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Original Article

Study on the effects of VPA combined with oncolytic HSV-1 on the proliferation and apoptosis of U251 cells in vitro

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Abstract: Objective: This study aims to observe the effects of sodium valproate acid (VPA) combined with herpes simplex virus type I (HSV-1) on the proliferation and apoptosis of tumor cell U251 in vitro. Methods: U251 glioma cells were treated by VPA, HSV-1 and VPA combined with HSV-1 respectively. The MTT assay was used to observe cell proliferation; the cell morphology was observed under a microscope; the rate of apoptosis of the cells was detected by flow-cytometry; cell migration was observed by inverted phase contrast microscope; the expression level of cyclin D1 in U251 cells was detected by western-blotting and the level of VEGF (vascular endothelial growth factor) secreted by U251 cells was detected by ELISA. Results: The phenomenon of cell atrophy, necrosis and shedding could be observed in some cells of the VPA group; cell swollen, large nuclear, and cell fusion were observed in some cells of the HSV-1 group; cytological morphological changes in the VPA + HSV-1 group were more obvious than that of HSV-1 group, the rate of U251 cell proliferation inhibition and apoptosis in the VPA + HSV-1 group was significantly higher than that of the VPA or HSV-1 group. Inhibition on the migration of U251 cells in the VPA combined with HSV-1 group is stronger than that of VPA or HSV-1 group. The expression levels of cyclin D1 and secretion levels of VEGF were significantly lower than that of the VPA or HSV-1 group ($P<0.01$). Conclusion: VPA combined with HSV-1 had a synergistic or superimposed killing effect on U251 cells in vitro, which was probably accomplished by reducing the expression level of cyclin D1 and the secretion level of vascular endothelial growth factor.

Keywords: VPA, HSV-1, glioma, proliferation, apoptosis

Introduction

Cerebral glioma is one of the most common malignant tumors in the central nervous system with high incidence, mortality and recurrence rate. Astrocytoma accounts for about 40% of cerebral glioma, it can be divided into four grades according to its malignant degree [1]. At present, the treatment methods of cerebral gliomas mainly include surgical treatment, chemotherapy, radiotherapy and combined treatment, but their effects of treatment are not ideal.

Oncolytic virus can infect tumor cells selectively and has little effect on normal cells, it showed high susceptibility, specificity, cytotoxicity and safety. It replicated and passaged largely in vivo and eventually lead to the demise of the tumor cells [2-4]. At present the commonly used oncolytic virus included oncolytic herpes simplex virus and oncolytic adenovirus etc. Herpes simplex virus type 1 (HSV-1) is a large double stranded DNA virus with a capsule. Its genome is 153 kb long and consists of 2 interconnected long segment (L) and short segment (S) linear double stranded DNA molecule [5]. Its specific killing tumor cells role is achieved by a number of gene mutations. Clinical trials of mutation virus G207 of HSV-1 have passed through the I phase and the survival time of patients was prolonged [6, 7]. NVI020 virus originated from HSV-1 could inhibit the growth and spread of tumor [8].

Histone deacetylase inhibitor (HDACi) is a new type of anti-tumor drugs with great potential

Table 1. The inhibition rate of U251 cells in different group (M ± SD, %)

Control	VPA	HSV	VPA + HSV
1.10±0.02	14.37±1.80*	41.16±1.16**	56.36±1.19**

*P<0.05 vs. control; **P<0.01.

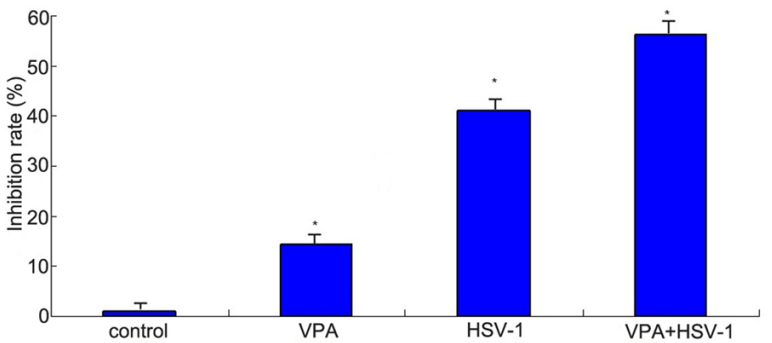

Figure 1. The inhibition rate of U251 cells in different group. *P<0.05 vs. control.

and can increase the cytotoxic effect of oncolytic virus on tumor cells. Valproic acid (VPA), a kind of HDACi acted on human cerebral glioma cells and increased the cytotoxicity and the yield of MGH2 and rQNestin34.5 [9]. VPA could inhibit the growth of bladder cancer cells by delayed cell cycle [10]. Mammalian target of rapamycin (mTOR) inhibitor combined with VPA can prevent the migration, adhesion and metastasis of prostate cancer cells [11].

In this study, we explored the effects of VPA combined with HSV-1 on the glioma cell U251 in vitro and its mechanism, which could provide a new clinical treatment mode for refractory malignant tumors.

Materials and methods

Cell culture

Human cerebral glioma cell line U251 was supplied by the research center of Wuhan University. U251 cells were cultured in DMEM (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) at 37°C in 5% CO_2.

Determination of virus titer

HSV-1 virus was supplied by Wuhan University. Suspension of HSV-1 (0.5 ml) was placed into culture flask with cells and cultured at 37°C. Plaque forming experiment was performed to determine the virus titer.

Cell morphology observation

The cells were seeded at density 1×10^5 cells/ml in 6-well plate and cultured at 37°C with 5% CO_2. They were divided into control group, VPA (2.0 mmol/L) group, HSV (10 MOI) group and VPA (2.0 mmol/L) + HSV (10 MOI) group with different treatment. Cellular morphological changes were observed under microscope after culture for 72 h.

MTT assay

The tested cells were seeded at density 2×10^4 cells/ml in 96-well plates. They were divided into control group, VPA group, HSV group and VPA + HSV group with different treatment. The cells were added 20 μL MTT (5 mg/ml) and the cells were incubated for an additional 4 h at 37°C. The culture medium was removed, 150 μL of DMSO were added to each well. With shaking at low speed for 15 min, the MTT solution was aspirated and optical densities (OD) of the supernatant were read at 568 nm using a Microplate Reader (Thermo Scientific). The experiments were repeated three times and the negative control was conducted using only cell-free culture medium. The inhibition rate (%) = (1-experimental group OD/control group OD) ×100%.

Flow cytometry analysis

Fluorescein Annexin V-FITC/PI double labeling was performed with the Annexin V-FITC Apoptosis Detection Kit (Beckman) to detect the apoptotic rate according to the manual. The cells were seeded at density 1×10^5 cells/ml in 6-well plate and divided into control group, VPA group, HSV group and VPA + HSV group. They were stained with Annexin V-FITC and PI and the apoptotic cells were determined with a FACS Calibur flow cytometer (BD Biosciences) and analyzed with CELLQUEST software (BD Biosciences).

Cell scratch assay

The cells were seeded at density 1×10^5 cells/ml in 6-well plate and cultured at 37°C with 5%

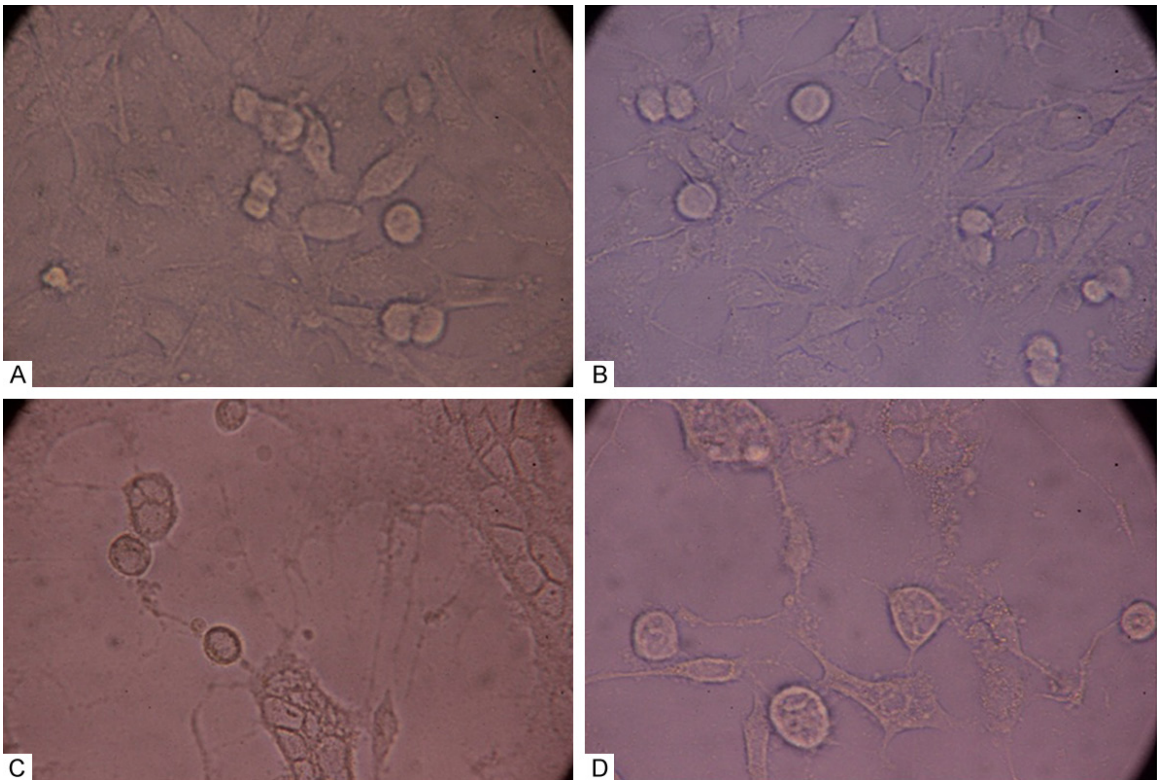

Figure 2. Observation of cell morphology. A. Control group; B. VPA group; C. HSV-1 group; D. VPA + HSV-1 group. There were more cells and cell morphology was spindle shaped or varied in control group; swollen cells and cell fusion phenomenon could be seen in HSV-1 group; some atrophy, necrosis or shedding cells could be seen in VPA group; the cell morphology in was similar with that of HSV-1 group but with a large number of cell necrosis.

Table 2. The apoptosis rate of U251 cells in different group (M ± SD, %)

Control	VPA	HSV	VPA + HSV
5.79±0.10	12.33±0.80*	36.22±0.93**	50.83±1.86**

*P<0.05 vs. control; **P<0.01.

CO_2. The bottom of plate was marked line when the cells covered the bottom. The plate was washed with PBS to remove the suspended cells. They were divided into control group, VPA group, HSV group and VPA + HSV group with different treatment. Cellular migration was observed under microscope after culture for 24 h.

The determination of Cyclin D1 with western blotting method

The cells were lysed using RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40 and 0.1% sodium dodecylsulfate) at pH 7.4, supplemented with protease inhibitors cocktail (10 g/mL leupeptin, 10 g/mL pepstatin A, 10 g/mL aprotinin and 1 mM of 4-(2-aminoethyl)-benzenesulfonyl fluoride) and phosphatase inhibitors (1 mM NaF and 1 mM Na_3VO_4). The cell lysates were centrifuged at 1000 g for 20 min at 4°C. The proteins were size-fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The membrane was then blocked with 5% skimmed dry milk for 2 h at room temperature. The primary antibody (1:1000) was added and incubated for 2 h at room temperature. They were washed with TBST and a HRP-conjugated secondary antibody (1:50000) was added and incubated at room temperature for 1 h. The membranes were developed using an enhanced chemiluminescence system after washing. The protein levels were normalized to β-actin control.

The levels of VEGF

The expression levels of VEGF in different group were detected using ELISA kits according to the protocol. OD values were read at 450 nm using a Microplate Reader (Thermo Scientific).

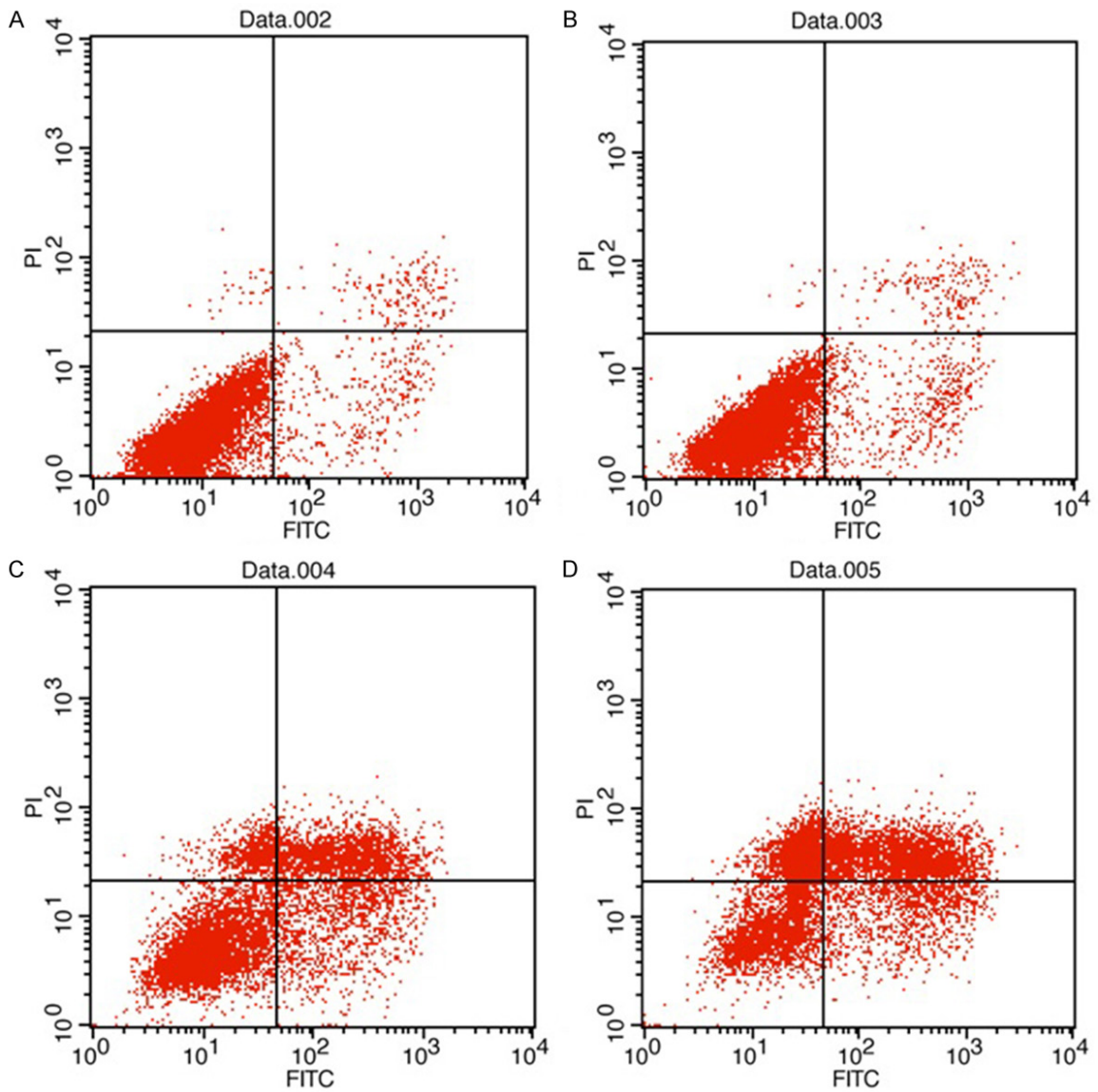

Figure 3. Flow cytometry analysis. A. Control group; B. VPA group; C. HSV-1 group; D. VPA + HSV-1 group.

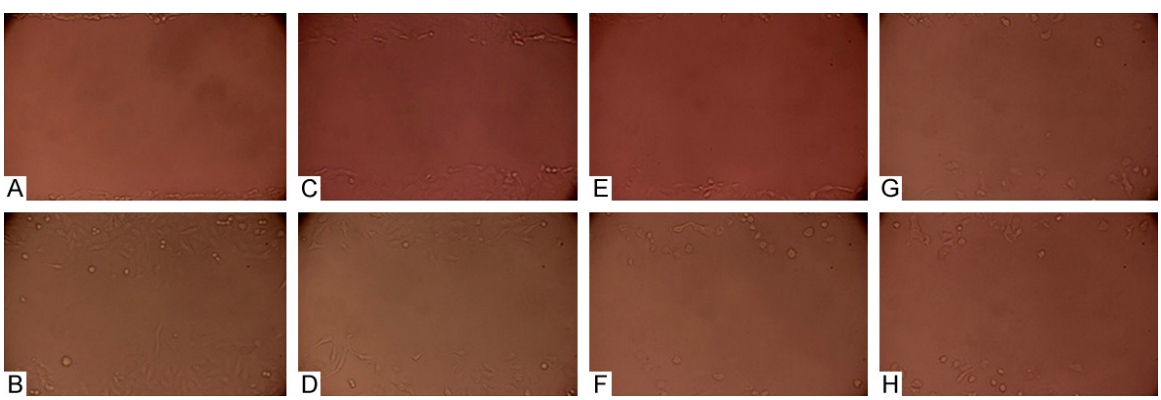

Figure 4. Cellular migration assay. A. Before treatment in control group; B. After treatment for 24 h in control group; C. Before treatment in VPA group; D. After treatment for 24 h in VPA group; E. Before treatment in HSV-1 group; F. After treatment for 24 h in HSV-1 group; G. Before treatment in VPA + HSV-1 group; H. After treatment for 24 h in VPA + HSV-1 group.

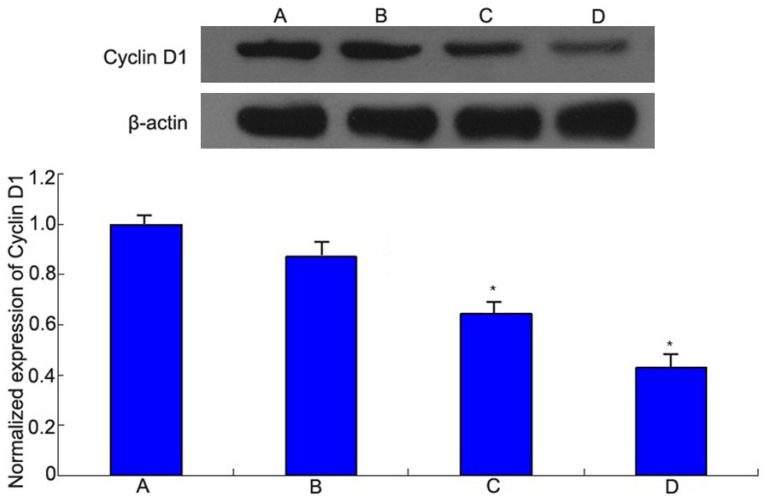

Figure 5. The determination of Cyclin D1 with Western blotting method. *P<0.05 vs. control.

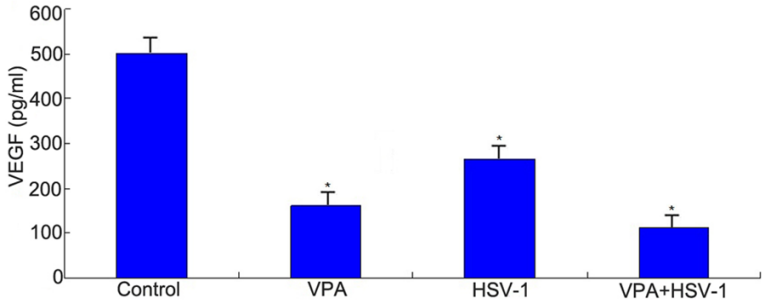

Figure 6. The levels of VEGF in different group. *P<0.05 vs. control.

Statistic analysis

All results were expressed as the mean ± SD. SPSS18.0 software was used to do statistic analysis. A t-test was used to compare among groups. P<0.05 was considered to indicate a statistically significant difference.

Results

U251 cell proliferation activity

The MTT results were shown in **Table 1** and **Figure 1**. They showed that the inhibition rate of U251 cells in VPA + HSV-1 group were higher than that of HSV-1 group and VPA group (P<0.01).

Cellular morphological changes

There were more cells and cell morphology was spindle shaped or varied in control group; swollen cells and cell fusion phenomenon could be seen in HSV-1 group; some atrophy, necrosis or shedding cells could be seen in VPA group; the cell morphology in VPA + HSV-1 group was similar with that of HSV-1 group but with a large number of cell necrosis (**Figure 2**).

Flow cytometry analysis

The apoptosis rate of U251 cells in different group was detected by flow cytometry. It showed that the apoptosis rate of U251 cells in VPA + HSV-1 group were significantly higher than that of HSV-1 group and VPA group (P<0.01, **Table 2** and **Figure 3**).

Cellular migration ability

There were aequilate no cell scratch lines in each group before treatment. After treatment for 24 h, migrated cells in control group were more than that of other groups, there was little migrated cell in VPA + HSV-1 group (**Figure 4**).

The levels of VEGF and Cyclin D1

The expression levels of Cyclin D1 in VPA + HSV-1 group were significantly lower than that of other groups (**Figure 5**) and it was similar about VEGF levels (**Figure 6**).

Discussion

Malignant tumors endanger human life safety and have become one of the common causes of death, which causing serious economic losses and casualties [12]. Malignant glioma is a malignant tumor derived from the epithelial cells of the nerve which caused by multiple factors and virus is one of the bio-factors [13]. However, improved or inactivated virus can also be used as a new therapy means of tumors. Researchers have paid more and more attention to the oncolytic virus. G207 derived from the HSV-1 was used for the treatment of intra-

cranial malignant tumors [14], HFI0 was used for the treatment of breast cancer [15] and NVI020 was used for the treatment of rectal cancer [16], their efficacy was obvious.

The mechanism and effect of HDACi on the malignant tumor cells are very complex. HDACi also acts on a number of non-histone proteins such as transcription factors, signal transduction factors and molecular chaperones [17]. Tumor angiogenesis and regulation disorder of cell cycle proteins (Cyclins) are the two most important factors in tumor development. Vascular endothelial growth factor (VEGF) can activate vascular endothelial cells to split and proliferate, and directly involved in tumor angiogenesis [18]. New vascular formation and (or) vascular endothelial cell proliferation is a very typical feature of malignant gliomas [19, 20]. Cyclins are the decisive factors in regulating cell cycle, which determine whether the cells can be further divided and propagated [21]. Over expression of Cyclin DI is a common phenomenon in human malignant gliomas [22, 23]. VPA is not only a traditional anti epileptic drug, but also a kind of HDACi. It was found that VPA could increase the activity of Caspase-3 and decrease Notch-I in vitro and inhibit the growth of liver cancer cells [24].

In this study we combined VPA and HSV-1 to act on U251 cells and found that VPA + HSV-1 inhibit the proliferation activity of U251 cells and improve their apoptosis. The ability to inhibit cell migration was significantly enhanced in VPA + HSV-1 group. The expression levels of Cyclin DI and VEGF in VPA + HSV-1 group decreased significantly. These results suggested that VPA combined with HSV-1 have some synergistic or overlapping killing effects on U251 cells in vitro by reducing the expression levels of Cyclin D1 and the level of VEGF, which could provide a basis for further research on biological treatment.

In a word, VPA combined with HSV-1 have some synergistic or overlapping killing effects on U251 cells in vitro, which could be through reducing the expression levels of Cyclin D1 and the level of VEGF.

Disclosure of conflict of interest

None.

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