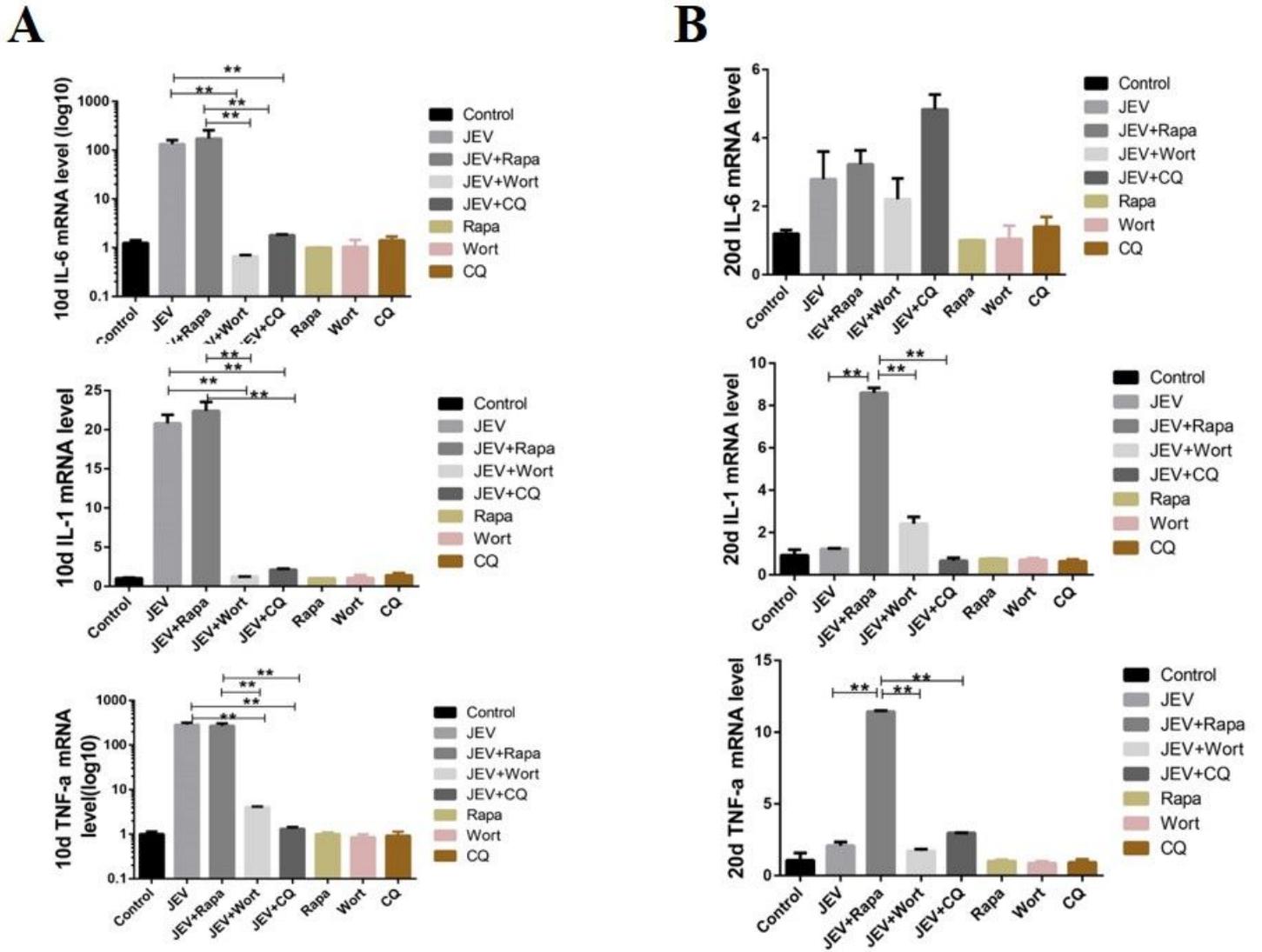


Figure 6

Autophagy inhibitors reduced the secretion of pro-inflammatory cytokines in the brain tissues of mice infected with JEV. Brain tissues were sampled at 10 d and 20 d after JEV infection, and total RNA was extracted. The expression levels of IL-6, IL-1 β , and TNF- α in the brain tissues of mice infected with JEV were detected using Q-PCR. Each error bar represents the standard deviations of 3 independent measurement results for 3 mice in a group. One-way ANOVA test was performed using Graph Pad Prism 6 software, ** $p < 0.01$, * $p < 0.05$, as compared with each group.

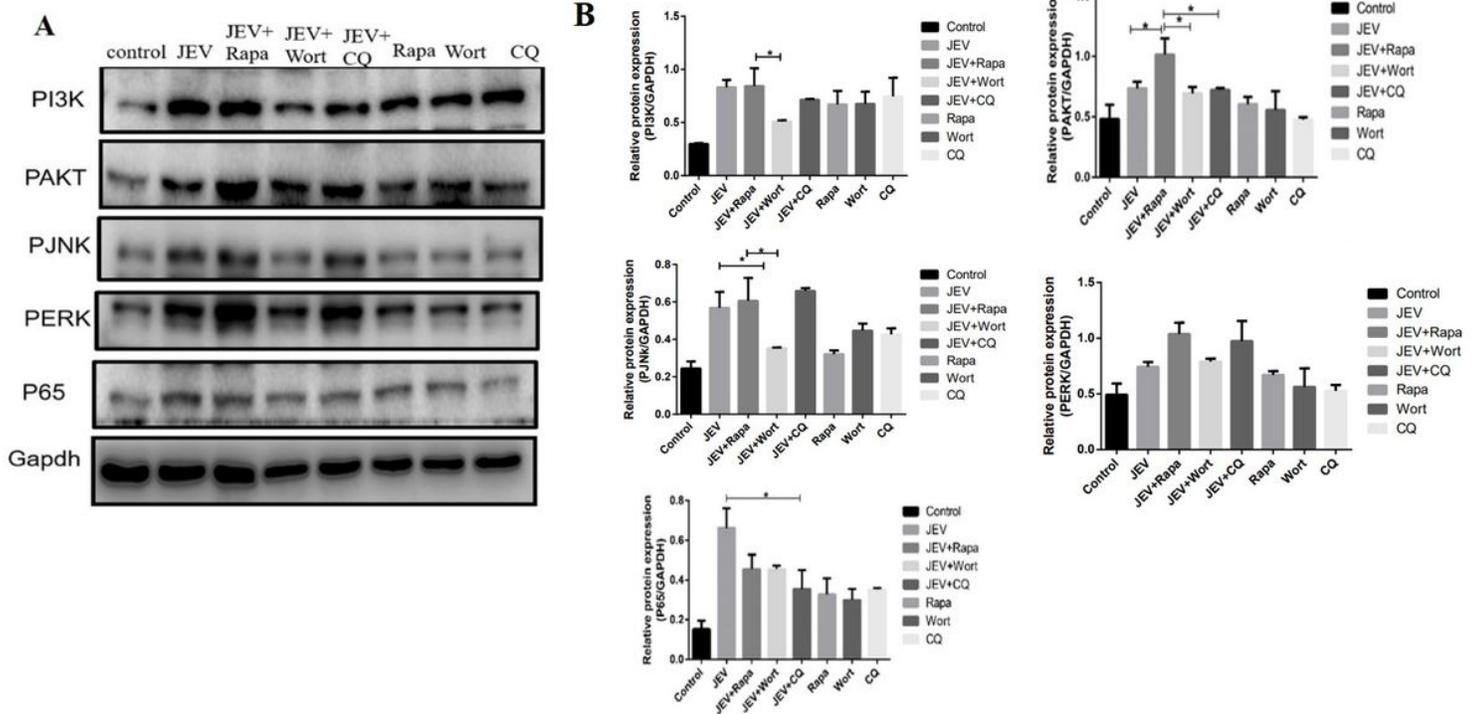


Figure 7

Autophagy inhibitors may reduce the secretion of inflammatory factors by reducing the activation of PI3K/AKT/NF- κ B pathway. Brain tissues were sampled at 10 d after JEV infection, followed by separation of cytoplasmic proteins and nucleoproteins. The expression levels of PI3K, P-AKT, P-JNK, P-ERK, and P65 proteins were quantitatively determined using western blot and scanning densitometry and normalized to GAPDH levels. A: a representative image. B: quantitative data. Each error bar represents the standard deviations of 3 independent measurement results for 3 mice in a group. One-way ANOVA test was performed using Graph Pad Prism 6 software, ** $p < 0.01$, * $p < 0.05$, compared with each group.

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