# HERPES SIMPLEX VIRUS 1 INHIBITS APOPTOSIS THROUGH A CASPASE-3-DEPENDENT PATHWAY IN PRIMARY CULTURES OF CORTICAL NEURONAL CELLS OF FETAL MICE

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*Received March 1, 2004; accepted February 17, 2005*

**Summary. –** We could induce apoptosis in primary cultures of cortical neurons of fetal mice with ceramide or sorbitol. The induction was accompanied by an increase in caspase-3 (CAS-3) activity and depolarization of the inner mitochondrial membrane of neuronal cells which both could be reversed by Herpes simplex virus 1 (HSV-1) infection. We conclude tha HSV-1 infection inhibited the apoptosis, induced in neuronal cells by sorbitol or ceramide, via a CAS-dependent pathway.

**Key words:** apoptosis; caspase-3; Herpes simplex virus 1; cortical neurons

## Introduction

Apoptosis is an autonomous cell death that is activated to eliminate superfluous, damaged, mutated or aged cells. The central components of apoptotic pathways are proteases of the CAS family, caspases (CASs) are present in inactive form in all nucleated cells. Their activity is balanced by specific activation and inactivation events, well established *in vitro* and in transformed cell lines. Upon activation, CASs cleave specific substrates and thereby mediate many typical biochemical and morphological changes in apoptotic cells, such as shrinkage, chromatin condensation, DNA fragmentation and plasma membrane blebbing.

Recent discoveries have significantly deepened the understanding of biochemical requirements of distinct apoptotics pathways. At least there are three signaling pathways related to CASs, namely the mitochondrial cytochrome *c,* death-receptor and endoplasmic reticulum pathway (Daniel, 2000).

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**Abbreviations:** CAS = caspase; HSV-1 = Herpes simplex virus 1; MEM = Minimal Essential Medium; p.i. = post infection; pNA = p-nitroaniline.

Osmotic stress has been shown to induce apoptosis in primary cultured chick embryo telencephalon neurons *in vitro* (Galvan and Roizman, 1998; Wang *et al*., 2001a). It is known that HSV-1 inhibits apoptosis in a cell type-specific and virus strain-specific mode (Galvan and Roizman, 1998). We have observed that HSV-1 can block (i) apoptosis induced in primary cultures of cortical neurons of fetal mice by non-specific stress agents such as thermal or osmotic shock (Wang *et al*., 2001a) and (ii) apoptosis in primary cultured cortical neurons of fetal mice *in vitro* induced by sorbitol or ceramide (Wang *et al*., 2001a,b).

It has been postulated that the mechanism of HSV-1 blocking of neuronal apoptosis might involve upregulation of Bcl-2 protein expression leading to extension of neuronal life span (Adams and Cory, 1998; Derfuss *et al*., 1998). It has been reported that (i) the HSV-1 *d*120 mutant-induces apoptosis by a CAS-independent pathway and (ii) the wild-type virus blocks the apoptosis induced by this pathway and the CAS-dependent pathway induced by osmotic stress. The block in the CAS-dependent pathway may occur downstream of CAS activation (Galvan *et al*., 1999). CASs exist normally in the cytoplasm in an inactive proenzyme form (Martin *et al*., 1995). Upon cleavage, typically by another CAS or an apoptosis regulatory protein, CAS is activated and cleaves downstream of the CAS or other apoptotic proteins (Swanton *et al.,* 1999).

Osmotic stress activates multiple signaling pathways and induces a rapid response that allows examination of various events in the cell death program (Rosette and Karin, 1996). Ceramide, a sphingolipid metabolite is supposed to play role in cellular apoptosis and differentiation. It induces apoptosis in various mammalian cell lines and, more recently, also in neurons. Although the mechanisms of ceramide-induced apoptosis has not been fully elucidated yet, it involves apparently a number of signal transduction pathways, namely proline-directed kinases, phosphatases, phospholipases, transcriptional factors and CASs. Taken together, an important multipotential role for this lipid in apoptosis is accepted (Toman *et al*., 2000).

Galvan *et al.* (1999) have demonstrated that HSV-1 infection can prevent the redistribution of cytochrome *c* and depolarization of inner mitochondrial membrane resulting from induction of apoptosis by osmotic stress. These results suggested that ceramide could also be an inducer of apoptosis and play an active role in regulating other aspects of mitochondrial function. Ceramide, one of major sphingosine-based lipid second messengers generated in response to oxidative stress is involved in induction of apoptosis (Andrieu-Abadie *et al*., 2001; Goswami and Dowson, 2000). Several extracellular conditions such as lack of oxygen due to ischemia or anemia and excessive membrane leakage should resulted in insufficient mitochondrial inner membrane potential.

Mitochondria, in addition to their role as the cell powerhouse, play a central role in the control of apoptosis. Mitochondria undergo two major changes during early stage of apoptosis. On one hand, the outer mitochondrial membrane becomes permeable to proteins, thus resulting in the release of soluble intermembrane proteins form mitochondria. On the other hand, the transmembrane potential of the inner mitochondrial membrane is reduced. These changes occur in most, if not all, types of apoptosis and can be utilized in detection of apoptosis at an early stage (Castedo *et al*., 2002; Ravangnan *et al*., 2002). Based on these results, the apoptosis in primary cultured cells induced by HSV-1 infection seems to be an early event.

In this study, we investigated CAS-3 activity in primary cultures of cortical neural cells of fetal mice, in which apoptosis was induced by ceramide or sorbitol and blocked by HSV-1 infection.

## Materials and Methods

*Cells.* Primary cultures of telencephalons were prepared from 14–16-day-old mouse embryos (C57BL/6) (Pettmann *et al*., 1979). Cortical cells were suspended in Minimal Essential Medium (MEM, Gibco, BRL) supplemented with 2 mmol/l glutamine, 0.1 mg/ml gentamycin and 10% of heat-inactivated fetal calf serum (the growth medium). The number of neural cells was evaluated by counting in a hemocytometer and their viability was determined by staining with Trypan Blue. Culture dishes coated with poly-L-lysine were seeded with 1 x $10^6$ cells/ml and incubated in 5% $CO_2$ and 95% humidity. The medium was changed after 3 days for a fresh one containing 10 mmol/l uridine and 5-fluoro-deoxyuridine for 24 hrs to stop cell division.. Vero cells were obtained from the Institute of Virology, Chinese Academy of Preventive Medicine, Beijing, P.R. China.

*Viruses.* HSV-1 Stoker stain was obtained from Institute of Virology, Chinese Academy of Preventive Medicine, Beijing, P.R. China. To obtain a virus stock, the virus was inoculated into Vero cell monolayer cultures and the latter were harvested 48–72 hrs post infection (p.i.) by freeze-thawing followed by low speed centrifugation to remove cellular debris. The virus suspension was stored at -80°C. Its titer was determined by plaque counting in Vero cells (Derfuss *et al*., 1998).

*Virus infection.* Neural cell cultures were washed with MEM, infected with the virus diluted in MEM at a multiplicity of infection (MI) of 0.1 PFU/cell or 1.0 PFU/cell and incubated for 1 hr. Then the medium was changed for the growth medium and incubation continued for 10 hrs.

*Induction and assay of apoptosis.* The neural cells were exposed either to 1.5 mol/l sorbitol or 100 µmol/l $C_2$-ceramide (Sigma) in Eagle's Minimum Essential Medium (MEM, Gibco, BRL) for 1 hr, the medium was changed for the growth medium and the incubation continued for 5 hrs.

*Flow cytometry.* Effects of apoptosis were evaluated by flow cytometry using the Flow TACS™ TUNEL Assay Kit. Mean values from triplicate assays were used.

All the incubations of cell cultures proceeded at 37°C

*Mitochondrial membrane potential assay.* Cells were harvested, rinsed with PBS, resuspended in 5 nmol/l Rhodamine 123 (Sigma), incubated at room temperature for 20 mins and subjected to flow cytometry using a FACScan flow cytometer (Becton Dickinson). Data for 2 x $10^6$ cells were collected, stored and analyzed by using the Becton Dickinson Cellquest software.

*CAS-3 assay.* Cells $10^6$ were harvested, rinsed with PBS, resuspended in a lysis buffer, placed on ice for 5 mins and centrifuged at 10,000 x g for 10 mins at 4°C. The supernatants were transferred to a 96-well microtiter plate and assayed spectrophotometrically for CAS-3 activity by using the ApoAlert Caspase-3 Assay Kit from Clontech (USA) and a BIO-RAD 1500 spectrophotometer. The assay consisted measurement of $A_{405}$ of detection of the chromophore *p*-nitroaniline (pNA) after cleavage from the labeled CAS-3 substrate DEVD-pNA. $A_{405}$ was read at 14 hrs. A known CAS-3 inhibitor, the synthetic tetrapeptide DEVD-fmk (Thornberry and Littlewood, 1998) was used in negative control for the CAS-3 ssaay.

*Statistical analysis* of results was performed by the *t*-test. The differences with $P \leq 0.05$ were considered significant.

## Results

### *Effect of HSV-1 infection on sorbitol- or ceramide-induced apoptosis*

We have shown previously that sorbitol can induce apoptosis in primary cultures of chick telencephalon neurons (Wang *et al.,* 2001). In this study, using TUNEL and flow

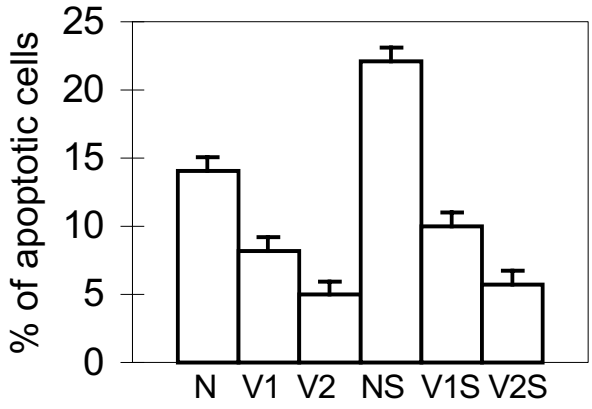


**Fig. 1**

**Effect of HSV-1 infection and sorbitol on the percentage of apoptotic cells in primary cultures of neuronal cells**

The assays were made by TUNEL and flow cytometry at days 3 p.i. Mock-infected cells (N); the cells infected at MI of 0.1 (V1); .the cells infected at MI of 1.0 (V2); the mock-infected and sorbitol-treated cells (NS); the cells infected at MI of 0.1 and treated with sorbitol (V1S); the cells infected at MI of 1.0 and treated with sorbitol (V2S).

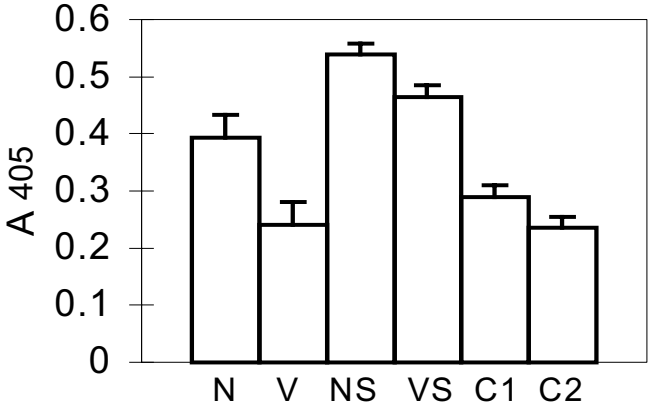


**Fig. 2**

**Effect of HSV-1 infection and sorbitol on CAS-3 activity of primary cultures of neuronal cells**

CAS-3 activity ($A_{405}$) was assayed at 14 hrs p.i. Mock-infected cells (N); infected cells (V); mock-infected cells treated with sorbitol (NS); infected cells treated with sorbitol (VS); mock-infected cells treated with sorbitol and exposed to DEVD-fmk (C1), (mock-infected cells treated with sorbitol but the substrate was omitted from the assay (C2).

cytometry for assaying the percentage of apoptotic cells, the apoptosis in primary cultures of cortical neurons from fetal mice induced by sorbitol could be blocked by HSV-1 infection (Fig. 1). The values of all the experimental groups differed significantly.

*Inhibition of apoptotic CAS activation by HSV-1 infection*

Morphological examination of cells for apoptosis by a blinded observation is a well-accepted and reproducible method of evaluating apoptosis (McGahon *et al*., 1995), but it provides only limited insights into the mechanisms by which apoptosis might occur. Therefore, we investigated the possibility of inhibition of CAS activation by HSV-1 infection during the apoptosis induced in primary cultures of mouse neurons by sorbitol. The values of all the experimental groups differed significantly. Fig. 2 shows that the sorbitol-induced apoptotic CAS-3 activity was markedly reduced by HSV-1 infection. Among controls, HSV-1 infection done as well as DEVD-fmk, an inhibitor of the CAS-3 activity reduced the CAS-3 activity to background values. All the differences observed were significant.

*Inhibition of depolarization of the inner mitochondrial membrane by HSV-1 infection*

We investigated whether HSV-1 infection could inhibit the depolarization of inner motichondrial membrane of cultured neuronal cells of fetal mice, an event that appears to be associated with some forms of apoptosis. Fig. 3 shows that the HSV-1 infection inhibited the depolarization of the inner mitochondrial membrane induced by sorbitol or ceramide.

## Discussion

The results of these studies have shown that the inner mitochondrial membrane potential was reduced in cells undergoing apoptosis induced by sorbitol or ceramide, while there was no obvious decrease in the inner mitochondrial membrane potential of cells infected with HSV-1, and that of cells infected with HSV-1 and exposed to sorbitol or ceramide. It can be concluded that virus blocked the depolarization of the inner mitochondrial membrane.

Thus these results differ markedly from others regarding the cells or virus strains used. Whereas we used primary cultures of mouse neurons and HSV-1 Stoker strain, others used SK-N-SH, HEp-2, Vero and Jurkat cell lines and HSV-1 F strain, mutant *d*120 or clinical isolates (Jerome *et al*., 1999). Although primary cultures of cortical neurons are more problematic in investigation and estimation of the course of apoptosis, they are closer to the condition *in vivo*. In general, primary cell cultures of neurons regardless of their type and origin (chick embryo telencephalon neurons or fetal mouse cortical neurons) represent an experimental approach of choice.

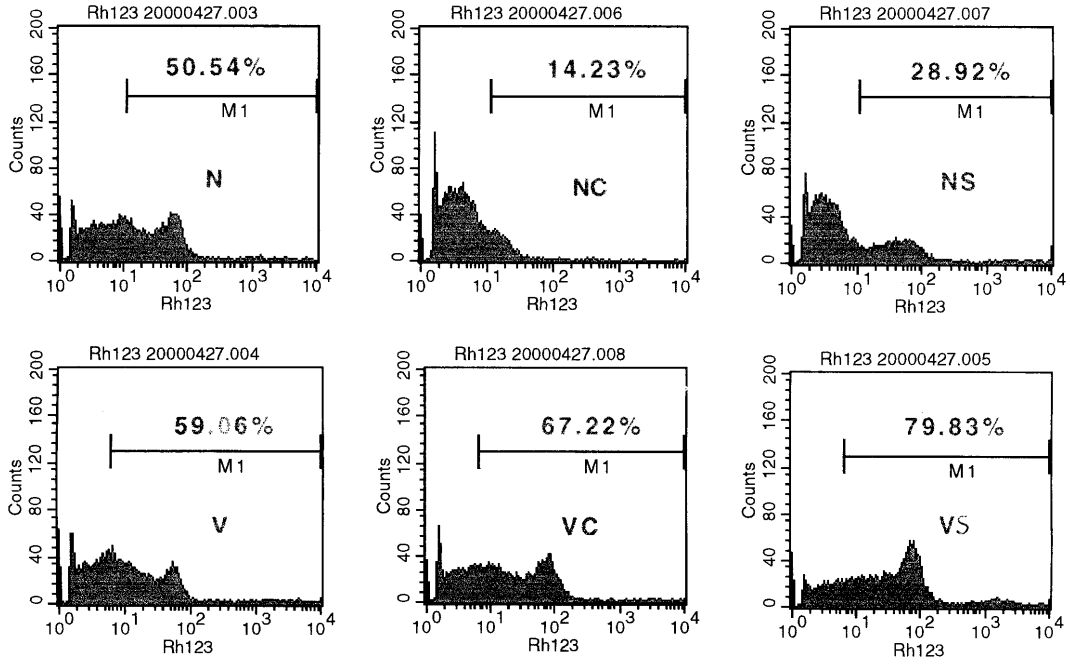


**Fig. 3**

**Effect of HSV-1 infection, sorbitol and ceramide on the potential of the inner mitochondrial membrane of primary cultures of neuronal cells**

The potential was assayed at 10 hrs p.i. The percentage of depolarized cells in individual experimental groups is shown in parentheses. Mock-infected cells treated with sorbitol (NS, 71.08%); (mock-infected cells treated with ceramide (NC, 85.77%); infected cells (V, 40.94%); infected cells treated with ceramide (VC, 32.78%); infected cells treated with sorbitol (VS, 20.17%).

In the studies on the interaction of HSV-1 and apoptosis, Zhou and Roizman (2000) had demonstrated that (i) the HSV-1 *d*120 mutant induced apoptosis by a CAS-independent pathway as CAS-3 was not activated and DNA fragmentation was not blocked by CAS inhibitors, even though the virus caused cytochome *c* release and depolarization of the inner mitochondrial membrane, (ii) the cells infected with wild-type HSV-1 did not exhibit any signs of apoptosis, (iii) the uninfected cells exposed to a higher concentration of sorbitol succumbed to CAS-dependent apoptosis as cytochrome c release and inner mitochondrial potential were blocked, CAS-3 was activated, and chromosomal DNA was fragmented, and (iv) although CAS-3 was activated in cells infected with wild-type HSV-1 and exposed to sorbitol, cytochrome *c* outflow, depolarization of inner mitochondrial membrane and DNA fragmentation were blocked. They concluded that HSV-1 mutant *d*120 induces apoptosis by a CAS-independent pathway, the wild-type virus blocks apoptosis induced by CAS-independent as well as CAS-dependent pathway (induced by osmotic shock). They suggested that the block in the CAS-dependent pathway may occur downstream of the CAS-3 activation step.

There are several reports indicating the ability of HSV-1 infection to protect cells against apoptosis induced by the virus itself, tumor necrosis factor α (TNF-α), antibody to Fas, C2-ceramide, and non-specific stress agents, such as osmotic (sorbitol) or thermic shock. Galvan and Roizman (1998) have shown that the HSV-1 mutant d120 induces apoptosis and wild-type virus blocks the realization of the apoptotic program triggered by expression of viral genes, Fas and TNF-α or non-specific stress agents. It is quite interesting that the mutant d120 lacking the genes encoding ICP4, the major viral regulatory protein induces a CAS-independent pathway of apoptosis in human SK-N-SH neuroblastoma cell line. However, the apoptosis induced by the same mutant in the HEp-2 cell line is CAS-dependent (Galvan *et al*., 2000).

In this study, we investigated CAS-3 activity in primary cultures of cortical neuronal cells of fetal mice, in which apoptosis was induced by ceramide or sorbitol and blocked by HSV-1 infection. The salient features of the data are as follows.

(i) The infection with laboratory-adapted strain of HSV-1 (Stoker strain) blocked the apoptosis induced by sorbitol or ceramide in primary culture of cortical neuronal cells.

(ii) CAS-3 activity could be detected in uninfected cells, in cells exposed to sorbitol, and in cells both infected and exposed to sorbitol. The CAS-3 activity in mock-infected

and sorbitol-treated cells was higher than that in uninfected cells. However, the CAS-3 activity in infected cells was lower than that in uninfected or uninfected and sorbitol-treated cells. CAS-3 activity in the cells undergoing apoptosis induced by sorbitol was inhibited by DEVD-fmk, an inhibitor of CAS-3. On the basis of these results we suggest that the apoptosis induced by sorbitol was CAS-3-dependent. CAS-3 activity was lower in the infected cells and in the infected and sorbitol-treated cells. Here we suggest that HSV-1 could block the CAS-3-denpendent apoptosis.

(iii) In the cells exposed to sorbitol or ceramide, the depolarization of inner mitochondrial membrane was higher in comparison with that in the cells without any treatment. However, the depolarization was unchanged in the infected cells or in the infected and sorbitol- or ceramide-treated cells.

On the basis of these results we can draw following conclusions. HSV-1 blocked the CAS-3-dependent apoptosis. This finding corresponds to those of other authors (Kohler *et al*., 2002; Shi, 2002; Vaughan *et al*., 2002). HSV-1 blocked also the depolarization of the inner mitochondrial membrane of primary cultures of fetal mouse neuronal cells induced by sorbitol or ceramide. The HSV-1 effects may be related to the virus virulence, time of infection and state of cells.

Further investigation of viral functions that block apoptotic pathways in host cells and pathogenesis of viral infections particularly in CNS may contribute to the progress in the therapy of these diseases (Distelhorst, 2002; Nicotera, 2002; Troy and Salvesen, 2002).

## References


Adams J, Cory S (1998): The bcl-2 protein family: arbiters of cell survival. *Science* **281**, 1332–1326.

Andrieu-Abadie N, Gouaze V, Salvayre R, Levade T (2001): Ceramide in apoptosis signaling: relationship with oxidative stress. *Free. Radic. Biol. Med*. **31**, 717–728.

Castedo M, Ferri K, Rouier T, Metivier D, Zamzami N, Kroemer G (2002): Quantification of mitochondrial alteration associated with apoptosis. *J. Immumol. Methods*. **256**, 39–47.

Daniel PT (2000): Dissecting the pathways to death. *Leukemia* **14**, 2035–2044.

Derfuss T, Fickenscher H, Kraft MS, Henning G, Lengenfelder D, Fleckenstein B, Meinl E (1998): Antiapoptosis activity of the herpesvirus saimiri-encoded Bcl-2 homolog: stabilization of mitochondria and inhibition of caspase-3-like activity. *J. Virol*. **72**, 5897–5904.

Distelhorst CW (2002): Recent insights into the mechanism of glucocorticosteroid-induced apoptosis. *Cell Death Differ*. **9**, 6–19.

Galvan V, Brandimarti R, Munger J, Roizman B (2000): Bcl-2 blocks a caspase-dependent pathway of apoptosis activated by herpes simplex virus 1 infection in HEp-2 cells. *J. Virol*. **74**, 1931–1938.

Galvan V, Brandimarti R, Roizman B (1999): Herpes simplex virus 1 blocks caspase-3-independent and caspase-dependent pathways to cell death. *J. Virol*. **73**, 3219–3226.

Galvan V, Roizman B (1998): Herpes simplex virus 1 induces and blocks apoptosis at multiple steps during infection and protects cells from exogenous inducers in a cell-type-dependent manner. *Proc. Natl. Acad. Sci. USA* **95**, 3931–3936.

Goswami R, Dowson G (2000): Does ceramide play a role in neural cell apoptosis? *J. Neurosci. Res*. **60**, 141–149.

Jerome KR, Fox R, Chen Z, Sears AE, Lee H, Corey L (1999): Herpes simplex virus inhibits apoptosis though the action of two genes, Us5 and Us3. *J. Virol.* **73**, 8950–8957.

Kohler C, Orrenius S, Zhivotovsky B (2002): Evaluation of caspase activity in apoptotic cells. *J. Immunol. Methods* **265**, 97–110.

Martin SJ, Gamble SR (1995): Protease activation during apoptosis: death by a thousand cuts? *Cell* **82**, 349–352.

McGahon AJ, Martin SJ, Bissonnette RP, Mahboubi A, Shi Y, Mogil RJ, Nishioka WK, Green DR (1995): The end of the (cell) line: methods for the study of apoptosis in vitro. *Methods Cell. Biol*. **46**, 153–185.

Nicotera P (2002): Apoptosis and age-related disorders: role of caspase-dependent and caspase-independent pathways. *Toxicol. Lett.* **127**, 189–195.

Pettmann B, Louis JC, Sensenbrenner M (1979): Morphological and biochemical maturation of neurons cultured in the absense of glial cells. *Nature* **281**, 378–380.

Ravangnan L, Roumier T, Kroemer G (2002): Mitochondria, the killer organells and their weapons. *J. Cell. Physiol.* **192**,131–137.

Rosette C, Karin M (1996): Ultraviolet light and osmotic stress: activation of the JNK cascade though multiple growth factors and cytokine receptors. *Science* **274**, 1194–1197.

Shi Y (2000): Mechanisms of caspase activation and inhibition during apoptosis. *Mol. Cell.* **9**, 459–470.

Toman R, Spiegel S, Faden A (2000): Role of ceramide in neuronal cell death and differentiation. *J. Neurotrauma* **17**, 891–898.

Troy CM, Salvesen GS (2002): Caspases on the brain. *J. Neurosci. Res.* **69**, 145–150.

Vaughan AT, Betti CJ, Villalobos MJ (2002): Surviving apoptosis. *Apoptosis* **7**, 173–177.

Wang JW, Wang DX, Feng ZJ, Wang RJ (2001a): Influence of HSV-1 infection on apoptosis of the primary cultured fetal mice cortical neurons. *Chin. J. Nneurol*. **34**, 39–42.

Wang JW, Wang DX, Feng ZJ,Wang RJ (2001b): Regulatory effect of Bcl-2 on the primary cultured mice cortical neurons with HSV-1 infection. *Chin. J. Exp. Clinic. Virol.* **15**, 228–230.

Wang RJ, Wang DX, Feng ZJ (2001): Osmotic stress induces neurons apoptosis. *Chin. J. Clinic. Neurosci.* **9**, 114–116.

Zhou G, Roizman B (2000): Wild-type herpes simplex virus 1 blocks programmed cell death and release of cytochrome c but not the translocation of mitochondrial apoptosis-inducing factor to the nuclei of human embryonic lung fibroblasts. *J. Virol.* **74**, 9048–9053.