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Original Article

Establishing cell model of herpes simplex virus type 2 latent infection and reactivation in SH-SY5Y cells

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Abstract: Objective: To establish cell model of herpes simplex virus type 2 latent infection and reactivation in SH-SY5Y cells. Methods: Effects of ACV on SH-SY5Y cells were observed after cells were treated with ACV concentrations of 20, 40, 60, 80, 100, 120, or 140 μmol/L, and also cells morphology was observed under phase contrast microscope after HSV-2 was inoculated into SH-SY5Y cells using MOI of 0.1, 1, 10 and 100. The optimum condition of temperature and heating duration were approached using temperature of 41°C, 42°C, 43°C, 44°C, 45°C and heating duration of 0.5 h, 1.0 h, 1.5 h, 2.0 h, 2.5 h to induce HSV-2 reactivation. The optimum concentration of Forskolin was also decided using 25, 50, 75, 100, 125 μmoL/L to reactivate the virus from latency. The viral gG gene and the LAT gene were used to verify HSV-2 latent infection and reactivation in cells by PCR and sequencing. The gG gene was quantitative detected by real-time PCR on the optimum state of latent infection and reactivation. The morphological changes were observed when the virus was reactivated from latency. Results: 60 μmoL/L ACV was the optimum concentration to establish latent state in SH-SY5Y cells. Virus titers between 1~10 MOI were the suitable infective dose of HSV-2 to establish cell model from latency to reactivation. In our research, the time of virus latency in SH-SY5Y cells could reach up to 14 d. Heat stress of 43°C, 1.5 h or Forskolin of 75 μmoL/L was the optimum condition to induce virus reactivation. Individual cells showed pathological changes after reactivation for 24 h and expanded after 48 h, as some cells fused into multinuclear giants with chromosome near the edge of the nuclear envelopes or broken nuclei. Viral LAT gene had expressed in SH-SY5Y cells latently infected with HSV-2 for 4, 6, or 8 d, while the gG gene had not expressed at all. Both LAT and gG genes expressed at 12, 24, and 36 h after heat- or chemical-induced reactivation. The gG gene expression after reactivation was 9.68 times higher than that in the latent period after normalizing to GAPDH expression. Conclusions: The cell model system of HSV-2 latent infection and reactivation in SH-SY5Y cells was established. The research provided usefulness for study on HSV-2 of latency, reactivation and pathogenic mechanism.

Keywords: HSV-2, SH-SY5Y cell, latency, reactivation

Introduction

Herpes simplex virus type 2 (HSV-2) is a common viral pathogen in humans. The HSV-2 genome is a linear double-stranded DNA molecule of approximately 152 kb divided into a long segment (L) and short segment (S) that comprise 82% and 18% of the viral DNA. Each gene consists of a unique sequence (U) in the middle and inverted repeat sequences on both ends, with GC accounting for 69%. The HSV-2 has 80 genes with 94 ORFs, encoding at least 84 distinct proteins [1, 2]. We established a HSV-2 latent infection and reactivation cell model in cultured human SH-SY5Y neuroblastoma cells in order to study the mechanisms of HSV-2 pathogenesis.

Materials and methods

Cell culture and viral propagation

Culture media, reagents, and major equipment: The 1640 medium for culture of SH-SY5Y human neuroblastoma cells was from Gibco (America) and fetal calf serum (FCS) from Hyclone (America). Other drugs used included trypsin (Gibco), acyclovir (ACV, Suppler) and Forskolin (Sigma, America). The SH-SY5Y human neuroblastoma cell line was maintai-

ned in a cell culture incubator (Shellab) and observed under an inverted fluorescence microscope with phase contrast optics (Nikon TE2000-E, Nikon, Japan).

Virus: The HSV-2 333 virus was supplied by the Institute of Virology, Chinese Academy of Preventive Medicine. Methods for virus anabiosis, inoculation into single-layered Vero cells, multiplication and virulence tests have been described previously [3].

SH-SY5Y human neuroblastoma cell culture: The SH-SY5Y cell line was purchased from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. Cells were cultured in 1640 medium supplemented with 10% fetal calf serum under a 5% CO_2 atmosphere. The medium was changed every two days until cells confluence rate reached 80%, when cells were split and subcultured.

Establishment of HSV-2 latent infection in cultured SH-SY5Y cells

The effect of the antiviral drug ACV on cultured SH-SY5Y cells: The culture solution was removed and cells were treated with 1-3 mL 0.25% trypsin for 2-5 min at 37°C, until cells attached to the culture flask became round and detached, observed with microscope. The same volume of 10% FCS RPMI1640 was added, and flasks were centrifuged at 500-1000 rpm for 1-2 min. Supernatant fluid was removed, 10% FCS RPMI1640 were added. Cells were made suspending by pipetting, with a density of $1.0\times10^5/cm^2$. Suspension was transferred to each of 24 wells (2 cm^2/well), with 1 mL in each. Supernatant fluid was removed when cells had attached to orifice after 24 h. 10% FCS RPMI1640 was added, accompanied by 100 μL of solution of ACV at 20, 40, 60, 80, 100, 120, and 140 μmol/L. Supernatant fluid was removed the next day. Each orifice was treated with 5 MOI 100 μL virus solution for 1 h at 37°C. Then RPMI 1640 plus 2% FCS was added and cells were incubated under 5% CO_2 at 37°C. Cells were observed at different time points. Viral particles were released into the cell medium after repeated freezing-thawing. The media containing virus particles was centrifuged at 10,000 rpm/min for 10 min at 4°C. The supernatant was collected and the tissue culture infective dose ($TCID_{50}$) of viral particles was calculated by the Reed-Munch formula.

The effect of different multiplicity of infection (MOI) levels on HSV-2 latency and reactivation in SH-SY5Y cells: The SH-SY5Y cells density was adjusted to approximately $1.0\times10^5/cm^2$ and cells were transferred to 24-well plates (2 cm^2/well), with 1 mL in each well. Supernatant was removed after 24 h incubation when cells became attached to flasks, and new RPMI 1640 medium containing 10% FCS was added with 100 μL of 60 μmol/L ACV (the optimal dose determined by dose response experiments described in Section 1.2.1). Supernatant was removed the next day. The cells in each well were then treated with 100 μL viral solution at 0.1, 1, 10 or 100 MOI for 1 h at 37°C. RPMI 1640 with 2.0% FCS was added, and cells were incubated under 5% CO_2 at 37°C. The cells were induced for 1.5 h at 43°C (the optimal heat induction protocol determined by induced response experiments described in Section 1.3.1) after 10 days incubation, the supernatant was collected at different time points and the viral $TCID_{50}$ was calculated by the Reed-Munch formula.

Morphology of SH-SY5Y cells infected by HSV-2 at latency: The SH-SY5Y cells density was adjusted to approximately $1.0\times10^5/cm^2$ and cells were transferred to each of 24 wells (2 cm^2/well), with 1 mL in each. After 24 h incubation until cell became attached, supernatant was replaced by new RPMI 1640 medium containing 10% FCS with 100 μL of 60 μmol/L ACV. Supernatant was removed the next day. The cells in each well were then treated with 100 μL viral solution with 5 MOI (the optimal MOI determined by dose response experiments described in Section 1.2.2) for 1 h at 37°C. RPMI 1640 with 2.0% FCS was added, and cells were incubated under 5% CO_2 at 37°C. The shapes of infected cells were observed under phase contrast microscope at different time points during exposure to the virus and ACV.

Induced reactivation of latent HSV-2 in SH-SY5Y cells

Heat induced reactivation of HSV-2 in SH-SY5Y cells: To observe the induction effect of different temperatures and durations, cells latently infected by HSV-2 for 8 days were placed in a temperature controlled water bath at 41°C, 42°C, 43°C, 44°C, or 45°C for 0.5 h, 1.0 h, 1.5 h, 2.0 h, or 2.5 h respectively. The supernatant was collected at different time points to calculate viral $TCID_{50}$ by the Reed-Munch formula.

Forskolin induced HSV-2 reactivation in SH-SY5Y cells: Cells latently infected by HSV-2 for 8 days were treated with 100 μL Forskolin at 25, 50, 75, 100, or 125 μmol/L for 1.0 h. The supernatant was collected at different time points and the viral $TCID_{50}$ of the supernatant was determined by the Reed-Munch formula. The shapes of infected cells were observed under phase contrast microscopy at different dose induced by forskolin.

Verification of latency and reactivation of HSV-2 in SH-SY5Y cells

The viral LAT gene and the gG gene of HSV-2 in cultured SH-SY5Y cells, latently infected for 4, 6, or 8 d, were detected by RT-PCR. Cells latently infected for 8 days were subjected to 43°C heating for 1.5 h and viral LAT gene and gG gene expression in harvested media were then detected by RT-PCR on 12 h, 24 h, and 36 h later. The PCR primers included: HSV-LAT gene was 5'-GCCAGACGTGCGTGCTCTGCACGAT (upstream primer) and 5'-TGTTGGTC TTTATC-ATAGAACAGAG (downstream primer), located in nucleotides 121, 343~121, 492 (the length of the amplified band was 150 bp); the gG gene primers was 5'-GACCCAAAGACGCACCC-ACA (upstream primer) and 5'-CCAAGGCGAC-CAGACAAACG (downstream primer), located in 139, 478~141, 988 (the length of the amplified band was 412 bp). The volume of PCR reaction system was 50 μL, consisting of 2 μL sample supernatant, 10 μL 5×PCR buffer (including Mg^{2+}), 4 μL dNTP mixture (at 2.5 mmol/L respectively), 0.5 μL DNA polymerase, 20 μmol/L upstream and downstream primers (1 μL respectively) and 31.5 μL ddH_2O. Reaction conditions were 94°C for 10 min of initial denaturation, followed by 30 main cycles at 94°C for 50 s, 65°C for 15 s, 72°C for 90 s, and a final extension at 72°C for 7 min. The amplicons from this reaction were detected and purified on a 2% agarose gels using electrophoresis. The sequences of the purified amplicons were assayed, whose result was then compared for BLAST homogenesis with Genbank.

Verification with Real-time PCR of latency and reactivation of the virus

Cells latently infected for 8 days were subjected to 43°C heating for 1.5 h (the optimal heat induction protocol determined experimentally) and viral gG gene expression in harvested media was then detected by real time RT-PCR at 12 h later. The GAPDH gene was used as the internal reference standard. cDNA was reverse transcribed and then viral gG gene expression was assessed by real time RT-PCR. The reaction conditions were 95°C for 10 sec; followed by 45 cycles of 95°C for 5 s and 60°C for 20 s. As the reaction ended, a standard curve of the Ct value from the GAPDH and target gene amplification was plotted in order to quantify sample mRNA.

Results

Effects of different ACV concentrations on SH-SY5Y cells

Cultured SH-SY5Y cells were treated with ACV concentrations of 20, 40, 60, 80, 100, 120, or 140 μmol/L. ACV with concentration lower than 60 μmol/L had no effect on cell morphology (**Figure 1A**, **1B**), whereas ACV with concentration higher than 80 μmol/L exhibited significant influence on appearance of cells, including cytolysis of membrane, rounding, plasmolysis and even cellular death (**Figure 1C-E**). Cells incubated in different concentrations of ACV over night were treated with 5 MOI virus. Change of virus titer versus time is shown (**Figure 2A**). The virus titer at ACV concentrations of 20, 30 or 40 μmol/L after inoculation for 4, 5, or 6 d respectively increased significantly, indicating cells had undergone acute cytolysis. Concentration of ACV higher than 50 μmol/L prevented reactivation and $TCID_{50}$ value was lower than 2.0, demonstrating that the virus remained latent. Cell morphology indicated mild cytolysis at 70 μmol/L ACV until the 8^{th} day inoculation. Therefore, ACV concentrations of 50-60 μmol/L were used to establish a HSV-2 latent state in SH-SY5Y cells. In the experiment, we chose 60 μmol/L ACV to perform all the following experiments.

Effects of different MOI levels on HSV-2 latency and reactivation in SH-SY5Y cells

Cultured SH-SY5Y cells in 24-well plates were incubated with 60 μmol/L ACV over night and then treated with 0.1, 1, 10, or 100 MOI virus solution. The change in media virus titer is shown (**Figure 2B**). In the presence of 100 MOI, the virus titer rose gradually from the 2^{nd} day and acute cytolysis had been observed by the 6^{th} day. By contrast, virus titer did not change in cells inoculated with 0.1 MOI after induction at 43°C for 1.5 h on the 10^{th} day. Heat induction of cultures inoculated with 1 or 10 MOI virus

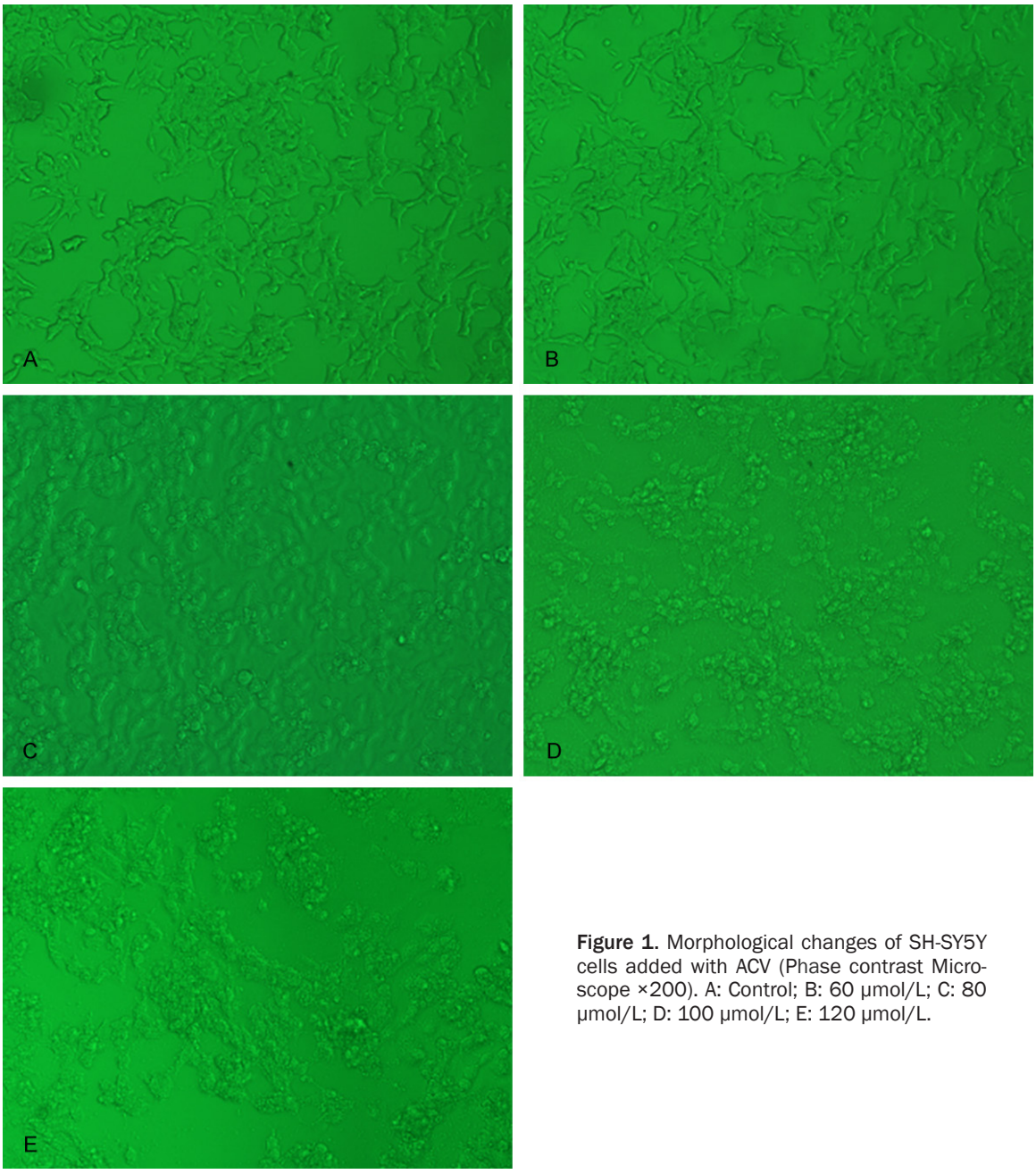

Figure 1. Morphological changes of SH-SY5Y cells added with ACV (Phase contrast Microscope ×200). A: Control; B: 60 μmol/L; C: 80 μmol/L; D: 100 μmol/L; E: 120 μmol/L.

caused significant rise in titer, indicating reactivation of latent HSV infection and acute cytolysis. As a result, virus titers between 1-10 MOI were used to construct the reactivation model. We chose 5 MOI as the infection level for all the following experiments.

Effects of the SH-SY5Y cell morphology during latent infection

Cultured SH-SY5Y cell remained in the latent state from the beginning of virus inoculate to the 14th day. **Figure 3** shows the cells shape after inoculation with 5 MOI virus titer on the 0, 6, 8, 10, 12, and 14 d. After the 14th d, most cells exhibited cytolysis, indicating that the longest latency period after infection with 5 MOI of HSV-2 was 14 days in SH-SY5Y cells.

Heat-induced reactivation of HSV-2 infection in cultured SH-SY5Y cells

Effects of heating duration on reactivation: The induction of HSV-2 reactivation was highly sensitive to heating duration. Cells latently infected for 8 d of HSV-2 were treated with

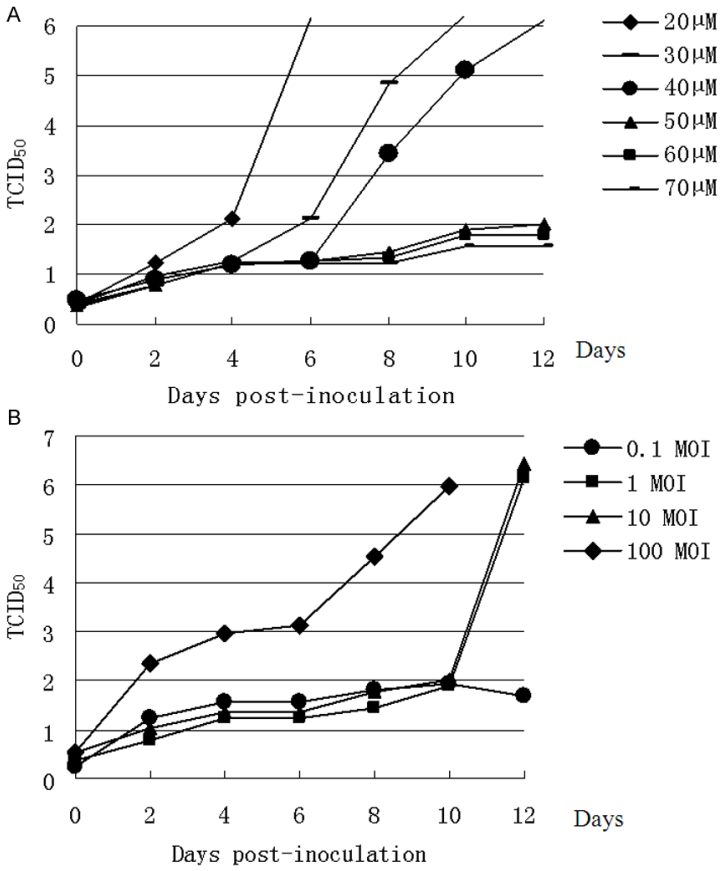

Figure 2. The effect of ACV and MOI on titers of HSV-2 in SH-SY5Y cell cultivation. A: The effect of ACV; B: The effect of MOI.

water bath at 43°C for different time (**Figure 4A**). For 0.5 or 2.5 h, there was no significant rise in virus titer, while for 2.5 h, most cells died. Heating duration of 1.0, 1.5, and 2.0 h appeared to reactivate virus, especially 1.5 h, which exhibited the most significant effect. So heating duration of 1.5 h was used for inducing reactivation.

Effects of heating temperature on reactivation: Cells latently infected for 8 d of HSV-2 were heated in a temperature-controlled water bath (**Figure 4B**). At 41°C or 45°C, there was no significant rise in virus titer, while at 45°C, most cells died. Temperatures of 42, 43 and 44°C appeared to induce reactivation, and both 42°C and 43°C have a better induce effect. So temperature of 43°C was used for inducing reactivation.

Induction of HSV-2 reactivation by forskolin

Cells latently infected by HSV-2 for 8 d were treated with forskolin of different concentrations (**Figure 4C**). Forskolin at 50-100 μmol/L significantly induced HSV-2 reactivation in SH-SY5Y cells, while 25 and 125 μmol/L had no obvious effect. We determined that 75 μmol/L was the optimal concentration for induction. **Figure 4C** also reveals that reactivation by forskolin was approximately 12-24 h slower than by heating.

Observation of the SH-SY5Y cell morphology during reactivation

Control SH-SY5Y cell cultures observed under phase contrast microscope exhibited typical monolayer growth with polygonal or fusiform somata and long, highly branched neurites (**Figure 5A**). Nuclei were round or oval and 1-2 nucleoli were clearly identified. There were binucleate cells, with abundant cytoplasm, and cells undergoing mitosis. Individual cells showed pathological changes after reactivation or 24 h: cells gradually rounded and swelled, nuclei also swelled, the nuclear membrane translocated and cells became dark under phase contrast microscopy (**Figure 5B**). The range of pathological changes expanded after 48 h of reactivation, as some cells fused into multinuclear giants with chromosome near the edge of the nuclear envelopes or broken nuclei (**Figure 5C**). Most cells detached from the substrate within 48 h and the multinuclear giant cells lysed after 72 h (**Figure 5D**).

Verification of HSV-2 latency and reactivation in SH-SY5Y cells

The supernatants from SH-SY5Y cells latently infected with HSV-2 for 4, 6, or 8 d were examined by PCR. Viral gene amplification revealed LAT gene had expressed in all the samples, while the gG gene had not expressed at all, indicating that the viruses were in the latent state (**Figure 6A**). Both LAT and gG genes expressed at 12, 24, and 36 h after heat- or chemical-induced reactivation (**Figure 6B**), evidence of

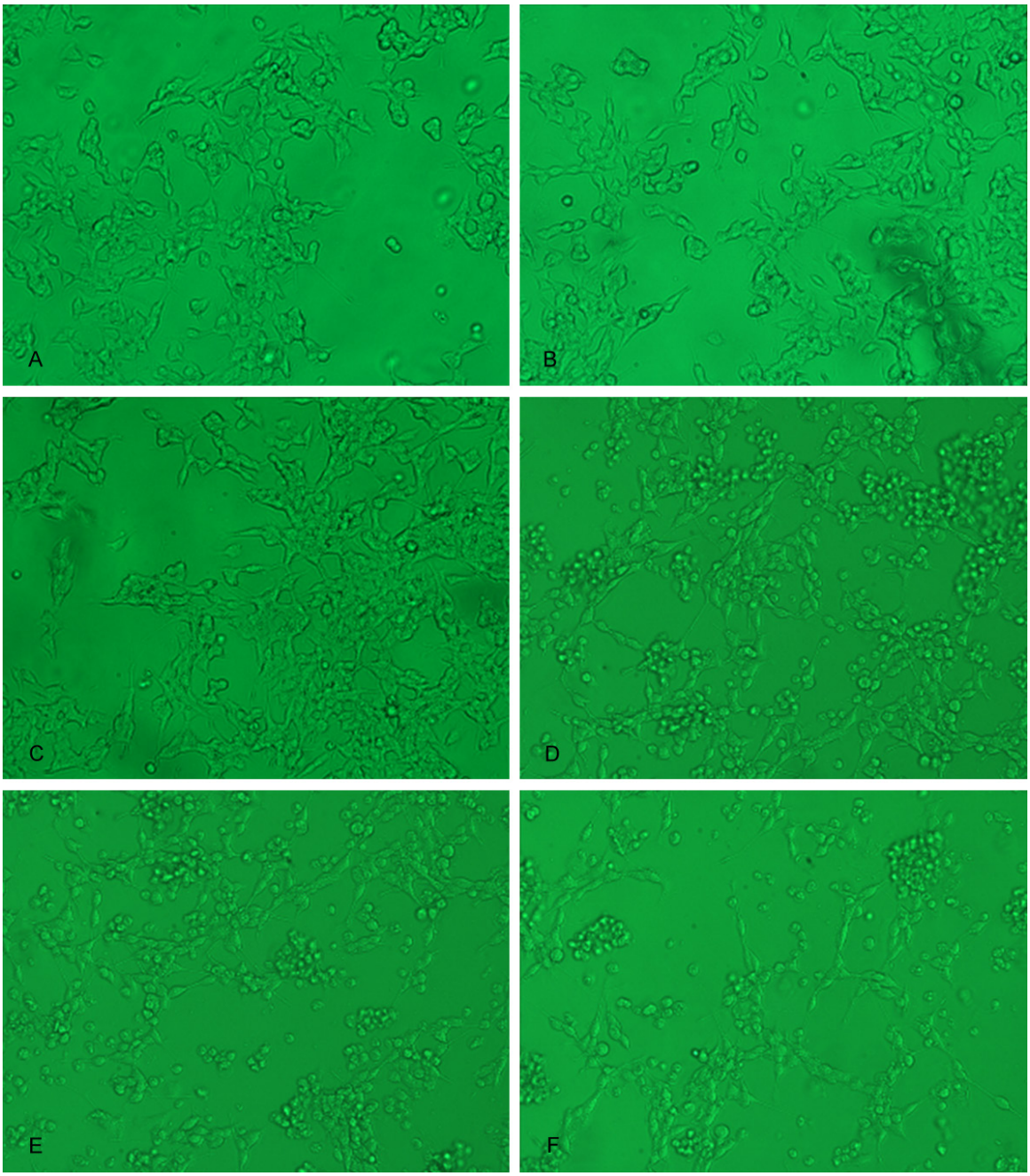

Figure 3. Morphological observation of SH-SY5Y cells latent infected with HSV-2 (Phase contrast Microscope ×200). A: 0 d; B: 6 d; C: 8 d; D: 10 d; E: 12 d; F: 14 d.

massive viral genome replication. Sequencing of PCR amplicons and BLAST searching in Genbank indicated that the amplified sequences were identical to the LAT and gG genes of HSV-2.

Verification of real-time PCR result

The expression of gG relative to GAPDH (the internal control) as revealed by real-time PCR is shown in **Tables 1** and **2**. The correction coefficient of GAPDH was 1.09 after relative quantification (**Table 1**). The gG gene expression after reactivation was 10.55 times higher than that in the latent period (**Table 2**) and 9.68 times higher after normalizing to GAPDH expression.

Discussion

The lack of a widely accepted cell model of HSV-2 latent infection has hampered research into the mechanisms of latency and reactiva-

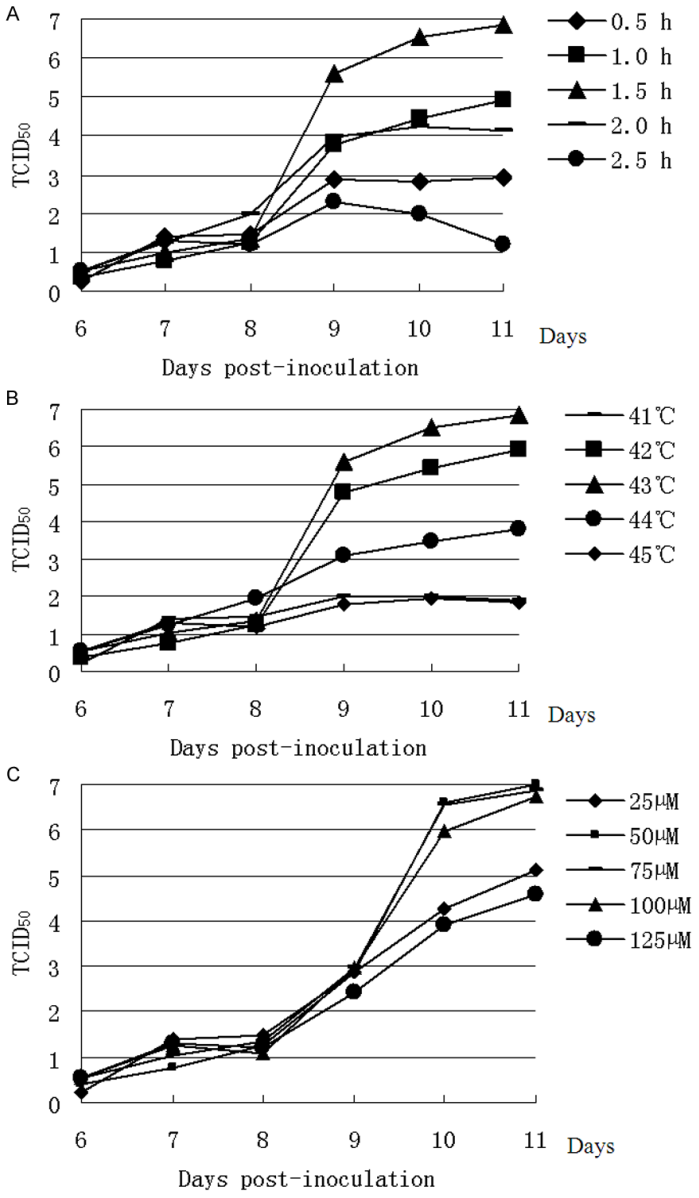

Figure 4. The effect of heating duration, temperature and Forskolin on titers of HSV-2 in SH-SY5Y cell cultivation. A: The effect of heating duration; B: The effect of temperature; C: The effect of Forskolin.

tion of HSV-2. While animal models have been employed to study the mechanism of HSV-2 latent infection and reactivation [4-9], there are several disadvantages. First, animals may have subclinical infection of other virus or bacteria, which can interfere with HSV-2 infection and leads to false positive. Moreover, animals acquired immunity through subclinical infection will be resistant to virus. However, immunity of isolated (non-immune) cells is not enhanced and thus experiments can be conducted on cultured cells in vitro. Second, expression of latency and reactivation varies among different species and is difficult to observe. Third, virus can infect only a limited number of neurons through injection on animals [7-9]. By contrast, cell model has several advantages: cell lines are highly available and analysis can be precisely quantified, so that it is easy to choose the most sensitive cells according to objective of the experiment. Moreover, cell model facilitates the study of virus reproduction in a single cell type and the relationship between virus and cell. Whereas inoculation effect on animal model is influenced by age, dosage and route of inoculation, cells can not only be replicated, but can also be cultured for a longer period of time. SH-SY5Y, which belongs to human neuroblastoma cell line SK-N-SH, has many characteristics of sympathetic ganglion and can be induced into neuron-like cells. The genome of SH-SY5Y cells is similar to that of human cells and has been widely used in researches of functional genomics and gene expression [10-13]. Overall, SH-SY5Y cell is a suitable human nerve cell model for HSV-2 latent infection and reactivation studies.

The latency-associated transcripts (LATs) are unique HSV genes that are mass expressed during latent infection of HSV and playing key roles in latent infection, maintenance and reactivation of HSV. Hence, it has become a hotspot to study HSV with LATs, both at home and abroad. Latency-associated transcripts, which locate in long repeated sequence of viral genome, include chief LATs (2.2 kb, 2.0 kb, 1.5 kb and 1.45 kb isoforms) and non-chief LATs (9.0 kb and 8.3 kb isoforms). These gene products gather in the host nucleus and cytoplasm during latent infection and in the cytoplasm during productive infection and latent reactivation [14, 15]. Experiments have con-

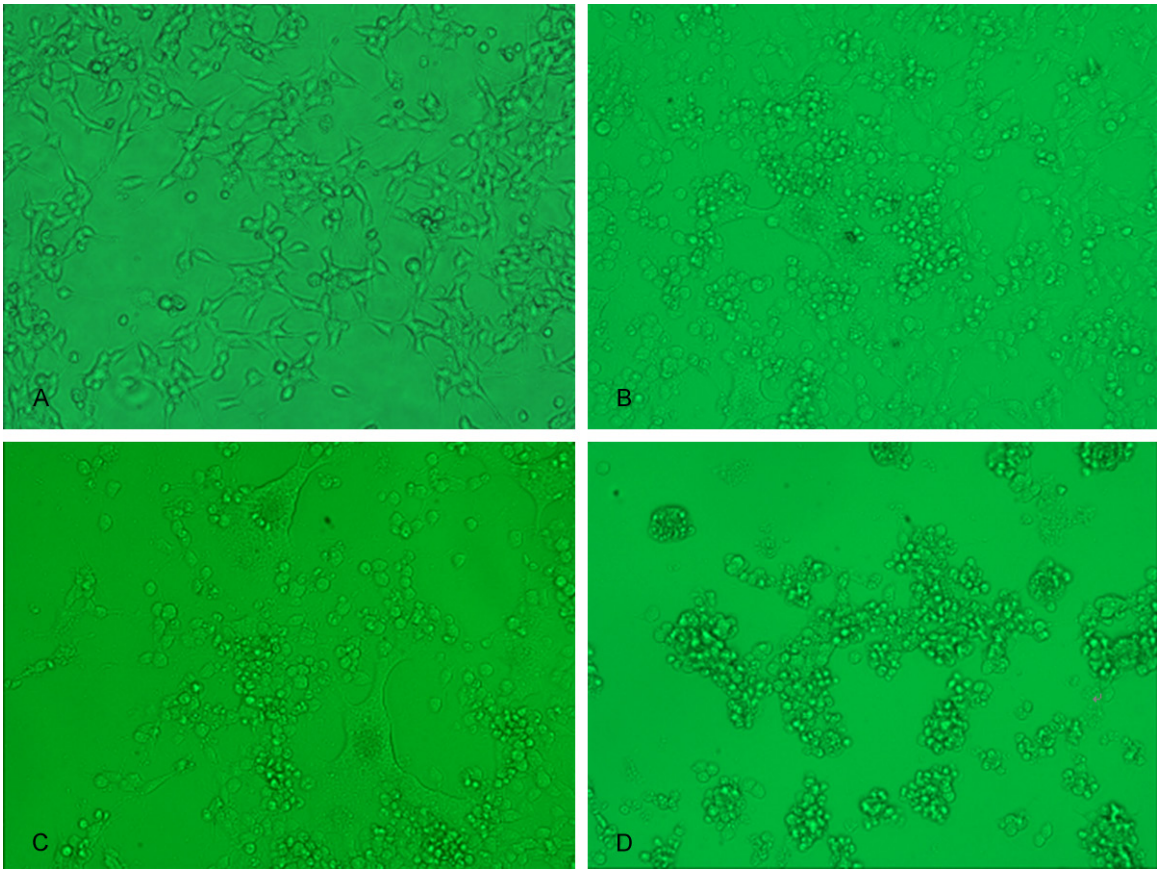

Figure 5. Morphological changes of SH-SY5Y cells latent infected and reactivated by HSV-2 (Phase contrast Microscope×200). A: Control; B: 24 h post-reactivation; C: 48 h post-reactivation; D: 72 h post-reactivation.

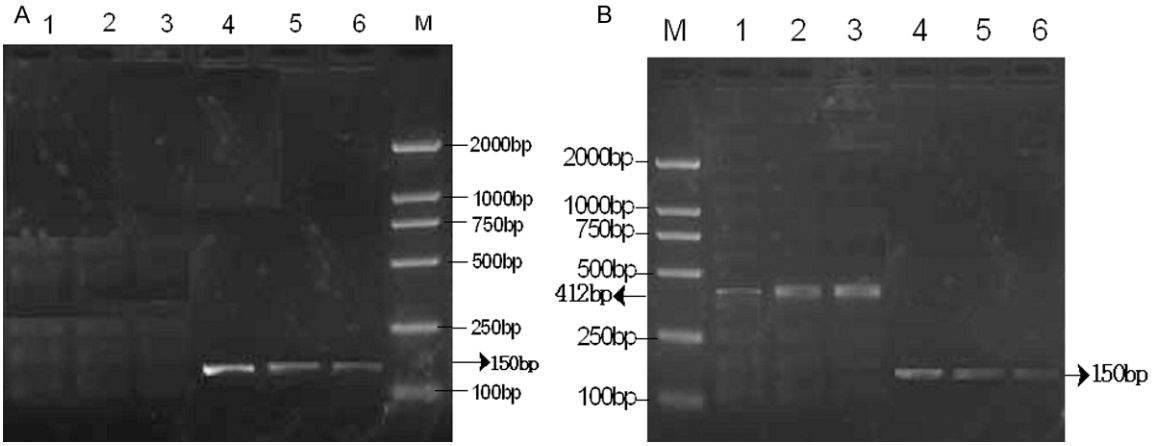

Figure 6. Electrophoresis of PCR products of HSV-2 LAT and gG gene. M: MDL2000 standard DNA ladder; A (latency): 1, 2, 3. gG gene on 4, 6, 8 d post latent infection; 4, 5, 6 LAT gene on 8, 6, 4 d post latent infection; B (reactivation): 1, 2, 3 gG gene on 12, 24, 36 h post reactivation; 4, 5, 6 LAT gene on 12, 24, 36 h post reactivation.

cluded that there are two HSV-2 LATs, the 9.0 kb non-chief LAT and the 2.2 kb chief LAT, which cannot be cut into smaller segments. So the 2.2 kb LAT is a marker of the latent state [16-18]. Kesari et al. [19] have summarized HSV-2 characters in the latency phase: First, HSV-2 can persist in the host while the host lacks of clinical features. Second, the virus can

Table 1. Date result of real time RT-PCR on GAPDH

Well	Sample Type	Repl	Ct	SQ
A01	Standard	1	19.39	1000.00
B01	Standard	2	22.18	100.00
C01	Standard	3	27.01	10.00
D01	Standard	4	31.83	1.00
E01	SAMPLE	A (latency)	20.45	402.02
F01	SAMPLE	B (reactivation)	20.30	436.54 (+1.09)

Table 2. Date result of real time RT-PCR on gG

Well	Sample Type	Repl	Ct	SQ
A02	Standard	1	22.07	1000.00
B02	Standard	2	25.25	100.00
C02	Standard	3	28.69	10.00
D02	Standard	4	32.37	1.00
E02	SAMPLE	A (latency)	30.47	3.30
F02	SAMPLE	B (reactivation)	26.95	34.80 (+10.55)

be reactivated from the latent state and produce infectious particles. Third, even in the absence of detectable virus antigen, the viral genome is still active. Fourth, LAT expression can be detected in the absence of cytolytic viral gene expression. We can draw several conclusions from the cell model established in our study. First, the virus can stay dormant (latent infection) in cells for at least 7 d under in the presence of an appropriate antiviral drug (ACV). During this time, there were no observable changes in cell morphology or physiology. Second, the virus can be reactivated quickly to produce cytolysis by certain induction factors (heat, forskolin). Third, few genes for virus structure or replication can be detected in the latent phase, while the LAT gene can be detected in the supernatant of cell culture. Both the LAT gene and other genes controlling viral replication and structure can be detected after reactivation.

We used different viral MOI (multiplicity of infection) levels to infect SH-SY5Y cells. The traditional concept of MOI originated from the study of bacteria infected by phages. The MOI is the ratio of phage to bacteria during infection, i.e. the average number of phage of each infected cell. The MOI is usually expressed as a ratio without units, but in fact it has a connotative unit-pfu number/cell, with the unit of the number of phages pfu. The virus MOI was developed later in viral researches and was defined as the ratio of the number of virus to cells during infection. However, because the reproducibility of pfu measurements is lower, $TCID_{50}$ or $TCID_{50}$/cell is considered better ways to express the virus infection unit. The $TCID_{50}$ is culculated as

$$\frac{\text{the volumme of viral solution (ml)} \times \text{virus titer } (TCID_{50}/\text{ml})}{\text{the volumme of cell suspension (ml)} \times \text{cell density (cell/ml)}}$$

We used $TCID_{50}$ to calculate viral titer because $TCID_{50}$ is one of the sensitive measures for detecting "virulence". When $TCID_{50}$ were measured, highly diluted cells can resist low virus counts, so the exposure time must be extended on condition that cells grow normally. As for HSV-2 inoculation, exposure time should not be over 10 d since HSV-2 is highly virulent and cells will soon die, detach and become round once infected.

Factors such as physical stimuli (heat [20, 21], UV [6]), chemicals (forskolin) [20, 22] and biological factor (pituitary adenylate cyclase activating polypeptide, PACAP) [23] can induce HSV reactivation from latency. In this research, with heating and forskolin, we successfully reactivated HSV-2 infection, which prompted cytolysis in SH-SY5Y cells. What's more, forskolin-induced reactivation was delayed by 12-24 h compared with heat induction.

In conclusion, the cell model system of HSV-2 latent infection and reactivation in SH-SY5Y cells was established. The research provided usefulness for study on HSV-2 of latency, reactivation and pathogenic mechanism.

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Disclosure of conflict of interest

None.

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