



Neuroprotective effects of Bcl-2 overexpression on nerve cells of rats with acute cerebral infarction

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ABSTRACT. We aimed to investigate the influence of lentiviral-mediated Bcl-2 overexpression in cerebral tissues of rats with acute cerebral infarction. Forty-five rats were randomly divided into sham, model, and treatment groups. The sham and model groups were administered a control lentiviral vector via the intracranial arteries 10 days before surgery, while the treatment group received lentivirus encoding a Bcl-2 overexpression vector. We induced cerebral artery infarction using a suture-occlusion method and analyzed the cerebral expression levels of apoptosis-related genes (caspase-3, Bax), total cerebral apoptosis, range of cerebral tissue infarction, and changes in nerve cell function

after 72 h. The Bcl-2-encoding lentivirus was well expressed in rat cerebral tissues. The treatment group had significantly higher expression levels of Bcl-2 than the other two groups. After cerebral infarction, the model group had significantly increased expression levels of caspase-3 and Bax protein in cerebral tissues than the sham ($P < 0.05$). Expression of these apoptosis-related proteins in the treatment group was obviously lower than that in the model group ($P < 0.05$), but significantly higher than in the sham group ($P < 0.05$). Compared to sham, neuronal apoptosis levels and infarction range of cerebral tissues was increased in the model and treatment groups; however, these values in the treatment group were significantly lower than that in the model group ($P < 0.05$). Importantly, the treatment group had significantly decreased neurological impairment scores ($P < 0.05$). In conclusion, Bcl-2 overexpression can decrease neuronal apoptosis in rat cerebral tissue, and thus is neuroprotective after cerebral ischemia.

Key words: Acute cerebral infarction; Bcl-2; Apoptosis

INTRODUCTION

Acute cerebral infarction has a high rate of incidence and fatality, and is becoming one of the most threatening diseases to human health (Arboix and Alio, 2012). When acute cerebral infarction occurs, the coronary artery is blocked, interrupting blood flow through the vessels supplying the brain and causing necrosis or malacia of cerebral tissues via ischemia. However, an ischemic penumbra exists as a reversible necrotic area between normal cerebral tissues and the ischemic center, where tissues present with irreversible functional impairment. Cells within the penumbra have normal structure and are morphologically apoptotic (Charriaut-Marlangue, 2004; Hsieh and Chiou, 2014). Cell apoptosis is a process by which genes regulate programmed cell death. B-cell lymphoma-2 (Bcl-2) is a protein that consists of 239 amino acids and functions primarily in inhibiting cell apoptosis (Roset et al., 2007; Theofilas et al., 2009). Currently, numerous studies indicate that the downregulation of Bcl-2 gene expression and protein levels in cerebral tissues of patients with acute cerebral infarction causes homodimerization of Bax, a member of Bcl-2 protein family, which results in significantly increased levels of apoptosis and aggravates the impairment of nerve function (Youle and Strasser, 2008; Wang et al., 2012). Therefore, inhibition of cellular apoptosis within the cerebral infarction penumbra could protect nerve function. Based on these observations and the role of Bcl-2 in inhibiting cellular apoptosis, the present study analyzed the influence of Bcl-2 overexpression, mediated by lentiviral delivery with especially high expression in cerebral tissues of rats with acute cerebral infarction, on nerve function and aimed to provide a theoretical basis for clinical treatment of acute cerebral infarction.

MATERIAL AND METHODS

Model establishment and treatment of rats with acute cerebral infarction

Sprague Dawley rats (180-220 g) were purchased from Slac Laboratory Animal Center, Shanghai, China. We utilized the model of medium-sized artery infarction in the brains of rats

established by Longa et al. (1989). Two hours after surgery, rats presented apathetic ipsilateral Horner's syndrome, contralateral forelimb sagging, adduction, and internal rotation and spontaneous circling to the affected side, consistent with successful induction of cerebral infarction. Rats that did not display these behaviors were excluded from the study. Rats were divided into three groups: sham, model, and treatment groups, with 15 rats in each group. In the sham group, rats' vessels were separated without line embolism. Ten days before surgery, the sham and model groups were administered a control lentivirus via the intracranial arteries while a lentivirus encoding Bcl-2 was administered to the treatment group. 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 (Eighth Edition, 2010; Bethesda, MD, USA). The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Affiliated Bayi Brain Hospital, The Military General Hospital of Beijing PLA.

Western blot analysis

Cerebral tissues were dissected out 72 h after the infarction, placed in liquid nitrogen, ground with a mortar, and boiled in 1X loading buffer in a metal bath at 100°C for 10 min. The sample was centrifuged at 12,000 rpm/min for 5 min and then used to conduct SDS-PAGE. Later, the protein was transferred onto a PVDF membrane and embedded with 5% skimmed milk powder, to be enveloped overnight with caspase-3 and Bax primary antibodies (Santa-Cruz, CA, USA), and rinsed three times with PBST. It was then incubated for 1 h with a HRP-coupled goat anti-mouse secondary antibody (ORI Gene, Beijing, China) at room temperature, washed 3 x 5 min with PBST, and luminescent liquid was added for color development and imaging. With β -actin (ORI Gene, Beijing, China) as an internal reference, the grey-computing software was used to analyze the grey scale of the targeted protein band, and calculate the relative expression of the targeted protein.

TUNEL analysis

Rats were sacrificed 72 h after infarction and cerebral tissues were dissected out, embedded in OCT, cut into 7- μ m thick slices with a freezing microtome, and then mounted on microscope slides. Next, we adhered to the manufacturer instructions of the TUNEL kit (Vazyme Biotech Co., Ltd., Nanjing, China) as follows: slides were fixed with a 4% paraformaldehyde solution (dissolved in PBS), treated with 2 mg/mL Proteinase K, and 100 μ L 1X Equilibration Buffer was applied to the specimen. After 30 min of incubation, the liquid was removed. TdT incubation buffer (50 μ L) was applied to the specimen for 60 min of incubation at 37°C. The specimen was then washed 3 times 5X min with PBS. The PBS solution around and on the back-side of the sample was then sequestered with filter paper. The sample was analyzed immediately under a fluorescence microscope (Olympus, Tokyo, Japan) at 400X magnification. Ten visual fields were randomly selected in the infarction and surrounding areas to assess the proportion of apoptotic to normal cells, which would then be taken as the cellular apoptosis index.

TTC staining analysis

Rats were sacrificed 72 h after infarction and cerebral tissues were dissected and sliced coronally. The slices were incubated into 2% TTC phosphate buffer at 37°C for 20 min

of staining, and then fixed with paraformaldehyde. Normal tissues were red while infarct tissues were white. They were separated under a microscope (Olympus, Tokyo, Japan), and their wet weights were obtained with an analytical balance. Infarction range is the percentage of mass of infarction tissues compared to that of normal cerebral tissues.

Analysis of rat neural function

Analysis of functional nerve impairment for the three groups was carried out 12, 24, and 48 h after surgery based on the Bederson standard stroke assessment scale (Belayev et al., 1996). According to this scale: no functional nerve impairment = 0, incomplete stretching of the right front paw = 1, circling to the left = 2, inclining = 3, inability to spontaneously walk and loss of consciousness = 4.

Statistical analysis

All data were analyzed with the SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA), and measured data are reported as means \pm SD. The comparisons of inter-group data were conducted with variance analyses, and pairwise comparisons between groups were carried out with LSD. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Comparison of Bcl-2 expression levels in cerebral tissues from rats in the three groups

As shown in Figure 1, the Bcl-2 vector was delivered by lentivirus in the treatment group, and Bcl-2 protein was expressed at high levels in the cerebral tissues of these animals when compared to the sham and model groups. The analysis of Bcl-2 protein levels for the three groups revealed that the treatment group had higher expression levels of Bcl-2 in cerebral tissues than both the sham and model groups ($P < 0.05$).

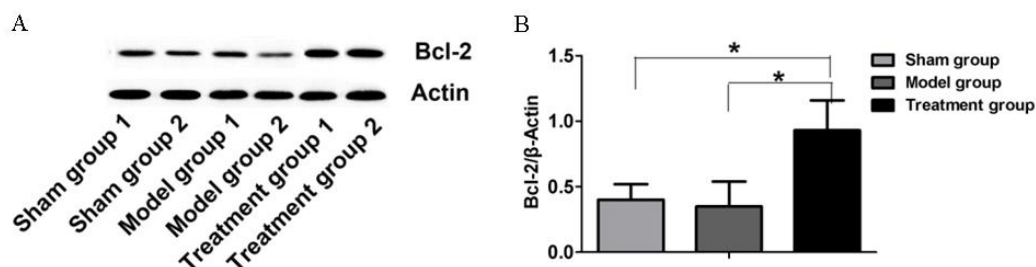


Figure 1. Bcl-2 protein in three groups. **A.** Western blot showed Bcl-2 protein in sham group, model group and treatment group; **B.** Quantitative analysis of Bcl-2 in sham group, model group and treatment group.

Comparison of caspase-3 and Bax levels in cerebral tissues from the three groups

Compared to the sham group, the model and treatment groups had significantly increased expression levels of caspase-3 and Bax proteins ($P < 0.05$). In rats with high expres-

sion levels of Bcl-2 mediated by lentivirus delivery (treatment group), the levels of caspase-3 and Bax protein in cerebral tissues were significantly lower compared to the model group ($P < 0.05$) (Figure 2).

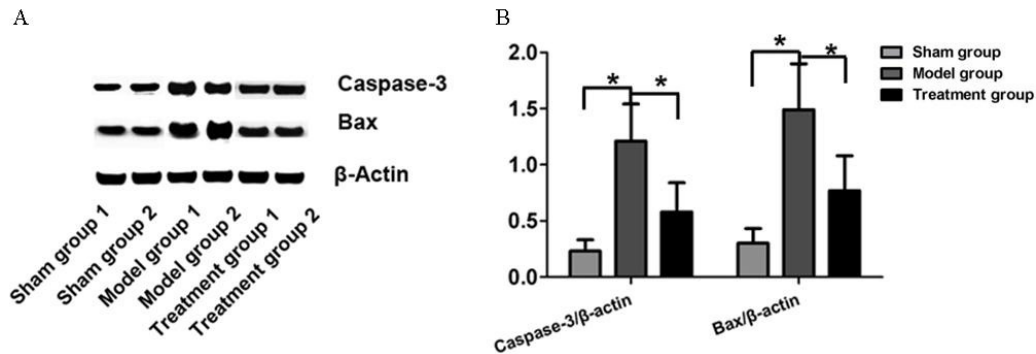


Figure 2. Caspase-3 and Bax protein in three groups. **A.** Western blot showed Caspase-3 and Bax protein in sham group, model group, and treatment group; **B.** Quantitative analysis of Caspase-3 and Bax in sham group, model group and treatment group.

Comparison of neuronal apoptosis in cerebral tissues and cerebral infarction range in the three groups

Cerebral tissue apoptosis and infarction range were markedly increased 72 h after infarction, while the amount of apoptosis and infarction range in cerebral tissues from animals overexpressing Bcl-2 were significantly smaller (Figure 3A-C). Quantitative analysis of apoptosis level and infarction range indicated that compared to the sham group, the model group had a significantly increased apoptosis index and infarction range in cerebral tissues ($P < 0.05$), while these were significantly lower in the treatment than the model group ($P < 0.05$) but still higher than in the sham group ($P < 0.05$) (Figure 3B-D).

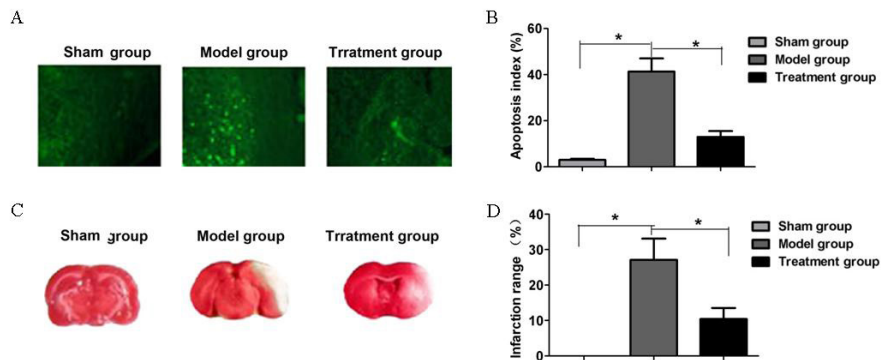


Figure 3. Comparison of apoptosis index and infarction range in three groups. **A.** TUNEL staining showed apoptosis of brain tissue in sham group, model group and treatment group; **B.** TTC staining showed infarction range of brain tissue in sham group, model group and treatment group; **C.** Quantitative analysis of apoptosis of brain tissue in sham group, model group and treatment group by TCC staining. **D.** Quantitative analysis of infarction range of brain tissue in sham group, model group and treatment group by TTC staining.

Comparison of nerve impairment degree in the three groups

As shown in Table 1, the evaluation of nerve impairment degrees of the three groups of rats suggested that the model and treatment groups had an obviously increased nerve impairment degree after ischemia than the sham group, but the treatment group had a markedly lower degree than the model group due to the overexpression of Bcl-2 ($P < 0.05$). However, the nerve impairment degree of the treatment group was still significantly higher than that of the sham group ($P < 0.05$).

Table 1. Comparison of nervous functional defects in three groups.

Groups	Cases	Pre-treatment	Post-treatment		
			24 h	48 h	72 h
Sham group	15	0.32 ± 0.17	0.29 ± 0.35	0.19 ± 0.17	0.10 ± 0.09
Model group	15	3.58 ± 0.99*	3.17 ± 0.69*	2.95 ± 0.67*	2.18 ± 0.61*
Treatment group	15	3.79 ± 1.12*#	2.17 ± 0.45*#	1.43 ± 0.27*#	0.49 ± 0.11*#

Compared with sham group, * $P < 0.05$; Compared with treatment group, # $P < 0.05$.

DISCUSSION

Apoptosis, the process of programmed cell death (PCD), is precisely regulated by many genes and may occur under certain physiological or pathological conditions; it is a vital mechanism for cells to maintain self-stability (Elmore, 2007; Ouyang et al., 2012). The recent finding of cellular apoptosis in the penumbra region after acute cerebral infarction is critical to treating acute cerebral infarction as this process is reversible and can be inhibited to recover cellular function. As a result, effective inhibition of apoptosis in this region is of great significance for reducing apoptosis of cerebral cells and impairment of nerve function (Oostveen et al., 1998; Tanaka, 2013). Bcl-2 is the main inhibitory protein in the regulation of cellular apoptosis. However, research on patients with acute cerebral infarction shows that the expression of Bcl-2 in serum is significantly decreased, which suggests that these patients have reduced anti-apoptotic function and cellular apoptosis is destabilized (Salakou et al., 2007; Liang et al., 2014). Accordingly, the reconstruction of a stable apoptotic environment in cerebral tissues may inhibit the apoptosis of cells in the penumbra region of cerebral infarction to protect cerebral tissues. Based on this idea, we analyzed the influence of Bcl-2 overexpression on cerebral tissue apoptosis in rats with acute cerebral infarction.

In this study, we used a lentiviral approach to over-express Bcl-2 in the cerebral tissues of rats. Western blot indicated that the expression of Bcl-2 in cerebral tissues from the treatment group was significantly increased, which suggests that overexpression of Bcl-2 in cerebral tissues using a lentiviral approach can be achieved. The experiment further verified the influence of the overexpression of Bcl-2 on the expression of other apoptosis-related genes in rats. Caspase-3 is the most critical direct apoptosis mediator in the downstream caspase cascade reaction, whose activation depends on the release of cyt-c, while the Bcl-2 family, including Bcl-2, can mediate the release of cyt-c via mitochondrion (Yang et al., 2013; Fan et al., 2014). Existing basic and clinical experiments and research indicate that levels of caspase-3 and Bax are significantly increased in cerebral tissues and peripheral blood of rats and patients with acute cerebral infarction (Manabat et al., 2003; Liu et al., 2012). We found that

after overexpression of Bcl-2, which can inhibit apoptosis, caspase-3 and Bax protein levels in cerebral tissues decreased significantly compared to that in the model group. Thus, high expression levels of Bcl-2 could inhibit cellular apoptosis in rat cerebral tissues. This finding was further confirmed by TUNEL staining.

High expression levels of Bcl-2 inhibit cellular apoptosis in cerebral tissues of rats with acute cerebral infarction (Qiao et al., 2012). The inhibition of apoptosis may lead to the reversal of penumbra apoptosis in cerebral tissues, resulting in a possible reduction of cerebral tissue infarction area. Our results demonstrate that the cerebral tissue infarct area of Bcl-2-overexpressing rats was smaller than that of the model group, which is consistent with the phenomenon that reduced apoptosis protein and apoptosis index corresponds to a reduction in penumbra apoptosis. Due to the small area of cerebral tissue infarction and reduced cellular apoptosis, the degree of functional nerve impairment in Bcl-2-overexpressing rats was found to be significantly lower than that in the model group. However, it is important to note that the inhibition of apoptosis by overexpressed Bcl-2 is limited given that treatment rats had significantly higher levels of apoptosis and infarction in cerebral tissues, as well as greater functional nerve impairment than rats in the sham group.

In summary, the overexpression of Bcl-2 in cerebral tissues of rats with acute cerebral infarction can significantly inhibit cerebral apoptosis, reduce cerebral infarction range, and protect nerve function. Our research provides a theoretical basis for the development and research of drugs for the treatment of acute cerebral infarction.

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