

Autophagy inhibitor treatment alleviates cerebral inflammatory responses in a mouse model of Japanese encephalitis

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Research

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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. Autophagy is a lysosomal degradation process that plays an important role in viral infection and cellular immunity. In vitro studies have shown that the Japanese encephalitis virus replication mechanism is related to the autophagy pathway. 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 observed histopathological changes in brain tissues of mice. 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) slow down the occurrence of neurological symptoms and reduce the prevalence of JEV-infected mice. As expected, autophagy inhibitors can inhibit the activation of the PI3K/AKT/NF- κ B pathway to alleviate cerebral inflammatory responses in mice, thereby protecting the mice from JEV-induced death.

Conclusions

Our study suggests that autophagy inhibitors wortmannin and chloroquine could attenuate the inflammatory response in the brain of JEV infected mice, providing a clinical basis for the treatment of Japanese encephalitis

Introduction

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 (Rapa) is a commonly used autophagy inducer that can promote the occurrence of autophagy by inhibiting the MTOR pathway [13–14]. Currently, autophagy inhibitors can be divided into early autophagy inhibitors and late autophagy inhibitors [15–16]. Early autophagy inhibitors, including wortmannin (Wort), LY294002, and 3-MA, can prevent the formation of the autophagosome, primarily by inhibiting the activity of class III Phosphatidylinositide 3 kinases [17–18]. Chloroquine (CQ), hydroxychloroquine (HCQ), bafilomycin A1, and lysosomal protease inhibitors inhibit autophagosome and lysosome fusion and/or prevent degradation of autophagic lysosomal material downstream (in the late stage) of autophagosome formation [19–20].

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 [21–23]. In the future, JEV is likely to become an emerging global pathogen [24]. JEV is a neurotropic virus whose clinical manifestations range from hyperthermia syndrome to multifocal central nervous system disease to death [25]. 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 [26]. 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 [27–28].

Due to the limited regeneration ability of nerve cells, autophagy plays a crucial role in protecting the central nervous system (CNS) and neurons from viral infections [3]. Autophagy, as a metabolic mechanism in the body, is not only directly beneficial to the removal of some damaged proteins and organelles but also plays an important role in reducing inflammasomes and NF- κ B activation and alleviating inflammatory responses in tissues [2]. JEV infection mainly causes excessive activation of microglia in mouse brain tissues and the subsequent secretion of a large number of pro-inflammatory cytokines, which causes severe inflammatory responses in brain tissues and ultimately neuronal necrosis [29]. It has been extensively reported that some viruses can escape autophagy or use autophagy to ensure their own replication, but limited information is available regarding the mechanism underlying autophagy in response to JEV invasion. This study employed the autophagy inducer Rapa, early autophagy inhibitor Wort, and late autophagy inhibitor chloroquine to regulate the autophagy pathway and investigate the effect of autophagy 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.

Establishment of the autophagy-regulated JE mouse model. Animal experiments were performed in accordance with the provisions of the Hubei Provincial Regulations on the Management of Laboratory Animals. A total of 305 BALB/c female mice aged 6 weeks were purchased from the Animal Experimental Center of Huazhong Agricultural University. They were divided into 8 groups: DMEM (0.1 mL) control group; JEV (10^5 PFU, 0.1 mL) infection group; JEV (10^5 PFU, 0.1 mL) + rapamycin (Rapa, 5 mg/kg, 0.2 mL) group; JEV (10^5 PFU, 0.1 mL) + wortmannin (Wort, 1 mg/kg, 0.2 mL) group; JEV (10^5 PFU, 0.1 mL) + chloroquine (CQ, 50 mg/kg, 0.2 mL) 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 [31]. 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 10 d or 20 d after drug administration and JEV challenge. 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% H_2O_2 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 $2^{-\Delta\Delta Ct}$ 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-1β | atgaaagacggcacaccac | 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: MAP LC3β (Santa Cruz Biotechnology), P62 (Servicebio), 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 LC3, P62, 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 ± 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

Autophagy inhibitors slowed the onset of neurological symptoms in JEV-challenged mice. Neurological symptoms in our autophagy-regulated JE mouse model were observed and scored. The duration of symptoms in the JEV + Rapa group was long (at 5–20 d after JEV challenge), and there were noticeable phenotypic symptoms of piloerection and arched back with motor dysfunction. The JEV group had a short duration of symptoms at 5–12 d after JEV challenge and showed significant neurological symptoms. The JEV + Wort group showed only mild mental depression and piloerection at 7–10 d after JEV challenge 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 JE incidence in the JEV + Rapa group was 65.5%, with the lowest survival rate. The JE incidence in the JEV group was 32.7%. The JE incidence in the JEV + Wort group was lower, with a survival rate of 90%. The survival rate in the JEV + CQ group was nearly 100%; and the DMEM control group and drug control group had zero incidence and mortality (Fig. 1C and Fig. 1D).

Autophagy inhibitors attenuated the relationship between autophagy and viral infection, and attenuated the damage of the subcellular structure level of the brain tissue of infected JEV mice. The brain tissues of mice in different treatment groups were subjected to transmission electron microscopy to observe the formation of autophagosomes in the brain, and the co-localization of the autophagy factor LC3 with JEV E protein was observed using tissue immunofluorescence. These experiments revealed single-membrane autophagosomes and mitochondrial damage in the brain tissues of mice in the JEV and JEV + Rapa groups, and single-membrane autophagosomes or double-membrane autophagosomes in the brain tissue of mice in the JEV + Wort and JEV + CQ groups without mitochondrial damage (Fig. 2A). Meanwhile, many cerebral neurons of the JEV and JEV + Rapa groups exhibited LC3 co-localization with the JEV E protein in the neuronal cytoplasm; however, no expression of LC3 and JEV E protein was observed in the cerebral neurons of the JEV + Wort group; the cerebral neurons of the JEV + CQ group markedly expressed LC3 but not the JEV E protein (Fig. 2B). There was no neuronal colocalization of LC3 with JEV E protein in the brain tissues of the control group (Fig. 2C).

Autophagy inhibitors reduce the distribution of JEV in the brain tissues of JEV-infected mice. We confirmed JEV distribution in the brain tissues of the JEV-challenged mice in different treatment groups. To this end, the mouse brain tissues were observed by serial sectioning combined with RNAScope staining and immunohistochemical staining. 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 at 10 d after JEV challenge, while the JEV + Wort and JEV + CQ groups had no JEV nucleic acids in the brain tissues. Immunohistochemical staining revealed that at 10 d after JEV challenge, 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. The DMEM and drug control groups did not have JEV antigen in the brain tissues (Fig. 3A). To further confirm that autophagy inhibitor treatment can alleviate JEV infection in JEV-challenged mice, Q-PCR was performed to detect the JEV load in mouse brain tissues at 10 d and 20 d after JEV challenge, revealing that at 10 d, the JEV and JEV + Rapa groups had a significantly higher JEV load than the JEV + Wort and JEV + CQ groups; at 20 d, the JEV + Rapa group had a significantly higher JEV load than the JEV, JEV + Wort, and JEV + CQ groups (Fig. 3B).

Autophagy inhibitors alleviate the inflammatory responses in the brain tissues of JEV-challenged mice. In order to determine mouse brain damage in different treatment groups after JEV challenge, mouse brain tissues were collected at 10 d and 20 d after JEV challenge, 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 vascular inflammatory responses to JEV infection and microglia proliferation in the late stage of infection. There were no noticeable inflammatory responses in the brain tissues of the JEV + Wort and JEV + CQ groups, but microglia proliferation was observed. Pathological changes in the brain tissues of the control and single administration groups were not evident (Fig. 4).

Autophagy inhibitors reduce the release of pro-inflammatory cytokines in the brains of JEV-challenged mice. To further confirm that autophagy inhibitors can alleviate inflammatory responses in mouse 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 mouse brain tissues of different treatment groups. Mouse brain tissues were collected at 10 d and 20 d after JEV challenge, 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 the peak of JEV infection, while the brain tissues of the control and single administration groups did not show marked changes (Fig. 5).

Autophagy inhibitors inhibit inflammatory response signalling in mouse brain tissues. Wort is an inhibitor of PI3K, and CQ can promote the aggregation of P62, an adapter protein for autophagic degradation, thereby weakening the activity of ERK proteins on the endoplasmic reticulum [30]. Mouse brain tissues were collected at 10 d after JEV challenge and subjected to protein extraction, followed by western blot analysis to evaluate the regulation of downstream signals by the PI3K/AKT and PERK pathways and the effects of these pathways 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 were upregulated ($P < 0.05$). However, no significant upregulation was observed for the PERK pathway (Fig. 6).

Discussion

In vitro studies have shown that JEV infection of cells induce autophagy, and autophagy has a positive regulatory effect on JEV replication [31]. It has also been reported that the autophagy protein LC3 has a negative regulatory effect on JEV replication [3]. In this study, a JEV mouse model was established and the mice were intraperitoneally injected with an autophagy inducer and inhibitor, aimed at identifying the effect of autophagy regulation 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 [32]. The results of this study showed that the JEV-challenged mice treated with autophagy inducers showed clinical manifestations of varying severity at 5–20 d after JEV challenge, including eye congestion and hind limb paralysis and circling at the peak of infection, as well as phenotypic symptoms of piloerection and arched back in the late stage of infection. JEV-challenged mice without any other treatment developed clinical symptoms at 5–10 d after JEV challenge, but recovered to normal in the late stage of infection. Some of the JEV-challenged mice treated with the autophagy inhibitor Wort developed early mild symptoms of mental depression, piloerection, and arched back. None of the JEV-challenged mice treated with the autophagy inhibitor CQ showed phenotypic symptoms. The JEV-challenged mice treated with Rapa had the highest disease prevalence, the JEV-challenged mice without any other treatment had a relatively high prevalence, the Wort-treated JEV-challenged mice had a survival rate of nearly 90%, and the CQ-treated JEV-challenged mice had a survival rate of nearly 100%. Cellular invasion of a pathogen depends on its ability to bind to the corresponding cellular receptor [33]. It has been

reported that autophagy can be activated as an innate immune mechanism to control infection after intracellular pathogen invasion [33–36]. This study preliminarily discovery of autophagy inhibitors Wort and CQ have certain protective effects on JEV infected mice.

TEM technology plays an important role in the study of autophagy, it is the only tool that can reveal the morphology of autophagy structures and all organelle structures with nanometer-scale resolution [37]. Special morphological structures during autophagy include early autophagosomes (AVi) and late autophagolysosomes (AVd). When cells undergo various stresses such as endoplasmic reticulum stress, oxidative stress, nutrition, hypoxia, and mitochondrial damage, autophagy levels are increased to cope with various stress conditions [3]. Therefore, autophagolysosomes were observed in the brain tissues of mice in the JEV group, JEV + Rapa group, and JEV + CQ group in this study. Wortmannin (wort) is an early autophagy inhibitor. It mainly prevents the formation of autophagosomes by inhibiting the activity of PtdIns 3kinases class III [20]. In this study, that no apparent autophagy structure was observed in brain tissue in the JEV + Wort group. It is also 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 [38]. This study showed that the neuronal cell membrane of brain tissue of mice in JEV group and JEV + Rapa group had LC3 positive signal distribution and co-localization with JEV-E protein, but the brain tissue of JEV + CQ group mice had only LC3 distribution and no obvious LC3 Co-localization with JEV-E. In addition, there were no significant LC3 and JEV-E positive signal distributions in the brain tissue of mice in the JEV + Wort group. These results indicate that inhibition of early autophagosome formation at the early stage of autophagy formation or fusion of late autophagosomes with lysosomes or degradation of autophagy cargoes in lysosomes are conducive to reducing JEV infection. 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.

JEV is mainly distributed in the cerebral cortex, basal ganglia, thalamus, mesencephalon, pons, and medulla oblongata [39]. The results of this study showed that in Rapa + JEV group mice, JEV was distributed in the cerebral cortex, thalamus or even the whole brain, while JEV in the brain tissues of JEV group mice was mainly distributed in the cerebral cortex and thalamus. JEV in the brain tissues of Wort + JEV group and CQ + JEV group mice was mainly distributed in the cerebral cortex. At 10 d after JEV challenge, the expression of JEV in the brain tissues of JEV-challenged mice treated with autophagy inhibitors was significantly lower than that of JEV-challenged mice without any other treatment and JEV-challenged mice with Rapa treatment, further confirming that autophagy inhibitors can reduce the degree of JEV infection in JEV-challenged mice.

As a neurotropic virus, JEV has a marked pathogenic effect on the brain tissues of the central nervous system [40]. 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, glial cell nodule formation, 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. JEV infection leads to excessive microglia activation and the subsequent release of numerous pro-inflammatory cytokines, resulting in an inflammatory storm [27–28]. In order to attempt to further confirm that autophagy inhibitors can alleviate the vascular inflammatory responses of brain tissues in JEV-challenged mice, we observed that IL-6, IL-1 β , and TNF- α were significantly downregulated in the brain tissues of JEV-challenged mice treated with autophagy inhibitors compared with those of the JEV and JEV + Rapa groups. Therefore, this study found that autophagy inhibitors can attenuate the degree of inflammatory response in brain tissues of infected JEV mice, which may be related to the degree of autophagy inhibitors attenuating the degree of virus infection in brain tissues of mice.

The PI3K/AKT pathway plays an important role in regulating various inflammatory responses [41]. 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 [42]. 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 [43]. During viral production, infected cells synthesize large quantities of viral proteins and unfolded or misfolded proteins, which results in endoplasmic reticulum stress [43–44]. The aggregation of viral proteins in the endoplasmic reticulum is known as the unfolded protein response (UPR) [44]. 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 [45]. In this study, the PI3K/AKT/NF- κ B and PERK/NF- κ B signalling pathways were investigated, and the results showed that autophagy inhibitors inhibited NF- κ B activation, mainly by inhibiting PI3K/AKT pathway activation, which reduced the degree of JEV infection in JEV-challenged mice, alleviating the inflammatory responses of brain tissues in JEV-challenged mice.

Due to the complexity of the body's response and the uncertainty of the pathogenic mechanism of Japanese encephalitis virus. We based on the results of this trial and the analysis of the theoretical knowledge, speculated that JEV may interact with autophagy during infection, or autophagy interacts with cellular immunity caused by viral infection, thereby weakening the degree of viral infection of brain tissue, which is in agreement with the results of the neurological symptoms and the JEV load in the brain tissue of mice in different treatment groups that we detected in this study.

Conclusions

In summary, this study revealed that autophagy inhibitors in mice can alleviate neurological symptoms of JEV-infected mice, mainly by inhibiting activation of the PI3K/AKT pathway to inhibit activation of NF- κ B, in turn reducing inflammatory responses in mouse brain tissues, which increases the survival rate of JEV-infected mice.

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

Availability of data and materials

All data supporting the conclusions of this manuscript are provided in the text and figures.

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.

Consent for publication

Not applicable.

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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References

1. Wang DY, Zhang JB, Jiang WK, Cao ZP, Zhao F, Cai TG, Aschner M, Luo WJ. The role of NLRP3-CASP1 in inflammasome-mediated neuroinflammation and autophagy dysfunction in manganese-induced, hippocampal-dependent impairment of learning and memory ability. *Autophagy*. 2017;13:914.
2. Deretic V, Levine B. Autophagy balances inflammation in innate immunity. *Autophagy*. 2018;14:243–51.
3. Sharma M, Bhattacharyya S, Nain M, Kaur M, Sood V, Gupta V, Khasa R, Abdin MZ, Vрати S, Kalia M. Japanese encephalitis virus replication is negatively regulated by autophagy and occurs on LC3-I- and EDEM1-containing membranes. *Autophagy*. 2014;10:1637–51.
4. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. *Nature*. 2011;469:323–35.
5. Dreux M, Gastaminza P, Wieland SF, Chisari FV. The autophagy machinery is required to initiate hepatitis C virus replication. *P Natl Acad Sci Usa*. 2009;106:14046–51.
6. Lee YR, Lei HY, Liu MT, Wang JR, Chen SH, Jiang-Shieh YF, LinYS, Yeh TM, Liu CC, Liu HS. Autophagic machinery activated by dengue virus enhances virus replication. *Virology*. 2008;374:240–8.
7. Tanida I, Fukasawa M, Ueno T, Kominami E, Wakita T, Hanada K. Knockdown of autophagy-related gene decreases the production of infectious hepatitis C virus particles. *Autophagy*. 2009;5:937–45.
8. Ke PY, Chen SS. Activation of the unfolded protein response and autophagy after hepatitis C virus infection suppresses innate antiviral immunity in vitro. *J C I*. 2011;121:37–56.
9. Yoon SY, Ha YE, Choi JE, Ahn J, Lee H, Kweon HS, Lee JY, Kim DH. Coxsackie virus B4 Uses Autophagy for Replication after Calpain Activation in Rat Primary Neurons. *J Virol*. 2008;82:11976–8.
10. Gannagé M, Dormann D, Albrecht R, Dengjel J, Torossi T, Rämer PC, Lee M, Strowig T, Arrey F, Conenello G, et al. Matrix Protein 2 of Influenza A Virus Blocks Autophagosome Fusion with

- Lysosomes. *Cell Host Microbe*. 2009;6:367–80.
11. Mercer CA, Kaliappan A, Dennis PB. A novel, human Atg13 binding protein, Atg101, interacts with ULK1 and is essential for macroautophagy. *Autophagy*. 2009;5:649–62.
 12. Ganley IG, Lam H, Wang J, Ding X, Chen S, Jiang X. ULK1.ATG13. FIP200 complex mediates mTOR signaling and is essential for autophagy. *J Biol Chem*. 2009;284:12297–305.
 13. Dunlop EA, Tee AR. mTOR and autophagy: A dynamic relationship governed by nutrients and energy. *Semin CellDev Biol*. 2014;36:121–9.
 14. Hwang JY, Gertner M, Pontarelli F, Court-Vazquez B, Bennett MV, Ofengeim D, Zukin RS. Global ischemia induces lysosomal-mediated degradation of mTOR and activation of autophagy in hippocampal neurons destined to die. *Cell Death Differ*. 2017;24:317–29.
 15. Yang YP, Hu LF, Zheng HF, Mao CJ, Hu WD, Xiong KP, Wang F, Liu CF. Application and interpretation of current autophagy inhibitors and activators. *Acta Pharmacol Sin*. 2013;34:625–35.
 16. Yang ZJ, Chee CE, Huang S, Sinicrope FA. The role of autophagy in cancer: therapeutic implications. *Mol Cancer Therap*. 2011;10:1533–41.
 17. Agarwal S, Mirzoeva S, Readhead B, Dudley JT, Budunov I. PI3K inhibitors protect against glucocorticoid-induced skin atrophy. *EBio Medicine*. 2019;41:526–37.
 18. Wu YT, Tan HL, Shui G, Bauvy C, Huang Q, Wenk MR, Ong CN, Codogno P, Shen HM. Dual role of 3methyladenine in modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase. *J Biol Chem*. 2010;285:10850–61.
 19. Rubinsztein DC, Gestwicki JE, Murphy LO, Klionsky DJ. Potential therapeutic applications of autophagy. *Nat Rev Drug Disc*. 2007;6:304–12.
 20. Mauthe M, Orhon I, Rocchi C, Zhou X, Luhr M, Hijlkema K, Coppes RP, Engedal N, Mari M, Reggiori F. Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. *Autophagy*. 2018;14:1435–55.
 21. Lord JS, Gurley ES, Pulliam JRC. Rethinking Japanese Encephalitis Virus Transmission: A Framework for Implicating Host and Vector Species. *Plos Neglect Trop D*. 2015;9:e0004074.
 22. Xu QQ, Zhu NW, Chen SL, Zhao P, Ren H, Zhu SY, Tang H, Zhu YZ, Qi ZT. E3 Ubiquitin Ligase Nedd4 Promotes Japanese Encephalitis Virus Replication by Suppressing Autophagy in Human Neuroblastoma Cells. *Sci Rep*. 2017;7:45375.
 23. Park SL, Huang YS, Lyons AC, Ayers VB, Hettenbach SM, McVey DS, Burton KR, Higgs S, Vanlandingham DL. North American domestic pigs are susceptible to experimental infection with Japanese encephalitis virus. *Sci Rep*. 2018;8:7951.
 24. Weaver SC, Reisen WK. Present and future arboviral threats. *Antiviral Res*. 2010;85:328–45.
 25. Sips GJ, Wilschut J, Smit JM. Neuroinvasive flavivirus infections. *Rev Med Virol*. 2012;22:69–87.
 26. German AC, Myint KSA, Mai NTH, Pomeroy I, Phu NH, Tzartos J, Winter P, Collett J, Farrar J, Barrett A, et al. A preliminary neuropathological study of Japanese encephalitis in humans and a mouse model. *T Roy Soc Trop Med H*. 2006;100:1135–45.

27. Potokar M, Jorgačevski J, Zorec R. Astrocytes in Flavivirus Infections. *Int J Mol Sci.* 2019;20:691.
28. Liu ZY, Yao XQ, Jiang WS, Li W, Zhu SY, Liao CR, Zou L, Ding RT, Chen JT. Advanced oxidation protein products induce microglia-mediated neuroinflammation via MAPKs-NF- κ B signaling pathway and pyroptosis after secondary spinal cord injury. *Journal of Neuroinflammation.* 2020;17:90.
29. Chen CJ, OU YC, Chang CY, Pan HC, Liao SL, Chen SY, Raung SL, Lai CY. Glutamate released by Japanese encephalitis virus-infected microglia involves TNF- α signaling and contributes to neuronal death. *Glia.* 2012;60:487–501.
30. Lee SJ, Duran A, Diaz-Meco MT, Pfluger PT, Pagès G, Pouyssegur J, Nogueiras R, Kim JY, Tschöp MH, Moscat J. A functional role for the p62-ERK1 axis in the control of energy homeostasis and adipogenesis. *EMBO Rep.* 2010;11:226–32.
31. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol.* 2011;12:21–35.
32. Ye J, Jiang R, Cui M, Zhu BB, Sun LQ, Wang YY, Zohaib A, Dong Q, Ruan XD, Song YF, et al. Etanercept Reduces Neuroinflammation and Lethality in Mouse Model of Japanese Encephalitis. *J Infect Dis.* 2014;210:875–89.
33. Joubert PE, Meiffren G, Grégoire IP, Pontini G, Richetta C, Flacher M, Azocar O, Vidalain PO, Vidal M, Lotteau V, et al. Autophagy Induction by the Pathogen Receptor CD46. *Cell Host Microbe.* 2009;6:354–66.
34. Hu BL, Zhang YN, Jia L, Wu HS, Fan CF, Sun YT, Ye CJ, Liao M, Zhou JY. Binding of the pathogen receptor HSP90AA1 to avibirnavirus VP2 induces autophagy by inactivating the AKT-MTOR pathway. *Autophagy.* 2015;11:503–15.
35. Chaumorcel M, Souquère S, Pierron G, Codogno P, Esclatine A. Human cytomegalovirus controls a new autophagy-dependent cellular antiviral defense mechanism. *Autophagy.* 2008;4:46–53.
36. Delpout S, Rudd PA, Labonté P, Messling V. Membrane Fusion-Mediated Autophagy Induction Enhances Morbillivirus Cell-to-Cell Spread. *J Virol.* 2012;86:8527–35.
37. Lucocq JM, Hacker C. Cutting a fine figure: On the use of thin sections in electron microscopy to quantify autophagy. *Autophagy.* 2013;9:1443–8.
38. Kuma A, Komatsu M, Mizushima N. Autophagy-monitoring and autophagy-deficient mice. *Autophagy.* 2017;13:1619–28.
39. Myint KS, Raengsakulrach B, Young GD, Gettayacamin M, Ferguson LM, Innis BL, Hoke Ch J, Vaughn DW. Production of lethal infection that resembles fatal human disease by intranasal inoculation of macaques with Japanese encephalitis virus. *Am Journal Trop Med Hyg.* 1999;60:338–42.
40. Lien JC, Wang CY, Lai HC, Lu CY, Lin YF, Gao GY, Chen KC, Huang AC, Huang SH, Lin CW. Structure analysis and antiviral activity of CW-33 analogues against Japanese encephalitis virus. *Sci Rep.* 2018;8:16595.
41. Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, Bilanges B. The emerging mechanisms of isoform-specific PI3K signalling. *Nat Rev Mol Cell Biol.* 2010;15:329–41.

42. Long XH, Wong CC, Tong L, Chu ESH, Szeto CH, Go MYY, Coker OO, Chan AWH, Chan FKL, Sung JJY, Yu J. *Peptostreptococcus anaerobius* promotes colorectal carcinogenesis and modulates tumour immunity. *Nat Microbiol.* 2019;4:2319–30.
43. Wang K, Li SF, Worku T, Hao XJ, Yang LH, Zhang SJ. Rab11a is required for porcine reproductive and respiratory syndrome virus induced autophagy to promote viral replication. *Biochem Biophys Res Commun.* 2017;492:236–42.
44. Wang QR, Xin X, Wang T, Wan JW, Ou YT, Yang ZB, Yu QJ, Zhu LT, Guo YL, Wu YS, et al. Japanese encephalitis virus induces apoptosis and encephalitis by activating the PERK pathway. *J virol.* 2019;93:e00887-19.
45. Lee YR, Kuo SH, Lin CY, Fu PJ, Lin YS, Yeh TM, Liu HS. Dengue virus-induced ER stress is required for autophagy activation, viral replication, and pathogenesis both in vitro and in vivo. *Sci Rep.* 2018;8:489.

Figures

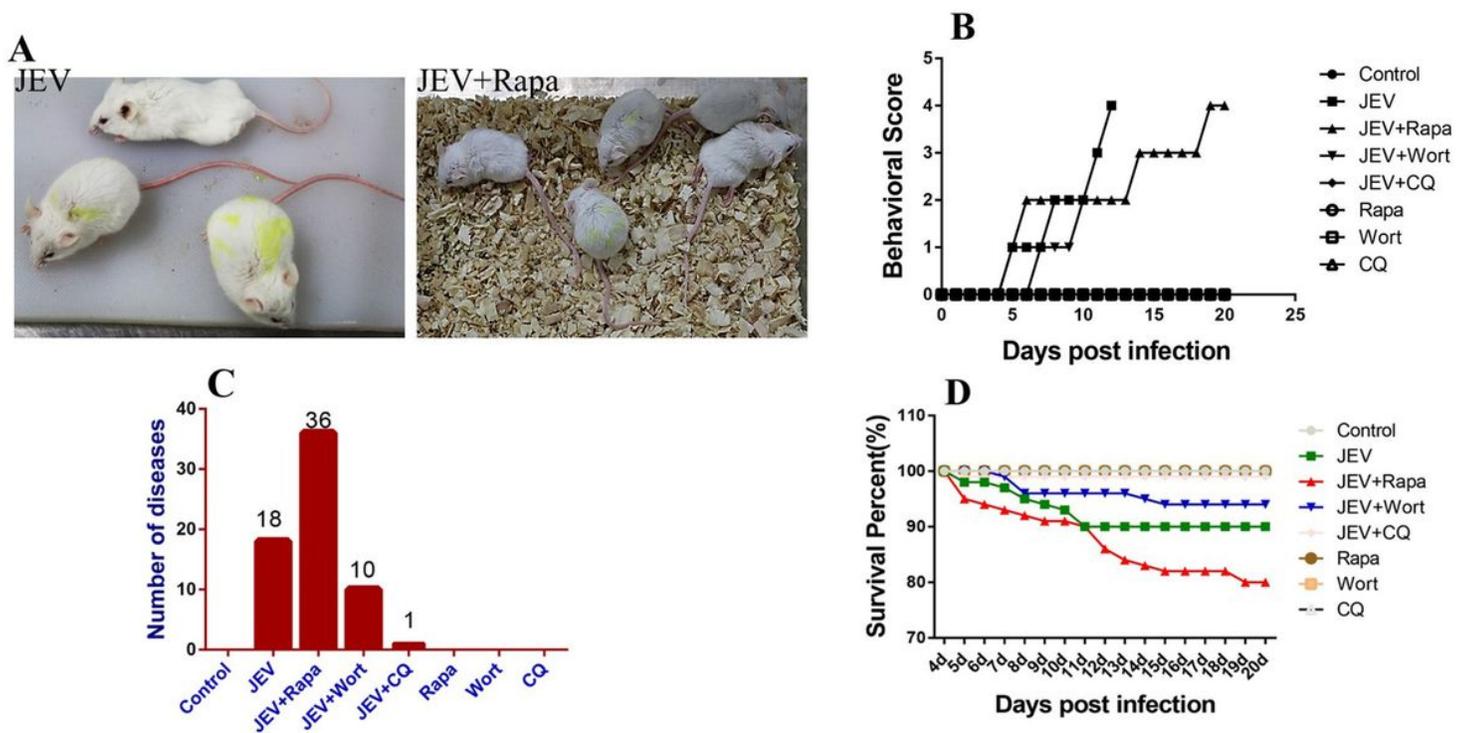


Figure 1

Autophagy inhibitors alleviated neurological symptoms in Japanese encephalitis virus (JEV)-challenged mice. A: the JEV and JEV+ Rapa groups developed neurological symptoms such as mental depression, piloerection, arched back, blinking, and hind limb paralysis at 5-12 d after JEV challenge; B: Behavioural scores showed that the neurological symptoms disappeared in the JEV group at 12 d after JEV challenge; the JEV+Rapa group developed neurological symptoms throughout the feeding process; 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. D: Statistical analysis of survival rate in mice after 20-d feeding.

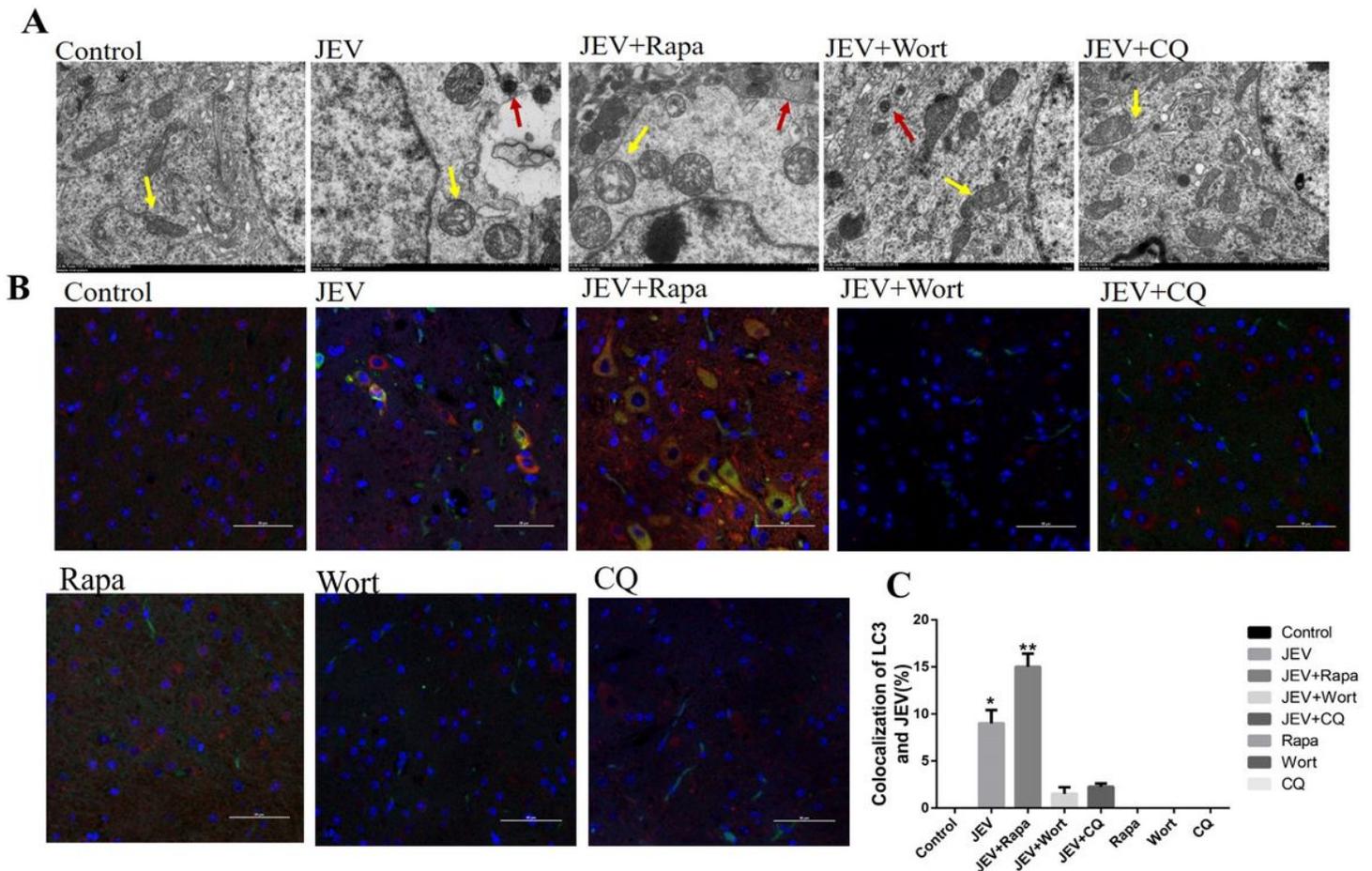


Figure 2

Inhibiting autophagy reduced colocalization of the autophagy factor LC3 and Japanese encephalitis virus (JEV) E protein in the brain neurons of JEV-challenged mice. A: Transmission electron microscopy (TEM) images of single-membrane or double-membrane autophagosome-like vesicles (red arrows) and mitochondrial structures (yellow arrows) in the brain tissues of different treatment groups with a scale bar of 2 μ m. B: Confocal immunofluorescence images of LC3 protein (red) and JEV E protein (green) with a scale bar of 50 μ m. C: Average number of cells with co-localized LC3 protein and JEV E protein in multiple fields of view. D: the average number of cells in which LC3 is present 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.

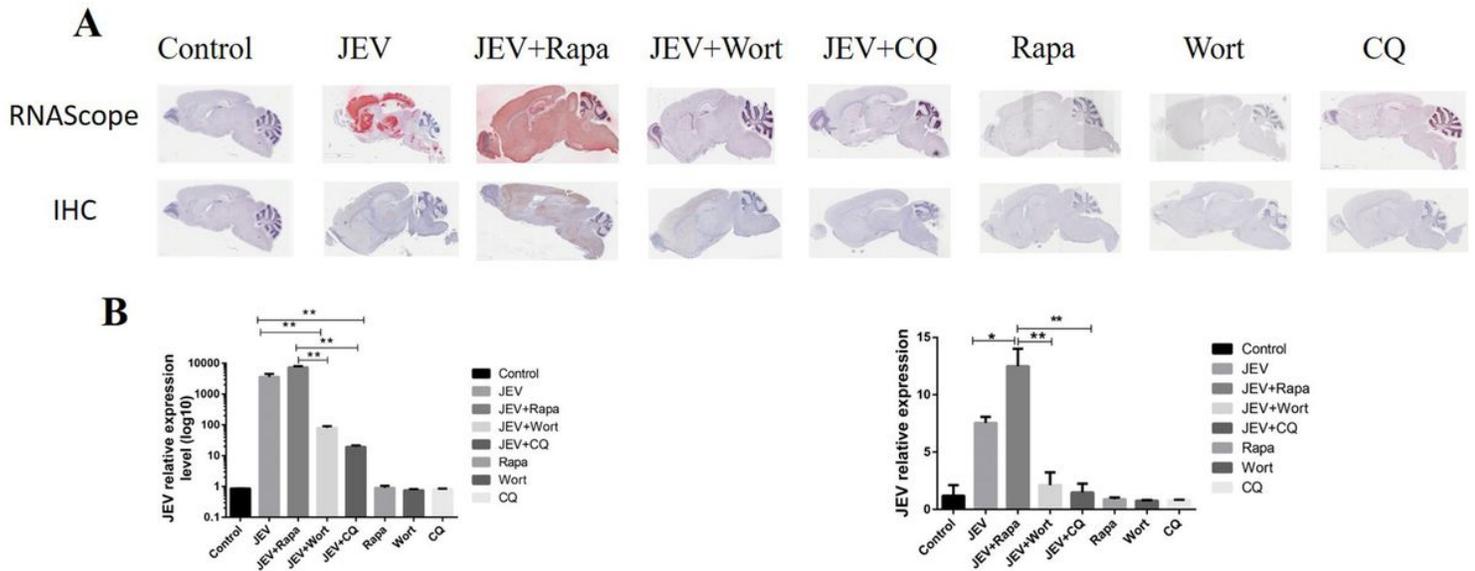


Figure 3

Autophagy inhibitors reduced the distribution of Japanese encephalitis virus (JEV) in mouse brain tissues. A: RNAScope staining and immunohistochemical staining revealed JEV in mouse brain tissues at 10 d after JEV challenge, as shown by the positive signals in red and brown (400 X). B: The JEV load in mouse brain tissues was evaluated using qPCR. 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.

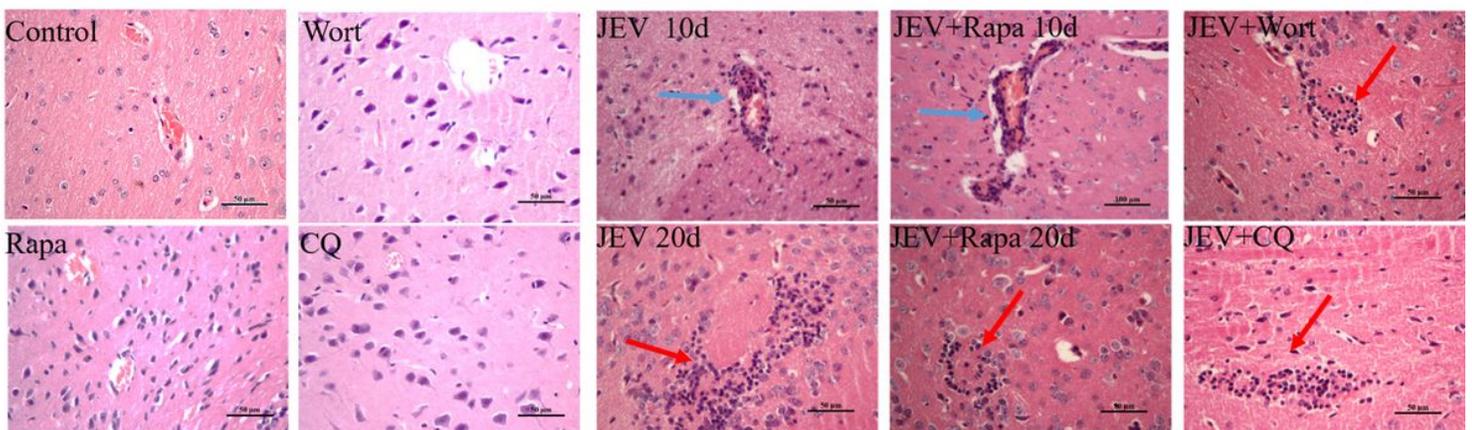


Figure 4

Autophagy inhibitor treatment alleviated histopathological changes in the brain tissues of Japanese encephalitis virus (JEV)-challenged mice. Significant perivascular cuffs were observed in the brain tissues of the JEV and JEV+Rapa groups at 10 d after JEV challenge (blue arrows). Glial cell proliferation

occurred in the brain tissues of the JEV and JEV+Rapa groups at 20 d after JEV challenge (red arrows). The JEV+Wort and JEV+CQ groups showed only glial cell proliferation after JEV challenge. The control and drug control groups showed normal brain tissue morphology (400X).

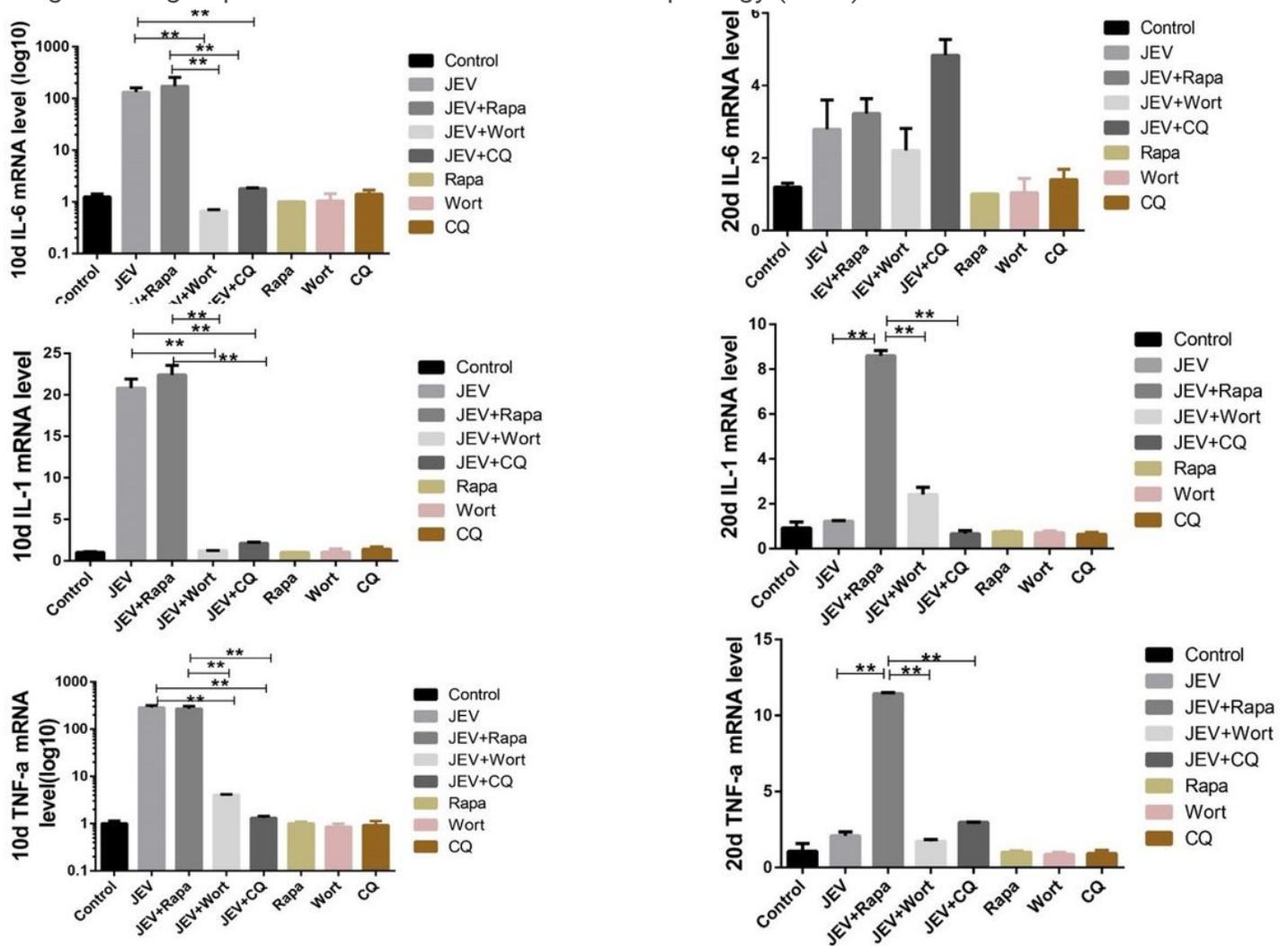


Figure 5

Autophagy inhibitor treatment reduced the secretion of pro-inflammatory cytokines in the brain tissues of Japanese encephalitis virus (JEV)-challenged mice. Brain tissues were sampled at 10 d and 20 d after JEV challenge, and total RNA was extracted. The expression levels of IL-6, IL-1β, and TNF-α in the brain tissues of JEV-challenged mice 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.

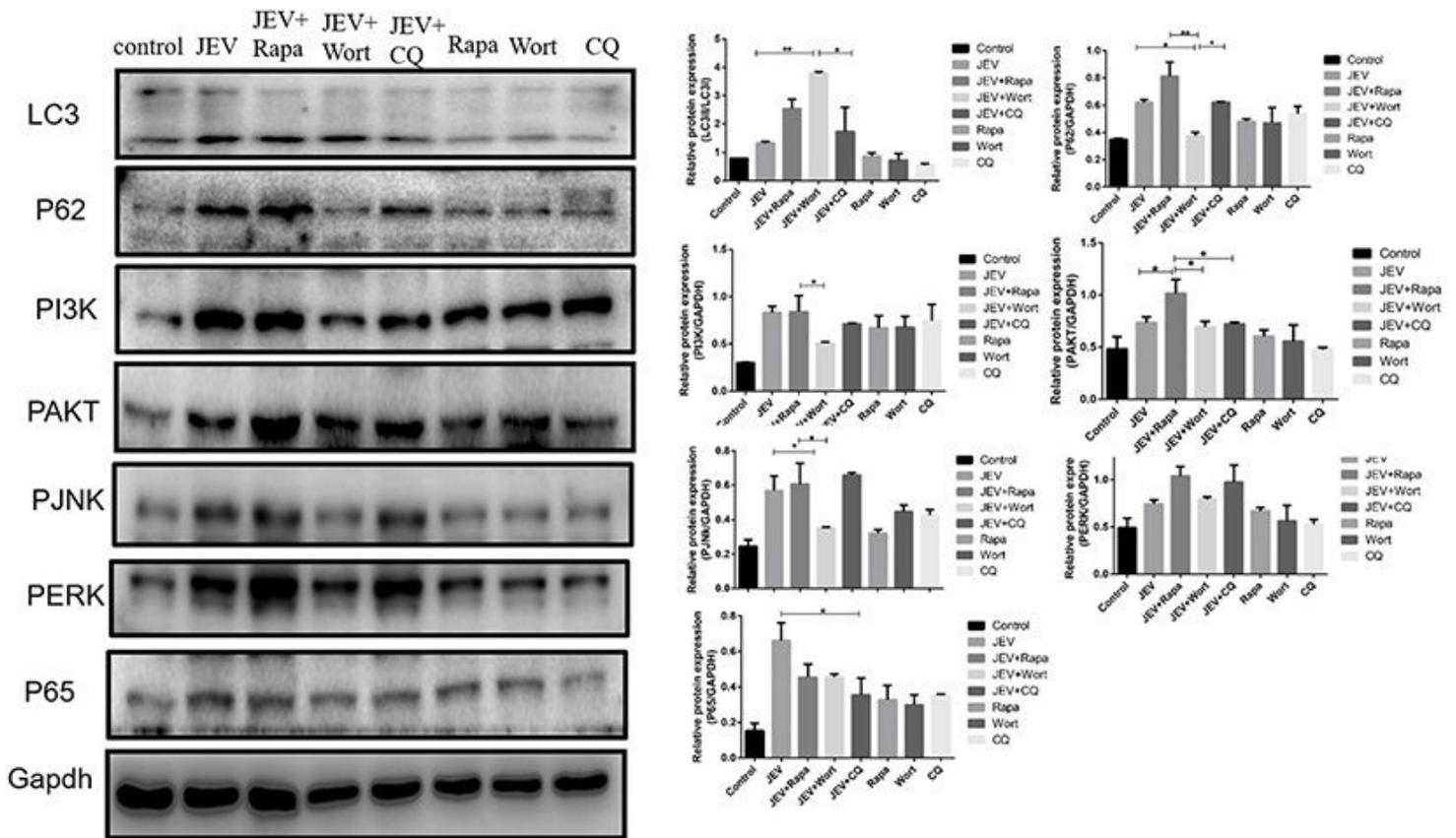


Figure 6

Autophagy inhibitor treatment reduced the secretion of pro-inflammatory cytokines primarily by inhibiting activation of the PI3K/AKT/NF-κB pathway in the brain tissues of Japanese encephalitis virus (JEV)-challenged mice. Brain tissues were sampled at 10 d after JEV challenge, followed by separation of cytoplasmic proteins and nucleoproteins. The expression levels of LC3, P62, PI3K, P-AKT, P-JNK, P-ERK, and P65 proteins were quantitatively determined using western blot and scanning densitometry and normalized to GAPDH levels. Left: a representative image. Right: 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.