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The Chinese herbal prescription JZ-1 induces autophagy to protect against herpes simplex Virus-2 in human vaginal epithelial cells by inhibiting the PI3K/Akt/mTOR pathway

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ABSTRACT

Ethnopharmacological relevance: The Chinese herbal prescription JieZe-1 (JZ-1) is based on the modification of Yihuang Tang, which was first described in Fu Qingzhu Nvke by the famous Qing Dynasty doctor Shan Fu as a treatment for leukorrheal diseases. As an in-hospital preparation, JZ-1 has been used in Tongji Hospital for many years to treat various infectious diseases of the lower female genital tract, including cervicitis, vaginitis, genital herpes and condyloma acuminatum. Our previous studies have shown that JZ-1 has curative effects on Candida albicans, Trichomonas vaginalis and Ureaplasma urealyticum infections.

Aim of the study: Genital herpes is among the most common sexually transmitted diseases (STDs) worldwide and is mainly caused by herpes simplex virus type-2 (HSV-2). Current therapies can relieve symptoms in patients but do not cure or prevent the spread of the virus. This study was designed to investigate the effect of JZ-1 on HSV-2 infection and its mechanism, which is based on autophagy induction, to provide new ideas and a basis for the study of antiviral drugs.

Materials and methods: Evaluation of the antiviral activity of JZ-1 was conducted by MTT assay and western blotting. Then, Western blot and immunofluorescence analyses, observations through transmission electron microscopy and experiments with the recombinant lentivirus vector mRFP-GFP-LC3B were used to monitor autophagic flux in VK2/E6E7 cells. To explore the mechanism by which JZ-1 regulates autophagy, western blotting and real-time quantitative PCR (qRT-PCR) were used to determine the expression of phosphoinositide 3-kinase (PI3K)/Akt/mTOR pathway proteins and to detect changes in critical molecules in the pathway after the application of a PI3K inhibitor. Additionally, the mRNA expression levels of inflammatory cytokines, namely, IL-6, IFN-α, IFN-β and TNF-α, were measured with qRT-PCR.

Results: HSV-2 infection inhibited autophagy in the VK2/E6E7 cells. Further study revealed that the activation of the PI3K/Akt/mTOR pathway induced by HSV-2 infection may result in the blocked autophagic flux and inhibited autophagosome and autolysosome formation. JZ-1 exhibited significant antiviral activity in the VK2/E6E7 cells, which showed increased cell vitality and reduced viral protein expression, namely, earliest virus-specific infected cell polypeptides 5 (ICP5) and glycoprotein D (gD). We found that JZ-1 treatment inhibited the upregulation of the PI3K/Akt/mTOR pathway proteins and promoted autophagy to combat HSV-2 infection, while PI3K inhibitor pretreatment prevented the enhanced autophagy induced by JZ-1. Moreover, JZ-1 attenuated the increase in inflammatory cytokines that had been induced HSV-2 infection.

Conclusion: Our results showed that JZ-1 protects against HSV-2 infection, and this beneficial effect may be mediated by inducing autophagy via inhibition of the PI3K/Akt/mTOR signaling axis.

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1. Introduction

Genital herpes is one of the most common, persistent and highly contagious sexually transmitted diseases in the world and is mainly caused by HSV-2. HSV-2 affects approximately 16% of the world’s population in the age range of 15–49 years (Looker et al., 2008), and it has been shown to increase the risk of human immunodeficiency virus type 1 (HIV-1) sexual acquisition (Freeman et al., 2006). Currently, antiviral drugs are mainly based on three nucleoside analogs: acyclovir, famciclovir and valacyclovir. These drugs significantly inhibit virus replication and improve symptoms in patients, but cannot cure the infection or prevent the spread of HSV-2, and the problem of drug resistance is becoming increasingly pronounced. Patients with impaired immune function may not benefit from the treatment. Besides, the HSV-2 vaccine efficacy trials with individuals with recurrent HSV-2 infection were mostly unsuccessful (Hofstetter et al., 2014). Therefore, the development of new antiviral drugs continues to be explored by scientists and technicians.

As a cellular defense mechanism, autophagy, a highly conserved catabolic pathway, has received increasing attention in the defense of microbes. Autophagy is a cell homeostasis process that is maintained by fusion of autophagosomes, consisting of intracellular protein aggregates and damaged organelles coated with a double layer of isolated membrane, withlysosomes to form autolysosomes. Autophagy reportedly removes damaged organelles and misfolded proteins, protects cells from nutrient deprivation and microbial infections, and prevents metabolic, heart and neurodegenerative diseases (He et al., 2017). Increasing evidence suggests that host cells attempt to control herpesvirus infection by activating autophagy (Cavignac and Esclatine, 2010). In addition, a number of studies have shown that viruses and intracellular bacteria have evolved to antagonize autophagy initiation or autophagosome maturation, evade autophagy recognition molecules and block autophagic flux (Levine et al., 2011; Liu et al., 2014). Since autophagy can promote innate and adaptive immunity and eliminate intracellular pathogens (xenophagy) (Deretic et al., 2013), enhancing autophagy may be a potential means of fighting viral infection, including herpes virus infection. While many studies on Herpesviridae subfamilies have focused on HSV-1, VAV and HSMV, few studies have been conducted on HSV-2 (Cavignac and Esclatine, 2010), which is the cause of genital herpes.

In traditional Chinese medicine (TCM), genital herpes and its complications fall under the categories of “sore of vulvae” and “erosion of vulva”. According to TCM theory, genital herpes is mainly caused by attack of the wind, dampness and heat, an overload of liver fire and kidney deficiency. It is well accepted that various factors may invade the human body to disturb the normal circulation of qi and blood and block the meridians, the damp heat of the liver meridian down to the genital area, eventually leading to a “sore of vulvae”. The onset stage is dominated by dampness and heat, while the remission stage is dominated by deficiency because recurrent attacks cause heat injury to body fluids, causing Yin deficiency. Therapeutically, it is essential to disperse the wind, resolve the dampness and clear the heat in the early stage, and strengthen the kidney and nourish the Yin during repeated attacks (Kuang et al., 2016). Yihuang Tang is a classic prescription composed of Dioscorea opposita Thunb. (Dioscoreaceae Rhizoma), Euryale ferox Salisb. (Euryales Semen), Phellodendron chinense Schneid. (Phellodendri Chinesis Cortex), Plantago asiatica L. (Plantaginis Semen) and Ginkgo biloba L. (Ginkgo Semen), which is mainly used for female leukorrheal diseases caused by spleen deficiency and damp heat. Some experimental, clinical, and observational studies have shown that Yihuang Tang exerts a satisfactory effect on genital mycoplasma infection caused by dampness and heat (Tan, 2017; Zhou and Gao, 2018) and damp-heat syndrome vaginitis (Wang et al., 2016). Genital herpes is mostly in the damp-heat syndrome stage, showing typical symptoms of dampness and heat. Therefore, strengthening the application of heat-clearing and dampness-resolving prescriptions is necessary. JI-1 is derived from Yihuang Tang, and is composed of Phellodendri Chinensis Cortex, Ginkgo Semen, Sorolarum nigrum L. (Solanum Nigrum), Taraxacum mongolicum Hand.-Mazz (Taraxaci Herbata), Thlaspi arvense Linn. (Herba Patriniae), Dictamnus dasycarpus Turcz. (Dictamni Cortex), Smilax glabra Roxb. (Smilacis Glabrae Rhizoma), Paeonia suffruticosa Andr. (Moutan Cortex), Mentha haplocalyza Briq. (Menthae Haplocalycis Herba) and Borneol Newman. Syntheticum.

As an external preparation, JI-1 has been used in Tongji Hospital for many years, and has had a definite effect on female genital tract infections, such as vaginitis and cervicitis. In addition, we have already conducted clinical trials of 310 patients whose symptoms include vaginal congestion, cervical erosion, abnormal leukorrhea, genital itching, and frequent urination to verify and confirm the protective effects of JI-1 on cervicitis caused by U. urealyticum (Wei et al., 2007, 2008). Our previous research showed that JieZe-2 (composed of JI-1 and the spermicide nonoxynol-9 (N-9) can prevent C. albicans and T. vaginalis infection in vivo and in vitro (Chen et al., 2009a, 2009b, 2009c). Taken together, these results suggest that JI-1 is a valid prescription for damp-heat syndrome, with an action similar to that of Yihuang Tang. Furthermore, some studies have shown that traditional Chinese medicines that can clear heat or remove dampness have an excellent effect on HSV-2 infection (Cheng et al., 2008a, 2008b; Chin et al., 2010; Sheng, 2010). Therefore, we investigated the effect of JI-1 on HSV-2 infection in vitro, and fond it to be a potent anti-HSV-2 herbal medicine. Our previous experiments showed that JI-1 decoction could protect human vaginal epithelial cells against HSV-2. The anti-HSV-2 effect of JI-1 is superior to that of berberine or penciclovir, and the effect is mainly achieved by augmenting the host cell defenses ability and blocking the adhesion and penetration of HSV-2 (Duan et al., 2019). In a model of HSV-2-infected VK2/E6E7 cells, the expression of the HSV-2 envelope protein gD increased gradually over time, and JI-1 reduced this gD expression but had no effect on HVE in the human vaginal epithelial cells infected by HSV-2 (Ai and Chen, 2017; Qiao and Chen, 2016). Moreover, the mechanism involved in the anti-HSV-2 action of JI-1...
may be related to the regulation of the Toll-like receptor signaling pathway and likely is promoted by the secretion of interferon and other downregulating inflammatory factors (Huang and Chen, 2018). However, the related antiviral mechanism of JZ-1 is not fully understood and needs further explanation. Recent studies have shown that HSV-2 blocks autophagic flux but host cells increase autophagy levels to inhibit viral infection in the later stages of the viral replication cycle (Kristen et al., 2015; Petrovski et al., 2014). Therefore, this study explored the underlying mechanisms of JZ-1 in treating HSV-2 infection by inducing autophagy. Our study provides the basis for further development of this formulation as a Chinese herbal therapeutic agent for the treatment or prevention of genital herpes caused by HSV-2 infection.

2. Materials and methods

2.1. Preparation of JZ-1

JZ-1 consists of 10 traditional Chinese medicines, and the details of the herbal ingredients are shown in Table 1. JZ-1 was prepared as indicated previously (Duan et al., 2019). Briefly, Phellodendri Chinensis Cortex was extracted twice and filtered. Moutan Cortex and Menthae Haplocalycis Herba were separately extracted, and the distillate was collected for use. The other herbs were boiled for 3 h, and the herbal mixture filtrate was concentrated to a relative density of 1.08–1.14. The Phellodendri Chinensis Cortex, Moutan Cortex and Menthae Haplocalycis Herba extracts were added to the herbal mixture described above. The final concentration was adjusted to 0.5–0.55 g/mL (crude herbs/extract). After cooling for 24 h, borneol (dissolved in anhydrous ethanol) was added, and then the stock solution was filtered through a 0.22-micron filter. Nine other drugs in addition to Borneolum Syntheticum were collected for use. The other herbs/extracts were boiled for 3 h, and the distillate was stored at -80°C after harvest. We infected VK2/E6E7 cells with HSV-2 for different exposure times (6, 12, 18, 24, 48 h), and 24 h post-infection was selected as the time point for the follow-up experiments based on the relevant results.

2.2. 3D-high performance liquid chromatography (3D-HPLC) of JZ-1

The primary chemical constituents of JZ-1 were identified by 3D-high performance liquid chromatography (3D-HPLC) method and were described in detail in a previous article (Duan et al., 2019).

2.3. Lentivirus, chemicals and reagents

The recombinant lentivirus vector mRFP-GFP-LC3B was produced by GENECHEM (Shanghai, China). GFP and mRFP fluorescent puncta indicate autophagosomes that has not fused with lysosomes. However, when the autophagosomes fuses with the lysosome, the GFP signal is sensitive to the acidic environment in the lysosome and is quenched; therefore, the mRFP punctum that do not emit GFP correspond to an autolysosome.

Rapamycin (Rapa), 3-methyladenine (3-MA) and bafilomycin A1 (Baf A1) were purchased from Selleck, and LY294002 was purchased from MCE (MedChemExpress, Shanghai, China). The following antibodies in the immunoblotting and immunofluorescence were used: anti-LC3B, anti-Atg5, anti-p-Akt, anti-Akt, anti-P70K, anti-p-mTOR, anti-mTOR, anti-gd and anti-ICP5 were obtained from Cell Signaling Technology. The antibody for SQSTM1/p62 was obtained from Proteintech, and anti-β-actin, 3-{4,5-dimethylthiazol-2-yl}-2,5-diphenyltetrazolium (MTT) and DMSO were purchased from Servicebio, Wuhan, China.

2.4. Cell culture and HSV-2 infection

The human immortalized vaginal epithelial cell line (VK2/E6E7) was purchased from the American Type Culture Collection, and the Vero cell line was obtained from the Chinese Type Culture Collection. The VK2/E6E7 cells were cultured in Cn-TPR medium (CELLnTEC, Switzerland), and the Vero cells were cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum. The cells were grown at 37°C in a 5% CO2 atmosphere.

HSV-2 strain 333 was propagated on a monolayer of Vero cells and stored at -80°C after harvest. We infected VK2/E6E7 cells with HSV-2 for different exposure times (6, 12, 18, 24, 48 h), and the absorbance was measured at 570 nm using a microplate reader (BioTek).

2.5. MTT assay

Cytotoxicity and anti-HSV-2 activity of JZ-1 were examined by MTT assays following the method described in a previous article (Duan et al., 2019). Briefly, exponentially growing VK2/E6E7 cells were seeded into 96-well culture plates for 24 h, then various concentrations (0.625, 1.25, 2.5, 5, 10 and 20 mg/mL) of JZ-1 were added to the VK2/E6E7 cells for another 24 h in the presence or absence of HSV-2. Afterwards, add 100 μL of 5 mg/mL MTT solution to each well, and incubate for 4 h at 37 °C. Discard the MTT solution and add 100 μL DMSO. Finally, the absorbance was measured at 570 nm using a microplate reader (BioTek).

2.6. Drug administration

The VK2/E6E7 cells were pretreated with 0.1 mM rapamycin for 6 h, 20 μM LY294002 for 2 h, and 2.5 mM 3-MA for one-half hour. Then HSV-2 was added in the absence or presence of 5 mg/mL JZ-1 for the experiments. Baf A1(100 nM) was cotreated with HSV-2 for 24 h.

2.7. Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from the VK2/E6E7 cells with TRIzol
3. Results

3.1. HPLC fingerprinting of JZ-1

Because of the complex components in the JZ-1 extract, HPLC fingerprinting was used to control the chemical components in the extract better. The results of HPLC fingerprint have been shown in a previous article (Duan et al., 2019), a total of six JZ-1 components were determined, namely berberine, paeoniflorin, neoaestinib, astilbin, paenol and dictamine.

3.2. HSV-2 induces the accumulation of LC3B-II in the VK2/E6E7 cells

To determine the appropriate timepoint for the virus infection, we exposed VK2/E6E7 cells to HSV-2 for different duration intervals (6–48 h). The Western blot results showed that, compared with that of the control group, the expression of endogenous LC3B-II in the HSV-2-infected cells was increased significantly at each corresponding time point, with the greatest increase observed 24 h after infection (Fig. 1A). Therefore, 24 h post infection was selected as the time point for the follow-up experiments.

3.3. Cytotoxicity and anti-HSV-2 activity of JZ-1

As shown in Fig. 1B and C, JZ-1 was cytotoxic at concentrations > 5 mg/mL, and at 1.25, 2.5, 5, 10, and 20 mg/mL JZ-1, the cells exerted anti-HSV-2 activity. Considering these results, we chose 5 mg/mL as the final concentration of JZ-1 to use in this study.

3.4. Autophagic flux is inhibited in HSV-2-infected cells

The microtubule-associated protein 1 LC3B is synthesized in the form of the LC3B precursor (pro-LC3B), which is proteolytically processed into the cytosolic LC3B-I isoform. When autophagy is induced, LC3B-I is modified into the phosphatidylethanolamine-conjugated form, LC3B-II, which specifically binds to autophagosome membranes. Changes in LC3B provide a handy marker of autophagy that can be monitored by immunofluorescence and western blotting. In addition, as autophagic flux increases, SQSTM1/p62, another autophagy marker, is recruited to autophagosomes (Klionsky et al., 2016). During the formation of the double-layer autophagy isolation membrane, the ubiquitin-like conjugate ATG12-ATG5 localizes to the autophagosome precursors, and plays an essential role, with LC3, in their development (Mizushima et al., 2001). Besides, the SQSTM1/p62 protein is the link between LC3 and ubiquitinated substrates, and both LC3 and SQSTM1/p62 serve as indexes of autophagic flux (Bjorkoy et al., 2005). To examine the effects of HSV-2 infection on autophagy, we examined the protein expression levels of LC3B, SQSTM1/p62 and Atg5. The results from the immunofluorescence analysis showed that LC3B was decreased in the HSV-2-infected cells (Fig. 2B), a result similar to that from the Western blot analysis.
(Fig. 1A), suggesting an accumulation of autophagic elements in the infected cells. Increased LC3B-II levels may be the result of autophagy activation or reduced autophagic turnover due to defects in the late stages of autophagic degradation. Was the increased LC3B-II level induced by HSV-2 infection due to the activation of autophagy or reduced autophagy-related protein turnover? Bafilomycin A1 (Baf A1) was used to determine the cause for the increased level of LC3B-II. Baf A1 is a V-ATPase inhibitor that inhibits autophagosome degradation by blocking the fusion of autophagosomes with lysosomes, and we used it to monitor changes in autophagic flux (Shacka et al., 2006).

The immunofluorescence and Western blot data showed that the numbers of LC3B puncta and the LC3B-II and SQSTM1/p62 protein expression levels were both decreased in the Baf A1+HSV-2 treated group compared with puncta numbers and expression levels in the Baf A1 group (Fig. 2), suggesting that autophagosome turnover was blocked in the HSV-2-infected cells. Consistently, the protein expression of autophagy-related Atg5 in the HSV-2-infected cells was decreased compared with that in the control group cells. To better study the effect of HSV-2 infection on autophagic flux, the VK2/E6E7 cells were transfected with the mRFP-GFP-LC3B lentivirus, which served as a reporter of autophagic flux (Kimura et al., 2007). Since the GFP signal is pH sensitive, this method can be used to quantify the number of

![Figure 1](image1.png)

**Fig. 1.** HSV-2 induces the accumulation of LC3B-II. (A) HSV-2 induces the accumulation of LC3B-II. (B) Cytotoxicity of JZ-1 in the VK2/E6E7 cells as detected by the MTT method. (C) Anti-HSV-2 activity of JZ-1 was detected by MTT method. P < 0.05(*), P < 0.01(**), and P < 0.001(***); (n=6).

![Figure 2](image2.png)

**Fig. 2.** Autophagic flux was inhibited in HSV-2-infected VK2/E6E7 cells. VK2/E6E7 cells were treated with HSV-2 for 24 h in the presence or absence of 100 nM Baf A1. LC3B-II, SQSTM1/p62 and Atg5 levels were detected by western blotting (A), and LC3B-II immunoreactivity was analyzed by immunofluorescence (B). (C) Results from the quantification of the Western blot shown in (A). P < 0.05(*), P < 0.01(**), and P < 0.001(***); (n=6).
autophagosomes and autolysosomes. The yellow spots represent overlapping red and green spots, indicating autophagosomes that have not fused with lysosomes, while the red spots correspond to autolysosomes (Klionsky et al., 2016). Twenty-four hours after infection, we found a marked increase in the number and proportion of yellow puncta in the cells infected with HSV-2 compared with the number and proportion in the nontreated cells (Fig. 3B). Taken together, these results indicate that HSV-2 blocks autophagic flux in VK2/E6E7 cells.

3.5. JZ-1 acts against HSV-2 infection and inhibits HSV-2-induced inflammatory response by inducing autophagic flux

The levels of HSV-2 structural viral proteins, such as ICP5 and gD, can be measured as representative of viral replicating events in the cell (Liu et al., 2016; Peretti et al., 2005), and gD, as a major component of the virion envelope, has been previously found to be essential for viral infection. The Western blot analysis showed that the expression levels of ICP5 and gD were increased in the HSV-2-infected cells, indicating enhanced viral replication. However, the expression levels of ICP5 and gD were decreased compared with that in the HSV-2 group, while in the 3-MA + HSV-2 group, the levels were further increased compared with that of the HSV-2 group. Moreover, the ICP5 and gD protein expression levels of the cells in the JZ-1 + 3-MA + HSV-2-treated group were higher than those of the cells in the JZ-1 + HSV-2 group (Fig. 5A and C), which indicated that the inhibition of autophagy weakens the anti-HSV-2 effect of JZ-1. Our results indicated that the activation of autophagy protected against HSV-2 infection and that the anti-HSV-2 effect of JZ-1 was mediated by the induction of autophagic flux.

Furthermore, HSV-2 infection induces the secretion of inflammatory cytokines, including IFN-α, IFN-β and proinflammatory cytokine IL-6, TNF-α, which are important innate immune factors against viral infection (Chew et al., 2009). Our results showed that the cytokines IFN-α, IFN-β, IL-6 and TNF-α were all significantly increased after HSV-2 infection, while JZ-1 attenuated this effect. Treatment with Rapa also decreased the release of IFN-α, IFN-β and IL-6, but treatment with 3-MA dramatically increased the secretion levels of these cytokines (Fig. 6A).

3.6. JZ-1 induces autophagy by inhibiting the PI3K/Akt/mTOR pathway

The kinase mTOR is a complex of two mTOR components, mTORC1 and mTORC2, which have different protein components (Bhaskar and Hay, 2007). mTORC1 is a major negative regulator of autophagy, and the PI3K/Akt/mTOR pathway is a major upstream major modulator of mTORC1 (Schmelzle and Hall, 2000). To investigate the mechanism by which JZ-1 induces autophagy, we assessed the changes in the classic PI3K/Akt/mTOR pathway in the VK2/E6E7 cells. The results from qRT-PCR assays and Western blot analyses showed that, compared with that of the control, the expression of PI3K and phosphorylation levels of Akt and mTOR (p-Akt and p-mTOR) in the HSV-2-infected cells were significantly increased, and these proteins all were decreased significantly in the JZ-1 + HSV-2 group (Figs. 5B, Fig. 7C).

LY294002 (LY) is a broad-spectrum inhibitor of PI3K, and it was found to completely suppress the activation of the PI3K-Akt-mTOR axis in cells (Han and Zhang, 2010; Wu et al., 2009). To explore whether the regulation of the PI3K/Akt/mTOR pathway is associated with autophagy induction, the VK2/E6E7 cells were pretreated with LY294002 for 2 h and then co-incubated with HSV-2 in the absence or presence of JZ-1. The results from the Western blot and qRT-PCR analyses showed

![Fig. 3. Autophagic flux was inhibited in HSV-2-infected VK2/E6E7 cells and JZ-1 induced autophagic flux in the HSV-2 infected cells. (A) TEM was used to detect autophagy structures in the VK2/E6E7 cells. (B) Transfection with mRFP-GFP-LC3B plasmids in VK2/E6E7 cells.](image-url)
that, compared with that of the HSV-2 group, the expression levels of PI3K, p-Akt and p-mTOR were significantly reduced in the LY + HSV-2 group, indicating that it indeed inhibited the PI3K/Akt/mTOR pathway (Figs. 6C, Fig. 7D). On this basis, we measured the expression levels of LC3B and Atg5 and found that, in the JZ-1 + HSV-2 + LY - treated group, the LC3B and Atg5 mRNA expression levels and the LC3B-II and Atg5 protein expression levels were not different from those in the LY + HSV-2 group (Figs. 6C, Fig. 7D). As expected, LY pretreatment eliminated the enhanced activity of JZ-1 on the autophagy. Based on the above results, it can be inferred that JZ-1 can induce autophagy in the VK2/E6E7 cells by inhibiting the PI3K pathway.

4. Discussion

HSV-2 is a linear, double-stranded DNA virus belonging to the subfamily of α-Herpesviridae. Similar to most viruses, HSV-2 infects
host cells following the key steps: virus entry, viral gene expression, viral DNA synthesis, and assembly/egress of progeny virions (Taylor et al., 2002). The virus is taken into the cell by direct fusion with the cell membrane (independent of pH changes) or by endocytosis mediated by specific cell receptors. Both gD and the ICP5 of HSV play essential roles in the two uptake processes described above (Spear et al., 2000). Regardless, the binding of viral glycoproteins to cell attachment sites, usually heparan sulfate proteoglycans (HSPGs), mediates the entrance of HSV-2 (Shukla and Spear, 2001). It has been reported that glycoproteins B and C (gB and gC) of HSV-2 are also necessary for the initiation of virus-to-cell attachment. Therefore, the expression levels of the related viral proteins can represent the degree of virus infection and the activity of antiviral drugs.

Autophagy is an evolutionarily conserved intracellular process involving the formation of a bilayer membrane structure called an autophagosome that phagocytoses cytoplasmic macromolecules and damaged organelles and delivers them to the lysosome for degradation and recycling. Autophagy can be divided into three classic types: chaperone-mediated autophagy, microautophagy and macroautophagy, the most extensively studied autophagy (simply referred to as autophagy) (Deretic et al., 2013). As one of the body’s immune mechanisms against pathogen infection, autophagy selectively recognizes and removes invading pathogenic microorganisms, such as bacteria and viruses (Sui et al., 2017). Upon induction, phagophores elongate and subsequently enclose a portion of the cytoplasm, leading to the formation of autophagosomes. The outer membrane of the autophagosome fuses with the lysosome (forming the autolysosome), in which the contents of the autophagosome are degraded. In this process, the Atg12–Atg5–Atg16 complex binds to the outer membrane and then dissociates upon completion of the autophagosome. Upon receiving the signal to induce autophagy, Atg7 and Atg3 mediate the conjugation of LC3-1 to the membrane lipid phosphatidylethanolamine to form LC3B-II. LC3B-II integrates into both the outer and inner membranes of the autophagosome and facilitates membrane elongation and closure. SQSTM1/p62 is selectively incorporated into autophagosomes through direct binding to LC3; at the end of autophagy, both proteins are effectively degraded. The total cellular expression level of SQSTM1/p62 inversely correlates with autophagic activity, while the LC3B-II level positively correlates with autophagic activity (Mizushima et al., 2010).

As mentioned in the Introduction, autophagy pathways and autophagy proteins play crucial roles in combating viral infections. Several studies have found that repression of autophagy led to increased viral replication and pathogenesis (Orvedahl et al., 2010), whereas its activation decreased virus infection (Clark et al., 2018).

Different from many RNA viruses that induce autophagy and use membrane structures to create scaffolds for virus replication or support the release of viral particles during viral replication, many DNA viruses inhibit autophagy to prevent their own intracellular degradation and phagocytosis or to damage MHC presentation during antigen processing (Paul and Munz, 2016), especially herpesviruses, such as the α-herpesvirus HSV-1 (Orvedahl et al., 2007), the murine γ-herpesvirus 68 (MHV-68) (E et al., 2009) and the β-herpesvirus HCMV (Mouna et al., 2016). However, few studies have addressed the changes in autophagy induced by HSV-2. Generally, increased LC3B-II levels with decreased SQSTM1/p62 levels are considered to be the result of increased autophagy. In our study, we found the same results: HSV-2 induces the accumulation of LC3B-II and increases the degradation of SQSTM1/p62. Does this finding indicate that HSV-2 infection induces autophagic flux? Through TEM observation, applying autolysosome inhibitor (Baf A1), and using the lentivirus vector mRFP-GFP-LC3B to track the LC3B

Fig. 6. JZ-1 inhibits the inflammatory response in the VK2/E6E7 cells caused by HSV-2 infection. (A) VK2/E6E7 cells were treated with HSV-2 and JZ-1 for 24 h in the presence or absence of 0.1 mM rapamycin, which was used as pretreatment for 6 h, or 2.5 mM 3-MA, which was used as a pretreatment for one-half hour. mRNA levels of IFN-α, IFN-β, IL-6 and TNF-α were determined by qRT-PCR. (B) VK2/E6E7 cells were treated with HSV-2 in the presence or absence of JZ-1, which was added for 24 h, and the PI3K, Akt, and mTOR mRNA levels were determined by qRT-PCR. (C) VK2/E6E7 cells were treated with HSV-2 and JZ-1 for 24 h in the presence or absence of 20 μM LY294002, which was used as a pretreatment for 2 h, and the mRNA levels of PI3K, Akt, LC3B, and Atg5 were determined by qRT-PCR. P < 0.05 (*) and P < 0.01 (**); (n=6).
marker, we assessed the true influence of HSV-2 on autophagic flux. Compared with that of the group treated with Baf A1 alone, the expression level of LC3B-II, SQSTM1/p62 and Atg5 in the HSV-2 + Baf A1 group was reduced. In lentivirus experiments, the number of unused autophagosomes was significantly increased in the HSV-2-infected cells. These data indicated that HSV-2 inhibits autophagic flux. As for explanations about LC3B-II being elevated while SQSTM1/p62 is decreased, may be degraded in pathways other than the autophagy pathway (Barth et al., 2010; Moscat and Diaz-Meco, 2009).

As an in-hospital preparation of Tongji Hospital (Approval Number: Z20103135), JZ-1 has been widely used for many years. JZ-1 is a prescription of Yihuang Tang. In Yihuang Tang, Dioscoreae Rhizoma, Euryales Semen strengthen the spleen and reinforce the stomach, tonify the kidney and inhibit leucorrhoea. Ginkgo Semen convergence and dehumidify, and can also arrest leucorrhoea. Phellodendri Chinensis Cortex clears heat and resolves dampness, thereby promoting blood circulation. In the JZ-1 prescription, Phellodendri Chinensis Cortex, Ginkgo Semen and removing blood stasis, thereby promoting blood circulation. In the JZ-1 prescription, Phellodendri Chinensis Cortex, Ginkgo Semen, Taraxaci Herba, Herba Patriniae, Moutan Cortex or their extracts have a protective effect during virus infection. Phellodendri Chinensis Cortex has various pharmacological actions, which have been widely prescribed to remove damp heat, eliminate dampness, counteract toxicity, purge pathogenic fire, relieve consumptive fever, diarrhea and other syndromes(Chen et al., 2017; Wang et al., 2019). Phellodendri Chinensis Cortex has the immunomodulatory effects of a broad-spectrum antiviral activity by inducing an antiviral state through a type I IFN stimulation mechanism (Kim et al., 2016). Ginkgo Semen, which can converge and eliminate dampness and cure leucorrhoea, is a potential phytopharmaceutical. As for inflammatory cells, beneficial effects of anti-inflammatory effects on inflammatory cells, beneficial effects on neuron degenerative diseases through the prevention of chronic oxidative damage and useful effects for the treatment of diseases related to the production of free radicals (Yoshikawa et al., 1999). In addition, Ginkgo Semen can markedly reduce the infectivity of viruses by preventing their adsorption onto host cells (Haruyama and Nagata, 2013). The functions of Solanum Nigrum are heat-clearing, detoxification and diuresis. Solanum Nigrum plays a role in viral clearance during natural HCV infection, and it might be an alternative treatment for chronic HCV infection (Javed et al., 2011). Taraxaci Herba has the ability to eliminate heat and toxins, as well as to reduce swelling, choleresis, diuresis, and inflammation, and it can inhibit viruses by suppressing viral nucleoprotein synthesis and polymerase activity(He et al., 2011). Herba Patriniae can clear heat and induce detoxification, eliminating phlegm and removing blood stasis, thereby promoting blood circulation. In the model of λ-lysogen, which is used to study the inhibitory effects of antisevere acute respiratory syndrome (SARS) conferred by traditional Chinese medicines, Herba Patriniae effectively quenched the free radicals generated in the process of λ-lysogenic cells by UV radiation.
which suggests that Herba Patriniae is a potential Chinese medicine against the SARS virus (Li et al., 2006). The function of Dictamnii Cortex to clear heat and eliminate dampness, induce detoxification, clear hurricane and itching. The function of Smilacis Glabrae Rhizoma is dehumidification and detoxification. Moutan Cortex has the function of clearing heat, cooling blood and removing blood stasis. It is used in TCM as a hepatoprotective and anti-inflammatory herb, which can effectively prevent the entry of HCV and the related Zika flavivirus (Behrendt et al., 2017; Poon et al., 2011). Menthae Haplocalycis Herba has the effect of dispelling wind and heat, relieving sore throat, promoting eruption. All these medicines are used together to treat abnormal leukorrhea and erosion of the vulva of genital herps caused by attack of wind, dampness, heat and toxin, and can achieve good antiviral effects.

Our results showed that JZ-1 could significantly reduce the expression of the viral proteins gD and ICP5 in HSV-2-infected VK2/E6E7 cells, suggesting that it also exerts a significant anti-HSV-2 effect. As the main active ingredient in Phellodendri Chinensis Cortex, berberine can induce autophagy by activating the AMPK pathway to inhibit mTOR signaling (Fan et al., 2015). Dandelion root extract has the potential to induce autophagy (Ovadje et al., 2012). It has been shown that paenonel (2-hydroxy-4'-methoxyacetophenone), isolated from the Moutan Cortex, can up-regulate autophagy by activating the AMPK/mTOR signaling pathway, and the autophagy inhibitor CQ notably attenuates paenonel-induced autophagy (Wu et al., 2017). Cao et al. proved that Ginkgo Semen leaf extract can restore autophagy through inhibition of mTOR signaling (Cao et al., 2017). Based on this evidence, we postulate that JZ-1 protects against HSV-2 by inducing autophagy, a supposition confirmed in our later experiments. The TEM images, the mRFP-GFP-LC3B lentivirus reporter observations and results from applying autolysosome inhibitor Baf A1 showed that HSV-2 induces autophagy flux. To confirm whether JZ-1 exerts antiviral effects by regulating autophagy, we treated cells with the autophagy inducer Rapa and inhibitor 3-MA, which are widely used at the early stage of autophagy. 3-MA inhibits class I phosphoinositide 3-kinases (PI3Ks) as well as class III PI3Ks. Class I enzymes generate products such as PtdIns (3,4,5) P3, which inhibit autophagic sequestration, whereas the class III product (PtdIns3P) generally stimulates autophagic sequestration. 3-MA has different temporal patterns of inhibition, causing long-term suppression of the class I PI3Ks but only transient inhibition of the class III form of the enzyme. Therefore, the correct concentration and incubation times are important for interpreting the final results. We conducted many experiments during concentration experiments and pretreated the cells with 2.5 mM 3-MA for one-half hour finally.

One hypothesis has suggested that the antiviral response of autophagy is controlled by the PI3K/Akt signaling pathway (Shelly et al., 2009), which is known as a key regulator of autophagy. Research has shown that HSV-2 infection results in the activation of PI3K and Akt in the host cells, and BX-795 (a PI3K inhibitor) treatment inhibited HSV-2-induced Akt phosphorylation and activation, thus decreasing viral replication (Su et al., 2017). mTOR is a downstream target of the PI3K/Akt pathway, and other studies have confirmed that the activation of the PI3K/Akt/mTOR pathway inhibits autophagy (Wullschleger et al., 2006). This evidence suggests that the PI3K/Akt/mTOR pathway may be a regulatory mechanism of autophagy during HSV-2 infection. To elucidate the molecular mechanisms underlying autophagy induction by JZ-1 in the HSV-2 infection model, we assessed the PI3K/Akt/mTOR pathway in our study. Our results showed that the autophagy induced by JZ-1 might be mediated upon inhibition of the PI3K/Akt/mTOR pathway. The use of LY, a specific inhibitor of PI3K, to block the JZ-1-induced autophagy confirmed this molecular mechanism. Some limitations of our study are noted. For example, after using LY, the expression of the HSV-2 virus proteins gD and ICP5 could have been further explored to clarify the relationship between the PI3K/Akt/mTOR pathway and the virulence of the infection and the replication rate of HSV-2.

5. Conclusions

The present study demonstrated that JZ-1 is effective in combating HSV-2 infection, and the potential mechanism is associated with inducing autophagy through inhibiting the PI3K/Akt/mTOR pathway. Our findings suggest that JZ-1 is a promising anti-HSV-2 formula to be further researched and developed.

Author contributions

Zhuo Chen conceived the idea and organized implementation. Qingqing Shao performed main experiments and analyzed data. Tong Liu completed the HPLC experiments. Qingqing Shao and Zhuo Chen were responsible for manuscript preparation and revision. Lijun Xu prepared the Chinese medicine formula JZ-1. Wenjia Wang, Qianni Duan and Tianli Liu assisted the experiment. Guanying Huang guided the subject.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2020.112611.

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