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Original Article

Genistein inhibits glioma cell proliferation and suppresses gD gene expression in HSV-1 infection

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Abstract: This study aims to investigate inhibitory effects of Genistein (GST) on abnormal changes of proliferation and apoptosis in Human Glioma cell (U251) induced by HSV-1. U251 cells were infected by HSV-1 at a multiplicity of infection of 5. GST, GST+HSV-1, HSV-1 and control group were set up. MTT assay, cell apoptosis and RT-PCR were performed to detect inhibitory effects of GST on abnormal changes of proliferation and apoptosis induced by HSV-1 in U251 cells. There were great differences among different groups ($P<0.05$). Compared to control group, cell proliferation were inhibited in 30 μg/ml GST and HSV-1 group ($P<0.05$). MTT values in 15 μg/ml GST+HSV-1 were higher than those in HSV-1 group ($P<0.05$). Cell proliferation were inhibited in both 3.75 μg/ml GST+HSV-1 and 30 μg/ml GST+HSV-1 group compared to HSV-1 group ($P<0.05$). In HSV-1 group, cells started to merge at 12 h post-infection, and cytopathic effects (CPE) were observed at 36 h post-infection. In 15 μg/ml GST+HSV-1 group, a few merges appeared at 24 h post-infection and increased at 36 h post-infection when most cells kept normal forms. Apoptosis rates in 15 μg/ml GST+HSV-1 group were lower than that in HSV-1 group ($P<0.05$). gD gene expression at 6 h post-infection in HSV-1 group and 24 h post-infection in 15 μg/ml GST+HSV-1 group. In conclusion, GST at appropriate concentration does not influence proliferation or apoptosis of normal cells, but could inhibit abnormality glioma cell proliferation and apoptosis, as well as gD gene expression in HSV-1 infection.

Keywords: Genistein, HSV-1, U251, proliferation, apoptosis

Introduction

As a kind of neurological DNA virus widely infected in people, Herpes Simplex virus (HSV) could be divided into HSV-1 and HSV-2 [1]. HSV-1 could cause herpes labiali in adult, kerato-conjunctivitis, as well as herpes simplex encephalitis.

In recent years, the potential of soybeans and legumes on prevention or treatment of chronic diseases (such as cardiovascular disease, osteoporosis and cancer) has attracted great interest in relevant fields. Isoflavones, as one kind of flavonoids, is considered to be benefit for these chronic diseases [2]. Isoflavones and related flavonoids have been demonstrated to have a pharmacological effect against a broad spectrum of virus both in vivo and in vitro [3-6]. Various research work have confirmed that Genistein (GST, **Figure 1**) can inhibit viral infection [3, 4]. However, there is rare contribution devoted on its inhibiting effect on the HSV-1 infection in human astrocyte cell. In this paper, human astrocytoma cells (U251) are selected as research object to investigate the inhibitory effects of GST on abnormal changes of proliferation and apoptosis induced by HSV-1, and the potential therapeutic role of GST in acute viral infection was elucidated.

Materials and methods

Materials

Human Glioma Cell line U251 (American Type Culture Collection, Manassas, VA, cell line number: 99522) was purchased from the Shanghai Cell Resource Center of the Chinese Academy of Sciences. Africa green monkey kidney cell lines (Vero) were preserved in our laboratory. HSV-1-SM44 from Beijing union medical college was amplified in Vero cells. When more than 80% cells have pathological change after HSV-1

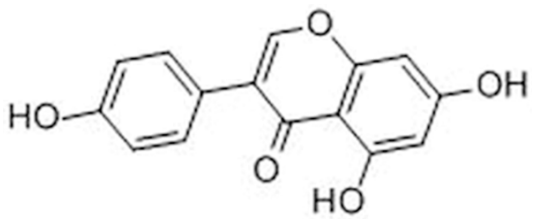

Figure 1. Chemical structure of genistein.

infection, virus was collected. The empty spot filamentous experiment was chosen to detect the degree of virus drops. Virus with 10^8 PFU/ml was stored at -86°C. Multiplicity of infection (M.O.I) of 5 was used in this study.

U251 cells were grown in tissue culture flasks in Roswell Park Memorial Institute (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every day. U251 cells were grown to anastomose every three days.

Cell culture

U251 cells were grown in tissue culture flasks in Roswell Park Memorial Institute (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every day. U251 cells were grown to anastomose every three days.

Analysis of cell viability by MTT assay

Genitein (Sigma aldrich, USA) was dissolved in DMEM. The U251 cells were inoculated to 96-well plates when grown to anastomose with cell concentration of 104 cells/well. GST group, GST+HSV-1 group, HSV-1 group, and control group were set up. After 24 h, the medium was removed. The GST group and GST+HSV-1 group were treated with 100 μl DMEM medium containing different concentrations of GST (3.75 μg/ml, 7.5 μg/ml, 15 μg/ml, 30 μg/ml) and 2% FBS. The HSV-1 group and control group were treated with same volume of DMEM containing 2% FBS. After 8 h, the GST+HSV-1 group and HSV-1 group were treated with HSV-1 (MOI = 5), while the GST group and the control group were treated with the same volume of DMEM containing 2% FBS. Cell viability and proliferation assay was performed by the colorimetric [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) assay according to the kit instruction (Ameresco, USA). The variation of the cell morphology was observed under an inverted olympus microscope. Yellow MTT is reduced to purple formazan, due to the metabolic activity of living cells. A total of 20 μl MTT (5 mg/ml) was added to each group before cells being obtained at 0, 24, 48, 72, and 96 h p.i (hours post infection), and allowed to metabolize for 4 h at 37°C. Blue crystals were dissolved in 150 μl DMSO (Sigma). Absorbance values were measured by microplate reader (DG3022, USA) at 492 nm, with a reference wavelength of 620 nm. Zero setting cells were set up (not containing cells) for a baseline references. At each time point, the performance was repeated in triplicate. The average value was taken for statistical analysis.

Analysis of cell apoptosis by flow cytometry assay

At 12, 24 and 36 h p.i (n = 3), the cells in GST (15 μg/ml) group, GST+HSV-1 (15 μg/ml) group, HSV-1 group and the control group were collected with the old culture medium and the adherent U251 cells, centrifuged, and discarded the old culture medium. Then, 100 μl DMEM containing 2% FBS was added in order to resuspend the cells, followed by labeling by addition of 100 μl of Nexin Reagent for 20 min at room temperature and avoiding light conditions. The Guava EasyCyteTM Cell Growth Kit (0500-1430, Guava technologies Inc, Millipore, USA) was applied to detect red and orange fluorescent signals of 5000 cells. The experimental results were shown with a four quadrant scatterplot Chart. At each time point, the performance was repeated in triplicate. The average value was taken for statistical analysis.

Analysis of HSV-1 gD gene by RT-PCR

U251 cells in GST+HSV-1 (15 μg/ml) group, HSV-1 group and the control group were collected at 6, 12, 24, 36 and 48 h p.i. Total RNA was extracted from cells using TRIzol reagent (Invitrogen, USA) and a UV spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing, China) was applied to determine RNA concentration and purity. cDNA was reverse transcribed using a AMV reverse transcription kit [Promega (Beijing) Biotech Co., Ltd, Beijing,

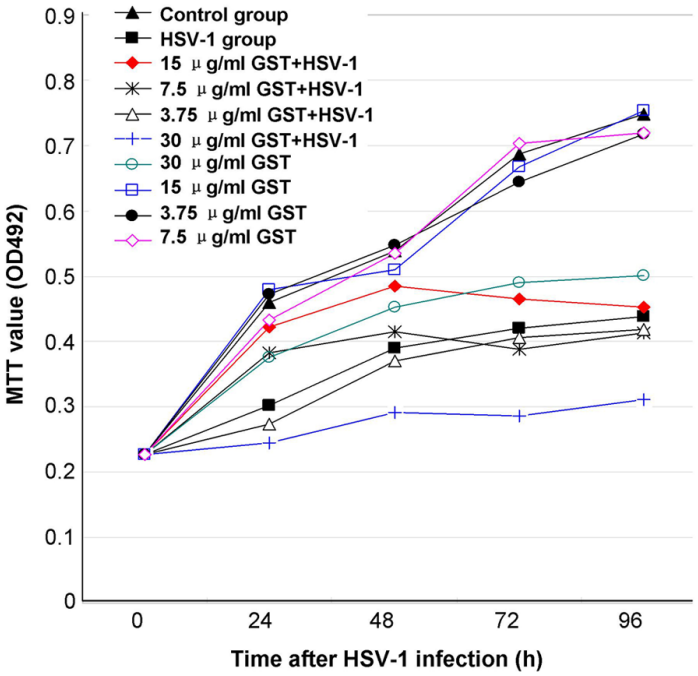

Figure 2. Proliferation variations of U251 in different groups.

China] in accordance with the manufacturer's instructions. The reaction product was preserved at -20°C. The primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., and designed according to the HSV-1 specific gene sequence of gD gene (GenBank accession no. X14112), β-actin (GenBank accession no. NM_001101.3). The primers were as follows: gD-F: GCAACTGTGCTATCCCCATCA, gD-R: CTCCGTCCAGTCGTTTATCTT, PCR product size was 221 bp. β-actin-F: TGGAACGGTGAAGGTGACAG, β-actin-R: GGCTTTTAGGATGGCAAGGG. PCR product size was 154 bp. RT-PCR was then performed for amplification of the target gene, under the following reaction conditions: initial denaturation at 94°C for 5 min, denaturation at 94°C for 40 s, primer annealing at 57°C for 40 s, elongation at 72°C for 30 s, all for 30 cycles, final elongation at 72°C for 10 min.

The PCR products were analyzed by 1.5% agarose gel electrophoresis, and the results were observed under ultra-violet illumination. Quantitative and qualitative analyses of the results were performed based on gray-scale values by the Quantity One imaging system (Bio-Rad Laboratories, Hercules, CA, USA). The gray-scale value ratios of the target gene as well as the internal reference and β-actin, were considered as the relative content of the mRNA of each gene. The experiment was performed in triplicate for each sample.

Statistical analysis

Statistical analyses were performed by using SPSS 19.0 (IBM, Armonk, NY, USA). Measurement data was expressed as the mean ± SD. Comparison between groups was performed with repeated measures ANOVA. The multiple comparisons were performed by LSD method, and the time effect was analyzed at the same time. $P<0.05$ was considered to be statistically significant difference.

Results

Inhibition effect of genistein on abnormal changes of proliferation induced by HSV-1 in U251 cells

To investigate the exact effect of GST on proliferation of normal and infected U251 cells, cell proliferations with various concentrations of drug and virus treatments were detected for by MTT assay (**Figure 2**). The results of MTT assay demonstrated the great differences among different groups ($F = 510.743$, $P<0.05$) and an obvious time depending effect ($F = 2243.2$, $P<0.05$). Compared with control group (without GST or HSV-1), inhibition of cell proliferation could be investigated in both 30 μg/ml GST group and HSV-1 group ($P<0.05$), while no obvious difference ($P>0.05$) was shown in groups with other concentration of GST (3.75 μg/ml, 7.5 μg/ml, 15 μg/ml). MTT values in 15 μg/ml GST+HSV-1 group were higher than those in HSV-1 group ($P<0.05$), while reverse results were found in 3.75 μg/ml and 30 μg/ml GST+HSV-1 groups ($P<0.05$). There was no significantly difference between 7.5 μg/ml GST+HSV-1 group and HSV-1 group ($P>0.05$) (**Table 1**; **Figure 2**). Therefore, we concluded that 15μg/ml GST could obviously inhibit abnormal changes of U251 cell proliferation, and this concentration was selected for further cell apoptosis and RT-PCR study.

Inhibition effect of genistein on the apoptosis induced by HSV-1 in U251 cells

Compared to control group, cells in HSV-1 group gradually merged after infection. The intercel-

Table 1. Proliferation changes of U251 in each group

Group	*Hour post infection*				
	0	24	48	72	96
Control group	0.226±0.020	0.460±0.018	0.539±0.022	0.687±0.016	0.748±0.019
HSV-1 group	0.226±0.020	0.302±0.023	0.39±0.026	0.421±0.018	0.483±0.016
3.75 μg/ml GST group	0.226±0.020	0.473±0.021	0.548±0.027	0.645±0.014	0.718±0.014
7.5 μg/ml GST group	0.226±0.020	0.433±0.022	0.535±0.011	0.704±0.015	0.720±0.024
15 μg/ml GST group	0.226±0.020	0.479±0.025	0.511±0.018	0.667±0.017	0.753±0.016
30 μg/ml GST group	0.226±0.020	0.375±0.016	0.452±0.019	0.490±0.011	0.501±0.025
3.75 μg/ml GST+HSV-1 group	0.226±0.020	0.273±0.019	0.371±0.014	0.407±0.015	0.418±0.013
7.5 μg/ml GST+HSV-1 group	0.226±0.020	0.383±0.023	0.415±0.020	0.389±0.018	0.413±0.017
15 μg/ml GST+HSV-1 group	0.226±0.020	0.422±0.028	0.485±0.022	0.466±0.013	0.453±0.021
30 μg/ml GST+HSV-1 group	0.226±0.020	0.245±0.015	0.292±0.024	0.287±0.027	0.311±0.014

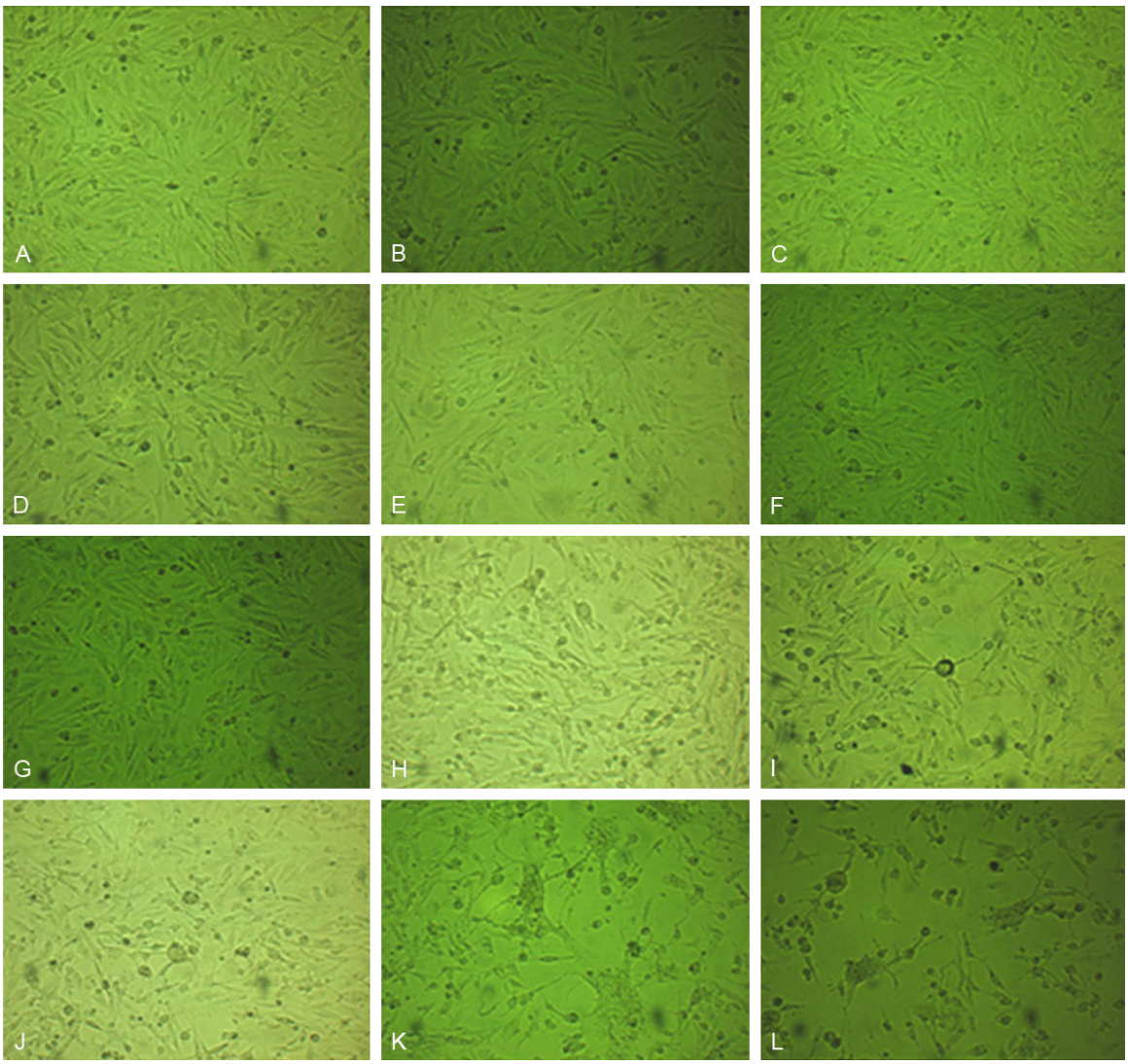

Figure 3. Morphological changes of U251 cells in each group (40×): A-C: 12 h, 24 h, 36 h control group cells. D-F: 12 h, 24 h, 36 h HSV-1 group cells; G-I: 12 h, 24 h, 36 h GST+HSV-1 group cells; J-L: 12 h, 24 h, 36 h GST group cells.

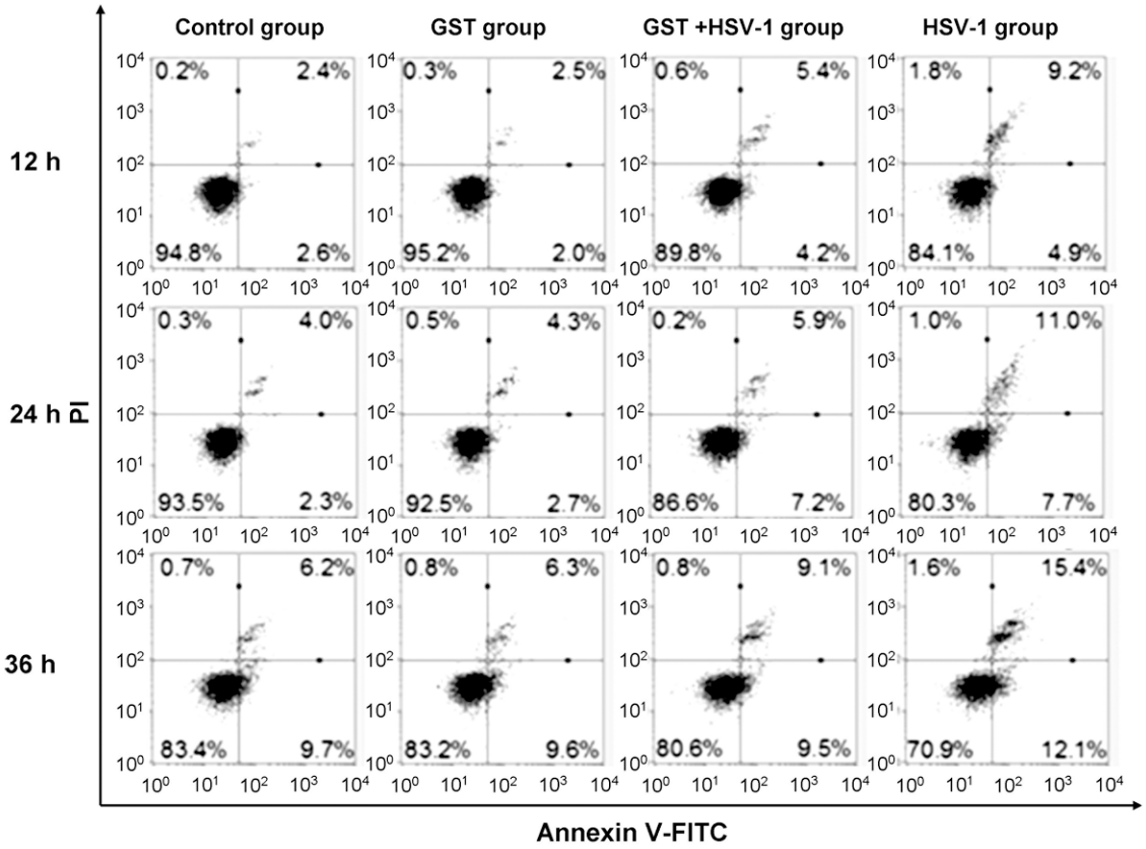

Figure 4. Apoptosis of U251 cells in each group analyzed by flow cytometry technology.

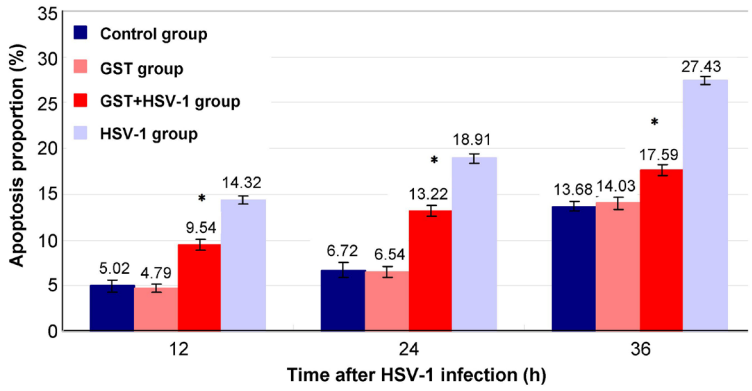

Figure 5. Changes of apoptosis proportion of U251 cells in each group. *P<0.05, compared to HSV-1 group cells in the same time scale.

lular space became larger, and the number of granules in cytoplasm increased. This group eventually showed typical cytopathic effect at 36 h p.i. while no apparent variation with clear outline and good refraction at 12 h p.i, was shown in 15 μg/ml GST+HSV-1 group. At 24 h p.i, it appeared some local small merge, and the merge increased at 36 h p.i. However, most of the cells were still in normal shape, indicating the unsuspecting protective effect of GST. There was no significant difference between the GST group and the control group (**Figure 3**).

Flow cytometric assay analysis

Cell apoptosis demonstrated the great differences among different groups ($F = 1788.58$, $P<0.05$) and an obvious time depending effect ($F = 766.478$, $P<0.05$). The apoptosis rate of GST+HSV-1 group at each time point was lower than that of HSV-1 group at the corresponding time ($P<0.05$), while no notable difference was detected between GST group and HSV-1 group ($P>0.05$) (**Figures 4**, **5**). Our data preliminary indicated that GST itself did not cause cell apoptosis, but could promote cell proliferation

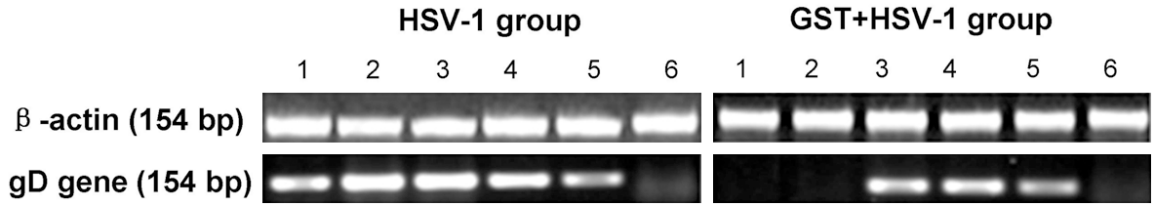

Figure 6. Expressions of gD gene in HSV-1 group and GST+HSV-1 group after infection. 1~5 represent HSV-1 group or GST+HSV-1 group infected after 6 h, 12 h, 24 h, 36 h and 48 h. 6 represents the control group.

and inhibit the apoptosis of U251 cells induced by HSV-1 infection.

Inhibition of gD gene expression by GST

The mRNAs of gD were firstly detected at 6 h p.i and 24 h p.i in HSV-1 group and 15 μg/ml GST+HSV-1 group, respectively (**Figure 6**). The expression of gD mRNA in the latter group remarkably decreased comparing to the former one. In both control group and GST group, gD mRNAs were not expressed. The results indicated GST inhibit gD gene expression during the early period of HSV-1 infection.

Discussion

As a neurotropic virus, HSV-1 lurks in neurons of the host and gets ready to be activated anytime, which is an important teratogenic pathogen causing nerve lesion of human. Although the exact mechanism for the central nervous system injury by HSV-1 infection is still unclear, animal experiments indicated that astrocytes could respond to a variety of disease [7]. Among various components of cells in central nervous system, astrocytes are particularly sensitive to HSV-1 and permissive to HSV-1 infection [8], suggesting the target cells of HSV-1 infection in central nervous system.

Because of the difficulty of the primary human astrocytes acquirement and their extremely restricted culturing condition, there is currently rare contribution related to the infection HSV-1 of on these cells. In this study, human astrocytoma cells, U251, were selected as the infection model, and the MTT assay, morphology, flow cytometry as well as RT-PCR were applied for confirming the inhibitory effects of GST on abnormal changes of proliferation and apoptosis induced by HSV-1. Our data would provide some new ideas for exploring clinical indications of GST.

Evidence has been shown that HSV-1 can infect human retinal pigment epithelial cells when multiplicity of infection (MOI) = 5 [9, 10], and majority of U251 cells infected under the same condition was observed in our previous study. Hence, we chose MOI = 5 as our experimental virus volume. Firstly, MTT assay was performed to detect GST effect on U251 cells proliferation. The results suggest significant statistical differences among different groups, along with a very significant time effect.

We found that MTT value was universally higher only in 15 μg/ml GST+HSV-1 group than that in HSV-1 group ($P<0.05$), as shown in **Figure 2**. The abnormality of cell proliferation was significantly inhibited in the same group after 48 h infection, suggesting that antiviral effects of GST may take effect on the early stage of HSV-1 infection. On the other hand, toxic effects of high doses of GST (30 μg/ml) on normal cells were also investigated, while lower doses had no significant impact on normal cells, indicating adequate doses of GST would neither promote nor inhibit cell proliferation, which guarantees single variable in this experiment.

We selected 15 g/ml GST for the morphology and flow cytometry characterization. The results showed that GST itself did not cause apoptosis of U251 cells. However, it could inhibit cell apoptosis induced by HSV-1 infection to some extent. RT-PCR results also revealed that within 12 hours of infection, GST effectively inhibit GD gene expression, the most well-known glycoprotein mark gene of HSV-1. Therefore, it could be inferred that antiviral activity GST for retarding virus infection would mainly work in the process of HSV-1 adsorption, entering host cells, as well as virus replication. Previous studied have confirmed that the GST could prevent HSV-1 from binding to host cell membrane, DNA replication, viral protein translation, as well as formation of virus envelope glycoprotein

complex, which were consistent with our results.

GST could play important roles in several cell functions, such as tyrosine kinase inhibitors (PTKs), estrogen receptor (ER), receptors or topoisomerase II inhibitors, etc. [11, 12] It has been reported that inhibition of PTK activity may prevent adenovirus, human herpes virus 8 (HHV8), Moloney murine leukemia virus (Mo-MLV), simian virus 40 (SV40), etc. into the host cell [13-16]. As PTKs is currently considered as major target for the mechanism of GST against viral infection, it plays the role mainly through the following three pathways: to compete with tyrosine kinase ATP binding site on ATP epidermal growth factor receptor [17, 18] to inhibit c-src [19, 20], which is one kind of tyrosine kinase participating mitogen-activated protein kinase (MAPK), and to activate p38 MAPK on transforming growth factor-β receptor level [21]. In addition, in order to regulate inflammation and immune response in threatened host body [13, 14, 16], GST also influences the expression of certain transcription factors and secretion of cytokines on cellular level.

In summary, human U251 cells were employed as HSV-1 infection model. It is proved that GST, as a potential antiviral drug, exhibits a promising prospect in suppressing abnormal cell proliferation and apoptosis induced by viral infection. The related mechanism is also been preliminary discussed. The results provide a theoretical basis for further study on anti-viral effect of GST on HSV-1 infected astrocytes.

Disclosure of conflict of interest

None.

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