Effect of baculovirus P35 protein on apoptosis in brain tissue of rats with acute cerebral infarction

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ABSTRACT. We explored the effect of baculovirus P35 protein on apoptosis in the brain tissue of rats with acute cerebral infarction (ACI). A rat model of middle cerebral artery infarction was created. The rats were randomly divided into sham, model, and treatment groups. Baculovirus P35 protein was injected into the intracranial arteries of the treatment group rats. The rats in the model group were given an equal volume of phosphate-buffered saline. The rats were sacrificed after 72 h and the brain tissue was separated. The levels of caspase-3, Bcl-2, and Bax mRNA, the brain cell apoptosis index, and the infarct size were determined. After 72 h, the levels of caspase-3 and Bax mRNA in the model and treatment groups were significantly greater than in the sham group, and the levels of Bcl-2 mRNA were significantly smaller (P < 0.05). The levels of caspase-3 and Bax mRNA in the model and treatment groups were significantly lower in the treatment group than in the model group, and the level of Bcl-2 mRNA was significantly greater (P < 0.05). Compared with the sham group, the brain tissue apoptosis index and the cerebral infarction area increased significantly in the model and treatment groups (P < 0.05). The brain tissue apoptosis index and cerebral infarction area in the treatment group were significantly lower than in the model group.
(P < 0.05). Baculovirus P35 protein can effectively inhibit brain cell apoptosis in rats with ACI. It delayed apoptosis and necrosis in subjects with ACI tissue and had a protective effect on brain tissue.

**Key words:** Acute cerebral infarction; Baculovirus P35 protein; Apoptosis

**INTRODUCTION**

Acute cerebral infarction (ACI) is a common clinical cardiovascular, cerebrovascular, and nervous system disease in the elderly. The incidence and mortality rates are high, and it has become a serious threat to human health. The pathological basis of ACI is as follows: acute cerebral ischemia occurs, focal cerebral microvascular endothelial cells are damaged, and the function of the blood-brain barrier is lost; thus, cell apoptosis and necrosis occur in the focal ischemic cerebral tissue (Hsieh and Chiou, 2014; Mokin et al., 2014). In the acute phase of cerebral infarction, neurons in the central belt of the ischemic region experience irreversible necrosis within several minutes. The corresponding symptoms of neural function defects appear, leading to neuron apoptosis and loss of biological function. Therefore, cell apoptosis plays an important role in the occurrence and development of acute cerebral infarction. Domestic and international research has shown that apoptosis levels in cerebral cells and neurons significantly increase in patients with ACI or in the cerebral tissue of animal models (Cevik et al., 2013; Olavarría et al., 2014). The baculoviruses were among the first viruses in which the ability to regulate the apoptosis behavior of the host was discovered. Research into the genes involved in apoptosis regulation could dramatically extend our understanding of the biological effects and molecular mechanisms of apoptosis caused by viral infection. The discoveries of the baculovirus P35 gene and P35 protein are important achievements in the field of anti-apoptosis. Their role in anti-apoptosis was discovered earlier this century and their wide-ranging effects have attracted researchers’ attention (Sahdev et al., 2010; Tian et al., 2010). Research has shown that the P35 protein can react with a certain conserved component in the apoptosis pathway, namely caspase. The P35 protein can directly inhibit active caspase and the activation of caspase in vivo. The research confirmed that caspase is also the central link in the apoptosis pathway. The P35 protein can inhibit the activities of caspase-1, -3, -6, -7, -8, and -10 in mammals. Caspase-3 can interact on the cell surface and play an important role in the mediation of cell apoptosis owing to its involvement in the receptor and mitochondrial apoptosis pathways (Yu, 2011; Yang et al., 2013; Fan et al., 2014). Based on the above theories, this study explored the inhibitory effect of baculovirus P35 protein on caspase-3 in the brain tissue of rats with ACI and cell apoptosis at an animal level. We aimed to provide a new therapeutic approach for the clinical treatment of ACI.

**MATERIAL AND METHODS**

**Establishment of ACI model and grouping**

Sprague Dawley rats (180–200 g) were purchased from the animal center of LAIKE Co., Shanghai, China. The rat models of the middle cerebral artery infarction were created using the Longa method (Yang et al., 2013). The rats showed listlessness, Horner’s syndrome...
on the same side, drooping of the contralateral forelimb, adduction and internal rotation, and spontaneous circling on the affected side for 2 h after the operation, which illustrated that model creation had been successful. If model creation failed, the rat was eliminated.

The experiment was divided into three groups: sham, model, and treatment groups, with 20 rats in each group. In the sham operation group, the vessel was separated but there was no suture occlusion. The rats in the model and treatment groups were operated on according to the model creation method. After the rats had been monitored for 3 h to verify the success of the model creation, the rats in the treatment group were immediately injected in their tail veins with 30 mg/kg baculovirus P35 protein. The rats in the model and sham operation groups were given an intravenous injection of an equal volume of phosphate-buffered saline. All indices were detected after 72 h. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA), 8th Edition, 2010. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Xinxiang Central Hospital.

Reverse transcription-polymerase chain reaction (RT-PCR)

The rats in the three groups were killed and brain tissue (0.1 g) was removed. The total RNA was extracted according to the manufacturer instructions of the RNA extract kit (TaKaRa, Dalian, China). The brain tissue was transferred to pre-cooled mortar. Liquid nitrogen was added to grind into powder. Cell lysate (400 µL) was added. After the samples had been repeatedly aspirated using a syringe with a No. 21-25 needle 8-10 times, they were transferred to a 1.5-mL centrifuge tube (provided with the kit). Neutralization solution (150 µL) was added and the samples underwent vortex oscillation for 15-30 s, followed by centrifugation at 12,000 g for 5 min at 4°C. The supernatant was removed to a 1.5-mL centrifuge tube. Iso-propanol (250 µL) was added and uniformly mixed. In accordance with the kit manufacturer instructions, the subsequent steps followed the centrifugal method. The evenly mixed liquid was transferred to a preparation tube, which was placed in a 2-mL centrifuge tube and centrifuged at 6000 g for 1 min at 4°C. The filtrate was discarded. The preparation tube was placed back in the 2-mL centrifuge tube. Washing liquid (500 µL) was added and the sample was centrifuged at 12,000 g for 1 min. The filtrate was discarded. The preparation tube was placed back in the 2-mL centrifuge tube. Desalination solution (700 µL) was added to the preparation tube, which was centrifuged at 12,000 g for 1 min. The steps were repeated and the filtrate was discarded. The preparation tube was placed back in the 2-mL centrifuge tube, which was centrifuged at 12,000 g for 1 min. The preparation tube was placed into the 1.5-mL centrifuge tube. Eluent (70-100 µL) was added to the center of the preparation tube, which was kept at room temperature for 1 min, centrifuged at 12,000 g for 1 min, and washed to obtain the RNA.

The following primers were designed according to the mRNA sequences of caspase-3, Bcl-2, and Bax provided by GeneBank: caspase-3-F: 5'-GGTATTGAGACAGACAGTGG-3'; caspase-3-R: 5'-CATGGGATCTGTTTCTTTGC-3'; β-actin-F: 5'-GCCGGAAATCGTGCGTAG-3'; β-actin-R: 5'-CGTCATACTCCTGCTTGCT-3'. The primers were diluted to 10 µM. First, the specificity and annealing temperatures of the primers were optimized. Then, the following reaction systems were prepared: 10 µL 2X SYBR Green general type quantitative PCR (qPCR) Master Mix (Roche, Basel, Switzerland), each with 0.6 µL upstream/downstream primers (10 µM) and 8.8 µL 1:100 diluted cDNA. The total reaction volume was
20 µL. The reaction mixture was centrifuged at 2000 g. The PCR was carried out as follows: pre-degeneration at 95°C for 30 s; degeneration at 95°C for 3 s; and annealing extension at 60°C for 30 s, for 30 cycles. The dissolution curve was constructed. Finally, the data were directly read from the ABI 7500 real-time fluorescence quantitative PCR instrument (Applied Biosystems, Foster City, CA, USA).

**Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-staining method**

The brain tissue slices of sacrificed rats from the three groups were taken, dewaxed by antigen repairing, and washed with PBST (phosphate-buffered saline with Tween 20) three times. The operation was carried out strictly according to the manufacturer instructions provided with the TUNEL kit (Roche). The magnification of the specimens was 400X. Ten visual fields were randomly selected in the infarction area and surrounding regions. The brain cell apoptosis index was calculated from the proportion of apoptotic cells.

**2,3,5-Triphenyltetrazolium chloride (TTC) analysis**

After the rats had been sacrificed, the olfactory bulb, the cerebellum, and the brain-stem were resected. The remaining tissue was made into coronal sections. The sections were placed into 2% TTC phosphate buffer (Sigma, St. Louis, MO, USA) and stained at 37°C for 20 min. They were then fixed using paraformaldehyde. The normal tissue was red and the infarction tissue was white; the infarction tissue was separated from the normal brain tissue under a microscope. The wet mass of the normal and infarction tissues was determined using an analytical balance. The infarction range was the infarction tissue mass expressed as a percentage of the total tissue mass.

**Statistical analysis**

All data were analyzed using the SPSS 17 statistical software (SPSS Inc., Chicago, IL, USA). The measured data are reported as means ± SD. The measured data were compared by analysis of variance between multiple groups. Comparisons between pairs of groups were carried out using the least significant difference method. P < 0.05 was considered to be statistically significant.

**RESULTS**

**Change of caspase-3, Bcl-2, and Bax mRNA levels in the rat brain tissue**

The levels of caspase-3, Bcl-2, and Bax mRNA in the brain tissue of the rats in the three groups were analyzed by real-time fluorescence quantitative PCR. The results showed that, compared with the sham group, the levels of caspase-3 and Bax mRNA increased significantly in the model and treatment groups (P < 0.05), while the level of Bcl-2 mRNA decreased significantly (P < 0.05). Compared with the model group, the levels of caspase-3 and Bax mRNA decreased significantly in the treatment group (P < 0.05), while the level of Bcl-2 mRNA increased significantly (P < 0.05; Figure 1).
Change of level of apoptosis in the brain tissue of rats in the three groups

This study analyzed cell apoptosis in the brain tissue of the rats in the different groups using TUNEL staining. The results are shown in Figure 2. Cell apoptosis was obvious in the model group. The number of apoptotic cells decreased significantly after treatment with the baculovirus P35 protein. The quantitative analysis of apoptosis in the three groups showed that, compared with the sham group, the level of apoptosis increased significantly in the model and treatment groups (P < 0.05). The level of apoptosis was significantly lower in the treatment group than in the model group (P < 0.05).

Figure 1. Change of caspase-3, Bcl-2, and Bax mRNA levels in the brain tissue of rats in the three groups. A. Caspase-3 mRNA in the sham, model, and treatment groups. B. Bax mRNA in the sham, model, and treatment groups. C. Bcl-2 mRNA in the sham, model, and treatment groups. *P < 0.05.

Figure 2. Change of level of apoptosis in the brain tissue of rats in the three groups. A. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining used to indicate apoptosis in the sham, model, and treatment groups. B. Quantitative analysis of the apoptosis index in the sham, model, and treatment groups. *P < 0.05.
Change of brain infarction range of rats in the three groups

Compared with the sham group, the infarction range was obviously greater in the model and treatment groups, and the difference was statistically significant (P < 0.05). The infarction range was significantly smaller in the treatment group than in the model group after treatment with baculovirus P35 protein (P < 0.05). However, compared with the sham group, the infarction range in the treatment group was still high, and the difference was statistically significant (P < 0.05; Figure 3).

**Figure 3.** Change of brain infarction range of the rats in the three groups. **A.** 2,3,5-Triphenyltetrazolium chloride (TTC) staining used to indicate infarction in the sham, model, and treatment groups. **B.** Quantitative analysis of the brain infarction range of rats in the sham, model, and treatment groups. *P < 0.05.

**DISCUSSION**

In ACI, depending on the degree of ischemia and hypoxia, necrosis and apoptosis may occur in the cells. The cells in the ischemic core area soon die as a result of serious hypoxia and hypoxia for an extended period. It is an irreversible process. In the ischemic penumbra
region, located around the ischemic focus, the degree of ischemia is relatively low. In addition, collateral circulation is established in time. A certain blood perfusion can be maintained after ischemic injury for a certain period of time. Although normal physiological function cannot be maintained, synthesis of some proteins and expression of some genes is possible. The route to cell death is usually cell apoptosis. Even if the function is damaged, it is still a reversible process. If the protective effects of a drug take effect in time, the apoptosis will be reduced, but the process of cell death will also be delayed. Once the blood supply is restored, death can be avoided. Otherwise, irreversible death will occur and the focal infarction will gradually extend to the surrounding tissue (Higgins et al., 2014; Power et al., 2014).

The P35 protein is an encoded protein that exhibits anti-apoptotic activity after the baculovirus invades host cells. It can inhibit cell apoptosis in mammalian, insect, and nematodermatophilic species, and is a widely effective inhibitor of apoptosis. Research has shown that P35 protein can combine the conserved constituent caspase in the apoptosis pathway and interact with it so as to inhibit its activity (Guy and Friesen, 2008; Brand et al., 2012). In vitro study results have shown that the P35 protein can form a complex that has steric stability with respect to its target molecules and can inhibit the activity of caspase in a competitive binding mode. Moreover, the P35 protein can be cut by the target caspase at a specific location; the P35 protein can then combine with caspase more strongly after cutting. The cleavage product can form the CED-3/ICE-P35 complex with CED-3/ICE protease, thereby inhibiting the action of CED-3/ICE, and further inhibiting cell apoptosis. Caspase-3, which is a member of the cysteine protease family, is the key enzyme involved in cell apoptosis. It can activate the cell apoptosis pathway by degrading intracellular substrates, which leads to cell apoptosis. Therefore, it is called a “molecular switch”. A large number of experimental studies have shown that caspase-3 plays an important role in the pathological process of myocardial and cerebral ischemia. It has attracted a great deal of attention, mainly for its role in the final common pathway of apoptosis (Yu et al., 2006; Kester and Nambu, 2011). The results of this study showed that the expression level of caspase-3 mRNA decreased significantly after the P35 protein was injected into the rats with ACI, which further confirms that the P35 protein has a certain inhibitory effect on the apoptosis pathway of brain tissue in rats following treatment.

The Bcl-2 family has the main switching role in regulating the cell death signaling pathway. Under normal conditions, the expression levels of Bcl-2 and Bax are relatively stable. However, when Bax is overexpressed, the number of Bax dimers increases significantly, the cells are stimulated, and apoptosis is initiated. When Bcl-2 is highly expressed, the Bax dimers dissociate and form more stable Bcl-2 and Bax complexes, resulting in cellular life extension (Walensky, 2006; Liang et al., 2014; Pan et al., 2014). Therefore, the proportion of Bcl-2/Bax plays an important role in maintaining the cell survival process. The results of this study showed that after the P35 protein was injected, the level of Bcl-2 mRNA significantly increased in the brain tissue of rats with ACI, while the level of Bax mRNA decreased significantly, so that the ratio of Bcl-2/Bax was more conducive to the survival of cells. It has been confirmed that the P35 protein can reduce the expression of Bax, thereby inhibiting cell apoptosis. In addition, the analysis results of the overall apoptosis level and infarction range in the brain tissue of the rats showed that the cell apoptosis ratio was significantly lower than in the model group after treatment with the P35 protein. Although the ratio was still significantly higher than that in the sham operation group, the P35 protein protected brain tissue to a large extent.
In short, baculovirus P35 protein can significantly inhibit cell apoptosis in the brain tissue of rats with ACI. This provides a solid theoretical basis for the use of P35 protein in the treatment of acute myocardial infarction.

Conflicts of interest

The authors declare no conflict of interest.

REFERENCES


