Lychee flower extract inhibits proliferation and viral replication of HSV-1-infected corneal epithelial cells

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Purpose: Herpes simplex virus type I (HSV-1) is capable of causing a wide array of human ocular diseases. Herpes simplex virus keratitis (HSK) is one of the most common viruses found in humans. Reactivation of HSV includes fever, trauma, emotional stress, and overexposure to ultraviolet (UV) radiation. Transmission typically occurs by direct contact with an open epithelial lesion or contaminated bodily secretions. Rarely, the virus is spread by contaminated materials such as towels or tissues. HSV belongs to the herpesviridae of the α-herpes virus subfamily (alphaherpesvirinae). Due to the different antigenic surface proteins, herpes can be divided into two serotypes: HSV type I (HSV-1) and HSV type II (HSV-2) [1]. HSV-1 often affects the eye and the oropharynx. HSV keratitis (HSK) is the most common cause of corneal blindness in the United States and the most common source of infectious blindness in the Western world [2,3].

Corneal cell abnormality is caused by herpetic keratitis that induces chronic inflammatory immune response that results in corneal scars and new blood vessel formation, leading to permanent vision damage. The Statens Seruminstitut rabbit corneal (SIRC) epithelial cells infected by HSV-1 and examined the antiviral capabilities of LFE.

Methods: SIRC cells were pretreated with different concentrations of LFE (0.2, 0.1, and 0.05 μg/ml) and then infected with 1 MOI of HSV-1 for 24 h. The cell viability or morphology was evaluated in this study. In addition, the supernatants and cell extracts were collected for Cell Counting Kit-8 (CCK), plaque assay, and western blotting.

Results: We found that HSV-1-induced cell proliferation is regulated through inhibition of the mammalian target of rapamycin (mTOR) and p70s6k phosphorylation in response to the LFE. In addition, the LFE enhanced the autophagy protein expression (Beclin-1 and light chain 3, LC3) and decreased the viral titers. These results showed the antiviral capabilities and the protective effects of LFE. Taken together, our data indicate that LFE has potential as an anti-HSK (herpes simplex keratitis) for HSV-1 infection.

Bacteria, virus, and pathogenic amoeba are three major pathogens of keratitis. Herpes simplex virus (HSV) is one of the most common viruses found in humans. Reactivation of HSV includes fever, trauma, emotional stress, and overexposure to ultraviolet (UV) radiation. Transmission typically occurs by direct contact with an open epithelial lesion or contaminated bodily secretions. Rarely, the virus is spread by contaminated materials such as towels or tissues. HSV belongs to the herpesviridae of the α-herpes virus subfamily (alphaherpesvirinae). Due to the different antigenic surface proteins, herpes can be divided into two serotypes: HSV type I (HSV-1) and HSV type II (HSV-2) [1]. HSV-1 often affects the eye and the oropharynx. HSV keratitis (HSK) is the most common cause of corneal blindness in the United States and the most common source of infectious blindness in the Western world [2,3].

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Lychee, a tropical and subtropical fruit tree, is native to Southeast Asia [6]. Our previous studies demonstrated that aqueous and ethanolic extracts of the dried flower contain abundant phenolic compounds and exhibit notable antioxidation [7,8], cardiovascular protection [9], hepatoprotection [10], and antiobesity effects [11].

Autophagy plays an important role in combating bacterial or viral infections [12,13]. The Lychee flower was shown to limit the replication, or enhance the degradation, of various viruses [14,15] in addition to its role in assisting the processing and presentation of pathogen antigens, boosting the host's adaptive immunity to infection. Autophagy is
initiated by mTOR inactivation to activate the Beclin-1 complex that assists with phagophore nucleation and elongation [16]. In addition, the light chain 3 (LC3) system is important for transport and maturation of the autophagosome. Once an autophagosome has matured, it fuses its external membrane with lysosomes to degrade its cargo [17]. Therefore, the objective in this study was to investigate the antiviral capabilities of the lychee flower extract on HSV-infected corneal epithelial cells.

**METHODS**

Materials and reagents: Glutamine, dimethyl sulfoxide (DMSO), EDTA (EDTA), leupeptin, phenylmethanesulphonyl fluoride (PMSF), (1X PBS: dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of NaHPO4 and 0.24 g of KH2PO4 in 1000 ml distilled H2O, pH 7.4), sodium chloride (NaCl), sodium nitrite (NaNO2), Triton X-100, and Tris were from Sigma Co. (St. Louis, MO). Dulbecco’s modified Eagle’s medium (DMEM), MEM/AHPLA medium, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Hyclone (Logan, UT).

Lychee flower extract: Fresh Lychee flowers were lyophilized with a freeze-dryer after being gathered from a farm in Taichung City, Taiwan [18]. The samples were extracted with ethanol (1/20, g/ml) for 24 h in the dark. After the mixture had been filtered, the solvent was evaporated to dryness on a rotary evaporator (Panchun Scientific Co., Kaohsiung City, Taiwan). The extract was then stored at −80 °C in an airtight bottle before use in the experiments. The components of the LFE were analyzed with high-performance liquid chromatography (HPLC). Five flavanoids [total amount, 102.73±5.50 mg/g of dried extract (mg/gDE)], including epicatechin (78.77±4.21 mg/gDE), rutin (19.05±1.02 mg/gDE), naringin (0.39±0.01 mg/gDE), quercitrin (2.22±0.12 mg/gDE), and neohesperidin (2.30±0.14 mg/gDE), and nine phenolic acids (total amount, 60.31±4.52 mg/gDE), including gallic acid (0.25±0.01 mg/gDE), gentisic acid (32.14±2.72 mg/gDE), chlorogenic acid (2.32±0.08 mg/gDE), vanillic acid (0.60±0.04 mg/gDE), p-coumaric acid (0.17±0.01 mg/gDE), ferulic acid (0.25±0.37 mg/gDE), syringic acid (0.88±0.04 mg/gDE), and p-anisic acid (1.06±0.05 mg/gDE), were identified in the LFE. In addition, proanthocyanidin A2 (the major proanthocyanidin) was present in the extract at a level of 79.31±2.95 mg/gDE. The LFE used in this study was extracted from the same one replicate.

Cell culture and virus: The SIRC rabbit corneal epithelia cell (ATCC® CCL-60™) were harvested in DMEM culture.
media (Hyclone, Logan, UT). Culture media were supplemented with FBS, 10% of the final volume (Hyclone), 100,000 U/l penicillin, and 10 ml/l streptomycin (Hyclone). Porcine kidney 15 (PK-15) cells were maintained in DMEM supplemented with 10% FBS. The stock of HSV-1 used in this study was amplified from the PK-15 cells, and the titer was determined with a standard plaque assay in the PK-15 cells. The cells were maintained at 37 °C in 5% CO₂ in a humidified atmosphere. Only SIRC cells between the third and sixth passages were used for all experiments. Cells at the higher passage indicated altered morphology compared to the ones at the third and sixth passages, and this might affect the response to treatment.

**Determination of cell viability:** The SIRC cells were pretreated with DMSO (0.1%) or various concentrations (0.2, 0.1, or 0.05 μg/ml) of LFE for 1.5 h and infected with 1 MOI of HSV-1 for 24 h. The cells were centrifuged at 13,000 ×g for 3 min at room temperature and the medium carefully removed from each well. Cell viability was determined with the Cell Counting Kit-8 (CCK-8, Donjindo Molecular Technologies, #CK04–11).

**Western blotting analysis:** The SIRC cells were pretreated with LFE (0.2, 0.1, or 0.05 μg/ml) for 1.5 h and then infected with 1 MOI of HSV-1 for 24 h. The infected cells were washed with PBS and lysed in a lysis buffer [50 mM Tris-HCl (pH7.5), 150 mM NaCl, 1% Nonidet P40, 2 mM EDTA (EDTA), 1 mM NaVO₃, 10 mM NaF, 1 mM dithiothreitol (DTT), 1 mM

![Figure 3](image-url) Figure 3. The effects of LFE in the expression of phosphorylated mTOR and p70s6k in HSV-1 infected SIRC cells. The cells pretreated with 0.05, 0.1 or 0.2 μg/ml of LFE for 1.5 h and then infected with HSV-1 at 1 MOI for 24 h. The protein levels were normalized against the β-actin signal. Bars are means ± standard error of the mean (SEM). A, B, C, D: bars with different letters differed (p<0.05).
phenylmethylsulfoney fluoride (PMSF), 25 μg/ml leupeptin] and kept on ice for 30 min. Cell lysates were centrifuged at 13,000 × g at 4 °C for 15 min, and the supernatants were stored at −80 °C until the analyses were performed. Protein concentrations were measured using a protein assay kit (Bio-Rad, Laboratories, Inc., Hercules, CA). Twenty micrograms of whole cell lysate proteins or nuclear lysate protein were resolved in the loading buffer, and then they were subjected to electrophoresis in 10% (V/V) polyacrylamide/sodium dodecyl sulfate (SDS) gels. The resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked in Tris-buffered saline (TBS)-Tween-20 solution containing 5% non-fat dry milk and incubated overnight at 4 °C with specific antibodies against pmTOR, pp70s6k, Beclin-1, LC3-I/LC3-II, or β-actin (Cell Signaling Technology, Billerica, MA). Proteins were visualized using goat anti-rabbit or mouse antibody conjugated to horseradish peroxidase and a chemiluminescence western blotting detection system (ECL Plus™ Western Blotting Reagents, Amersham Biosciences, Boston, MA). The protein band densities were quantified using Alpha Imager 2200 software (Alpha Innotech Co., San Leandro, CA).

Statistical analysis: The values are expressed as the mean ± standard deviation (SD). The significance of the difference from the respective controls for each experimental test condition was analyzed by using a Student t test for each paired experiment. A p value of less than 0.05 was regarded as a statistically significant difference.

Figure 4. LFE up-regulates Beclin-1, LC3-I and LC3-II expression in HSV-1 infected SIRC cells. Cells pretreated with 0.05, 0.1 or 0.2 μg/ml of LFE for 1.5 h then infected with HSV-1 at 1 MOI for 24 h. The protein levels were normalized against the β-actin signal. Bars are means ± standard error of the mean (SEM). A, B, C, D: bars with different letters differed (p<0.05).
RESULTS

Cell viability of HSV-1-infected SIRC cells: The cell viability of the HSV-1-infected SIRC cells was observed and determined with the Cell Counting Kit-8 after HSV-1 infection at 0.1, 1, or 10 MOI (Figure 1). The morphology of the SIRC cells infected with HSV-1 (0.1, 1, 10 MOI; Figure 1B–D) showed a round and swelling shape (Figure 1A). Additionally, the cell viability of the SIRC cells in the HSV-1-infected groups was significantly higher than those in the control group (p<0.05; Figure 1E). As shown in Figure 1E, 1 MOI of HSV-1 contained the highest cell viability and had no toxic effect on the SIRC cells. Thus, the 1 MOI of HSV-1 was used in subsequent experiments.

Effect of LFE on cell viability in HSV-1-infected SIRC cells: To study whether LFE causes toxicity in HSV-1-infected SIRC cells, the cell viability was tested at various concentrations of LFE in HSV-1-infected SIRC cells with the Cell Counting Kit-8 (Figure 2). As shown in Figure 2, the cell viability was significantly enhanced in the HSV-1 infection when compared with the negative control (mock). However,

Figure 5. The inhibition of LFE on HSV-1-infected cells was evaluated with a standard plaque assay in PK-15 cells. A: The number of plaques in the Lychee flower extract (LFE) group was lower than those in the dimethyl sulfoxide (DMSO) group. B: The titer declined in the LFE group. Bars are mean ± standard error of the mean (SEM). * p<0.05 when compared with the DMSO group.
Effects of LFE on the expression of pmTOR and pp70s6k in HSV-1-infected SIRC cells: The mammalian target of rapamycin (mTOR) is a serine-threonine kinase that plays an important role in the regulation of cell proliferation and protein synthesis through the activation of its downstream target ribosomal p70s6 kinase (p70S6K). The phosphorylation of cell proliferation–related proteins, mTOR (pmTOR) or p70s6k (pp70s6k) were parameters to evaluate the effects of LFE on HSV-1-infected SIRC cells (Figure 3). The cells were pretreated with LFE and then infected with HSV-1 for 24 h. The protein levels were normalized against β-actin. After the HSV-1 infection, the levels of phosphorylated mTOR and p70S6K were increased in the SIRC cells (Figure 3A). The expression of pmTOR in all LFE supplemental groups was significantly lower than that in the HSV-1-infected group (HSV-1-infected cells without the LFE treatment; p<0.05; Figure 3B). However, the expression of pp70s6k in the 0.1 or 0.2 μg/ml HSV-1-infected LFE group was significantly lower than that of the HSV-1-infected group (p<0.05; Figure 3C). These results demonstrated that the LFE treatment partially decreased the phosphorylation of mTOR and p70S6K. The effect of LFE on the cell proliferation in SIRC cells was further studied.

Effects of LFE on Beclin-1, LC3-I, or LC3-II protein expression in HSV-1-infected SIRC cells: In view of the ability of LFE to attenuate the cell proliferation of induced HSV-1, we investigated the effects of LFE on autophagy-related proteins, light chain 3 (LC3), and Beclin-1. Protein expression of Beclin-1 or LC3 was analyzed to evaluate the effects of LFE on HSV-1-infected SIRC cells (Figure 4). The expression of Beclin-1 in the LFE groups was significantly higher than that in the HSV-1-infected group (p<0.05; Figure 4B). In addition, the expression of LC3-II in the 0.1 or 0.2 μg/ml LFE group was higher than that in the HSV-1-infected group (p<0.05; Figure 4C).

LFE inhibited HSV-1 replication in SIRC cells: To identify the antiviral activity of LFE, we infected the SIRC cells with HSV-1 in the presence or absence of LFE and measured the viral titers with standard plaque assays in PK-15 cells. Virus yield in the presence of LFE was reduced two- to threefold compared to that of the HSV-1 infection group (DMSO; Figure 5A,B). The results demonstrated that LFE inhibited HSV-1 replication in the SIRC cells.

DISCUSSION

HSV is one of the most common viruses found in humans. Reactivation of HSV is commonly observed in humans with hypoimmunity. In the present study, the results indicate that the rabbit corneal epithelial cells (SIRC cells) can be used successfully to set up a screening platform of HSV-1 infection in the cornea as an in vitro model (Figure 1 and Figure 2).

There are reported uses of natural extracts to treat viral infections; results showed that natural extracts could be considered a potential anti-inflammatory agent for DNA virus infections [19,20]. In addition, a previous study showed that LFEs modulate inflammatory processes through the inactivation of nuclear factor kappabeta (NF-κB), extracellular-signal-regulated kinase (ERK), and Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway [14]. The Lychee flower should have the potential to be exploited as a functional food for anti-inflammation activity.

In a standard plaque assay in PK-15 cells, the replication of HSV-1 can be inhibited by an LFE supplement (Figure 1). Thus far, there is no effective treatment for viral infections in the cornea. In this study, rabbit corneal epithelial cells were infected with HSV-1 to result in increasing cellular proliferation (Figure 2). HSV-1 infects host cells to produce more viruses. However, Figure 3 shows that cell viability declined in the LFE treatment. The results indicate that the proliferation of HSV-1-infected cells was inhibited by the LFE.

The mTOR and p70S6K are important downstream targets of Akt/protein kinase B (Akt/PKB), controlling cellular activities such as cell proliferation, protein synthesis, etc. The mTOR is activated by several stimuli such as growth factors, nutrients, and stress [21]. As a central element signaling cell growth and enhancing protein translation, the mTOR, when inhibited, induces autophagy. Moreover, as a critical feedback mechanism, reactivation of mTOR terminates autophagy and initiated lysosome reformation [22]. Autophagy can be induced by a virus infection to result in cell degeneration. Autophagy is also an important mechanism for maintaining cell viability in times of starvation, stress, or infection [23]. McFarlane et al. [23] reported when human fetal foreskin fibroblasts were infected with HSV-1, autophagy could be induced by the presence of foreign DNA within cells.

Beclin-1 was among the first mammalian autophagy effectors [24]. Through its interaction with class III phosphatidylinositol-3-phosphate kinase (PI3KIII/Vps34), Beclin-1 regulates autophagy. A recent study suggested that in later stages of HSV-1 infection, autophagy or an autophagy-like...
process may function in producing processed HSV-1 antigens for presentation on major histocompatibility complex I (MHC I) [25]. The α-herpesvirus HSV-1 encodes the ICP34.5 protein that plays a major role in HSV-1 neuropathogenesis in part through antagonizing autophagy by directly binding to Beclin-1 [26]. This report suggesting that the ICP34.5-mediated inhibition of the host cell protein translation shutdown may be more significant than inhibition of autophagy in the regulation of HSV-1 growth in some cultured cells [27]. In contrast to LC3, a marker of final autophagosome formation, Beclin-1 participates in the early stages of autophagy, promoting the nucleation of the autophagic vesicle and recruiting proteins from the cytosol [28].

In this study, we found that HSV-1 decreased Beclin-1 and LC3-II expression (Figure 4). Results demonstrate that HSV-1 regulates autophagy in SIRC cells. Additionally, the study showed that pretreatments of LFE caused the induction of Beclin-1 and LC3-II via inhibition of mTOR and p70s6k phosphorylation, both of which are involved in viral replication.

Autophagy is also involved in host immunity against pathogen infection [29]. Furthermore, autophagy enhances the presentation of viral antigens by dendritic cells during the infection of Sendai and vesicular stomatitis viruses [30].

In summary, our study demonstrated that the LFE could be beneficial in the prevention of viral infectious diseases and is potentially an antivirus replication candidate for virus infection. As most of the current antiviral therapies are initiated after the appearance of keratitis, and drugs often cannot prevent damage to the underlying epithelial tissue [5], pretreating a patient with a high risk of HSV reactivation (i.e., trauma, emotional stress, immunocompromised) with LFE or perhaps applying LFE as an additional therapy to existing antiviral therapy may be clinically useful.

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