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Effects of recombinant activated coagulation factor VII on apoptosis and expressions of Bcl-2 and Bax in rats with intracerebral hemorrhage

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Abstract. – OBJECTIVE: To investigate the effects of recombinant activated coagulation factor VII (rFVIIa) on apoptosis and the expressions of B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) in rats with intracerebral hemorrhage (ICH).

MATERIALS AND METHODS: A total of 90 8-week-old male Sprague-Dawley (SD) rats with similar weight were selected and randomly divided into normal group (n=30), ICH control group (n=30), and rFVIIa treatment group (n=30). Five days later, hematoxylin-eosin (HE) staining was applied to observe pathological changes in rat brain in three groups. Cell apoptosis in rat brain was detected at 6 h, 12 h, 24 h, 48 h, 72 h, and 120 h, respectively. The relative expression levels of Bcl-2 and Bax in brain tissues were measured via fluorescence quantitative Polymerase Chain Reaction (qPCR) and Western blotting, respectively.

RESULTS: Compared with those in ICH control group, rats in rFVIIa treatment group had fewer degenerated and necrotic nerve cells and milder pathological changes in the marginal zone. The number of apoptotic cells in ICH control group and rFVIIa group was gradually increased in a time-dependent manner, and achieved the peak at 72 h. The number of apoptotic cells in treatment group was significantly lower than that in ICH control group after 24 h ($p<0.05$). Both fluorescence qPCR and Western blotting results proved that in comparison with ICH control group, rFVIIa group had a higher relative expression level of Bcl-2 ($p<0.05$) and a lower expression level of Bax ($p<0.05$).

CONCLUSIONS: Apoptosis mechanism may be involved in secondary brain injury after ICH. RFVIIa may have an important protective effect on neuronal injury after ICH by promoting the expression of Bcl-2 and inhibiting the expression of Bax protein.

Key Words:
Recombinant activated coagulation factor VII, Intracerebral hemorrhage, Bcl-2, Bax

Introduction

Intracerebral hemorrhage (ICH) is a disease with high incidence and death rate. The continuous expansion of secondary hematoma and the edema of brain cells around the hematoma often occur within a few days after ICH. Since a relatively high incidence rate of adverse complications is observe in secondary brain injury after ICH, the effective treatment is urgently needed[1]. How to effectively reduce nerve damage after ICH and improve the life quality of affected patients are the focus of clinical research. The pathogenesis of ICH is relatively complex, in which the research on the interaction of various physiological and pathological changes and the molecular mechanism involved in the complications related to ICH is still lacked. In addition, the possible treatment methods for reducing complications are still relatively deficient. Therefore, reducing or preventing the generation of nerve cell apoptosis is an important measure for cerebral ischemia and hypoxia injury reduction and brain function recovery. Sufficient recombinant activated human coagulation factor VII (rFVIIa) has been widely used for the control of acute massive hemorrhage of haemophilia with inhibitory factors in clinical practice[2]. Scholars[3-5] have found that rFVIIa is very effective in controlling the increase of ICH intracranial hematoma and rapidly correcting abnormal coagulation function. Moreover, Friso et al[6] discovered that rFVIIa may be associated with the susceptibility of coronary artery disease. The above study shows that rFVIIa factor may play an important role in the occurrence and development of coagulation disorders of ICH. Currently, it is considered[7] that B-cell lymphoma 2 (Bcl-2) family proteins are involved in multi-step gene regula-

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tion of apoptosis. Among Bcl-2 family, Bcl-2, and Bcl-2-associated X protein (Bax) are closely related to apoptosis and expressed in the central nervous system. Edema of brain cells after ICH has certain influence on the content of Bcl-2 and Bax. This study constructed a rat model to investigate the effects of rFVIIa on apoptosis and the expressions of Bcl-2 and Bax in rats with ICH.

Materials and Methods

A total of 908-week-old male Sprague-Dawley (SD) rats weighing 210-260 g were selected. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) kits (Boster, Wuhan, China), rabbit anti-mouse Bcl-2 and Bax polyclonal antibodies, streptavidin-biotin complex (SABC) kits and diaminobenzidine (DAB) coloring kits (Thermo Fisher Scientific, Waltham, MA, USA) were used for detecting cell apoptosis. Chloral hydrate, phosphate-buffered saline (PBS) and other reagents were domestic pure grade products. Rat brain stereotaxic instrument (Tokyo, Japan) was used for *in vivo* experiment. This study was approved by the Animal Ethics Committee of the Affiliated Hospital of Nantong University Animal Center.

Grouping

These 90 rats were randomly divided into three groups, namely normal group, ICH control group, and rFVIIa treatment group. ICH control group and rFVIIa treatment group were further divided into 6 h, 12 h, 24 h, 48 h, 72 h, and 120 h subgroups, with 5 rats in each subgroup.

Preparation of ICH Models

Rats were weighed and given 10% chloral hydrate for anesthesia, fixed in the rat brain stereotaxic instrument and routinely disinfected. Then, an incision (about 10 mm long) was cut along the head midline to expose the anterior fontanelle. 50 **μL** in coagulable rat blood was collected and injected into the left caudate nucleus. After that, the incision was sutured. Rats in rFVIIa treatment group were intramuscularly injected with rFVIIa 5 min after the operation. Rats in normal group did not receive any drug and operative treatment.

Brain Specimen Collection and Histological Examination

Three groups of rats were anesthetized with 10% chloral hydrate and decapitated. Then, their brains were taken and fixed with 4% paraformaldehyde. After that, brain tissue samples were prepared, sectioned, subjected to hematoxylin-eosin (HE) staining, and observed under an optical microscope for histological changes.

Apoptosis Detection

Brain tissue sample sections were immersed in 3% H_2O_2 at room temperature for 10 min. Sections were immersed in labeling solution and blocking solution, and incubated with biotinylated anti-digoxin antibody for 30 min of reaction. Then, sections were counterstained with SABC and DAB, mounted and observed under the microscope. Under the microscope, cells with brown granules in the nucleus were apoptotic cells. For each section, the number of apoptotic cells was counted in five high power fields (× 400) in the bleeding side of the cortex, and the average was taken as the final number of apoptotic cells in the section.

Fluorescence Quantitative Polymerase Chain Reaction (qPCR) Analysis

Brain tissues were subjected to liquid nitrogen grinding, and sample ribonucleic acid (RNA) extraction was carried out using TRIzol (Invitrogen, Carlsbad, CA, USA). All operating steps were carried out in strict accordance with the instructions. 1 μg RNA was taken and subjected to reverse transcription reaction according to the instructions of reverse transcriptase kits to obtain complementary deoxyribonucleic acid (cDNA). The concentration of cDNA was adjusted, and the messenger RNA (mRNA) levels were determined using the CFX 96 PCR instrument (Bio-Rad, Hercules, CA, USA) according to the instructions of SYBR® Premix Ex Taq™ II kits (TaKaRa, Otsu, Shiga, Japan). The corresponding primer sequences were shown in Table I.

Table I. Primer sequences.

Gene name	Primer sequence
Bcl-2	5'-3' ATCCTGTGCTGCTATCC
	3'-5' CGTCCACGTTCTTCATT
Bax	5'-3' GGATGCGTCCACCAAGAA
	3'-5' GGGACATCAGTCGCTTCAGT
β-actin	5'-3' GTGGACATCCGCAAAGAC
	3'-5' GAAAGGGTGTAACGCAACTA

Western Blotting Analysis of the Expressed-Protein

Brain tissues were placed in liquid nitrogen for grinding, put on ice for 30 min of lysis, and centrifuged to collect the supernatant. Then, part of the supernatant was taken, and the protein content of each sample to be tested was adjusted to 100 μg. After that, the supernatant taken was added with 5× reducing sample loading buffer and boiled in boiling water for 10 min. Next, above sample solution was slowly added into the spotting hole of a prepared 10% polyacrylamide gel using a microsyringe. Samples were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at a voltage of 80 V. After the completion of electrophoresis, the sample solution was subjected to wet transfer at a voltage of 40 V for 0.5 h. Then, target protein in the gel was transferred onto the nitrocellulose (NC) membrane. After that, membranes were washed with eluent for at least 3 times (10 min/time). Protein was blocked with skim milk at 4°C overnight. Primary antibody (diluted at 1:500) was added for incubation at room temperature for 2 h, and secondary antibody (diluted at 1:1000) was added for incubation at room temperature for 1 h. Lastly, fluorescence substrate was added, followed by squashing and imaging in a darkroom. Image J software was used to quantify the imaging results.

Statistical Analysis

All data were processed using Statistical Product and Service Solutions (SPSS) 16.0 software (Chicago, IL, USA). Measurement data were expressed as ($\bar{x}$±s). The *t*-test was employed for mean comparisons. The χ^2-test was adopted for enumeration data. Comparison between groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). For correlation analyses, $p<0.05$ suggested that the difference was statistically significant.

Results

HE Staining in 3 Groups of Brain Tissues After Treatment

In normal group, the cortex of rats showed a lot of neurons, abundant cytoplasm, fine nuclei and clear nucleoli without necrotic cells. In ICH control group, there were only a few glial cells and no neurons in the cortical hemorrhage center after reoperation. In addition, sparse neurons, large intercellular space, clear demarcation between the membrane and the surrounding area were observed. A large number of degenerated and necrotic neurons were found in the marginal zone of hemorrhage. In rFVIIa treatment group, the rat hemorrhage center became smaller, the degeneration and necrosis of nerve cells in the marginal zone were decreased. Besides, the relatively normal morphology of most surviving cells, and mild pathological changes were observed (Figure 1).

Comparison of The Number of Positive Apoptotic Cells Between ICH Control Group and rFVIIa Treatment Group

In normal group, no apoptotic cells were found in the cerebral cortex of rats. With the extension of time, the number of apoptotic cells was gradually increased and peaked at 72 h in ICH control group and rFVIIa treatment group. Meanwhile, the number of apoptotic cells in treatment group was significantly lower than that in control group after 24 h ($p<0.05$) (Figure 2).

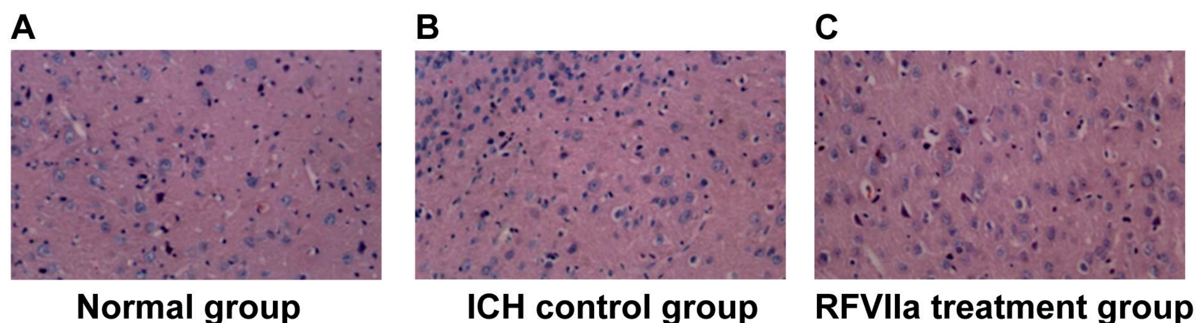


Figure 1. HE staining results in 3 groups of brain tissues after treatment. In comparison with ICH control group, rFVIIa treatment group has decreased degeneration and necrosis of peripheral nerve cells and milder pathological changes in rats (Magnification: 40×).

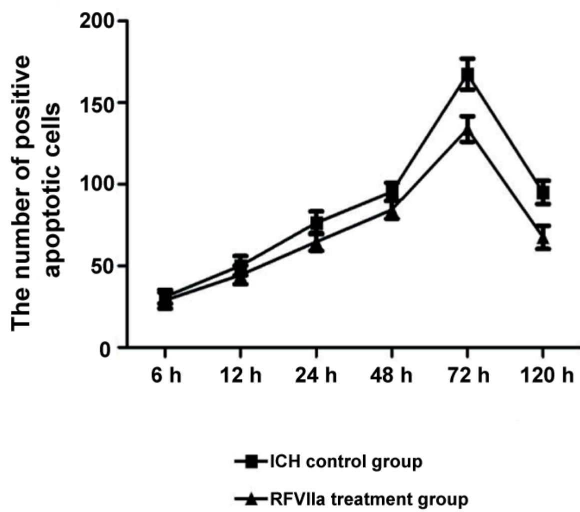


Figure 2. Number of apoptotic nerve cells at different time in ICH control group and rFVIIa treatment group (*$p<0.05$).

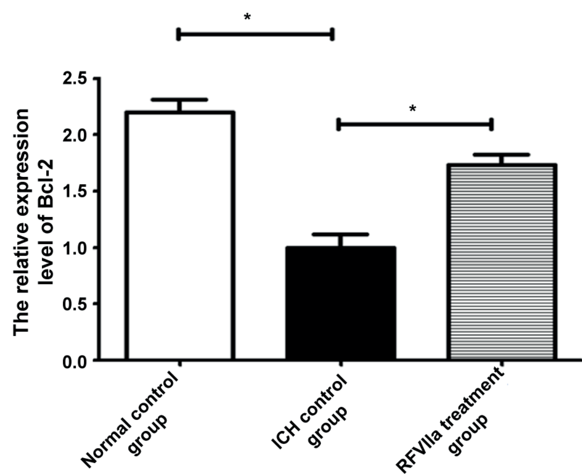


Figure 3. Results of qPCR. The relative expression level of Bcl-2 in rFVIIa group is higher than that in ICH control group ($p<0.05$) and lower than that in normal control group ($p<0.05$).

Comparison of Bcl-2 Expression Level Among Three Groups

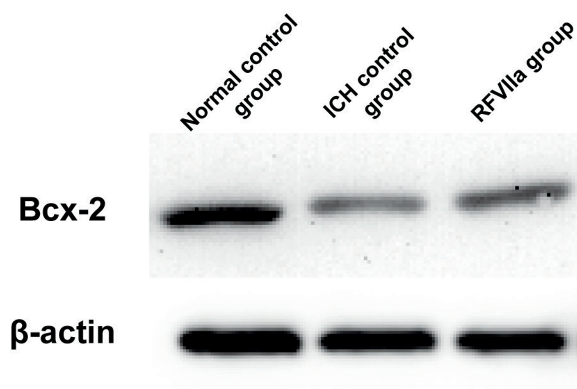
The results of fluorescence qPCR (Figure 3) and Western blotting (Figure 4) confirmed that the relative expression level of Bcl-2 in rFVIIa group was higher than that in ICH control group ($p<0.05$) and lower than that in normal control group ($p<0.05$).

Comparison of Bax Expression Level Among Three Groups

The results of fluorescence qPCR (Figure 5) and Western blotting (Figure 6) proved that the relative expression level of Bax in rFVIIa group was lower compared with that in ICH control group ($p<0.05$). However, Bcl-2 expression was increased compared with that in normal control group ($p<0.05$).

Discussion

ICH is one of the most common life-threatening brain-related diseases in elderly patients in the world, which has high incidence and death rates and critical condition[7,8]. Despite aggressive treatment has been carried out, some related neu-



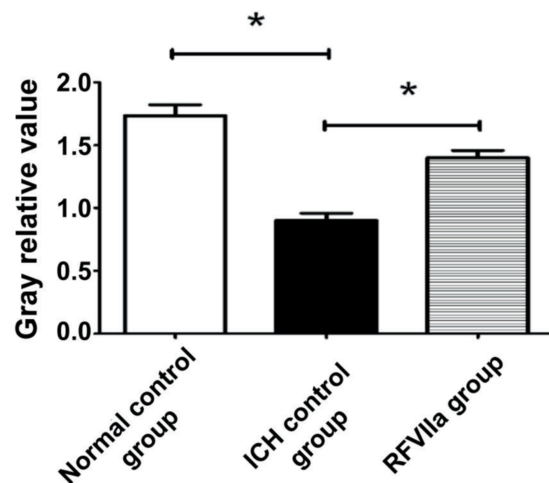


Figure 4. Results of Western blotting. The relative expression level of Bcl-2 in rFVIIa group is higher than that in ICH control group ($p<0.05$) and lower than that in normal control group ($p<0.05$).

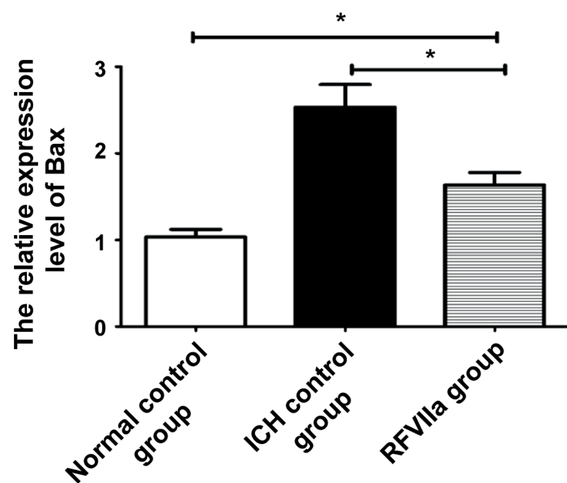


Figure 5. Results of qPCR. The relative expression level of Bax in rFVIIa group is reduced compared with that in ICH control group ($p<0.05$), but it is elevated compared with in normal control group ($p<0.05$).

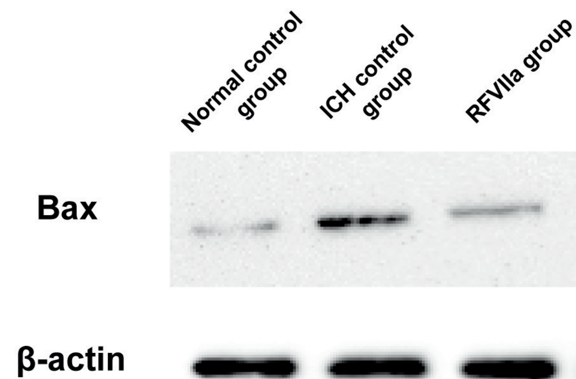


Figure 6. Results of Western blotting. The relative expression level of Bax in rFVIIa group is reduced compared with that in ICH control group ($p<0.05$), but it is elevated compared with in normal control group ($p<0.05$).

rological deficits are often maintained, seriously affecting the quality of life of patients. Therefore, studying its pathogenesis and effective treatment methods genetically have become the hotspot and focus in the research of the disease.

The mechanism of ICH injury is very complex, including mechanical damage, hematoma compression and occupancy and inflammatory reaction around blood clots after ICH. Inflammation after ICH is considered to be an important cause of delayed neuronal death[9-11]. Authors[12-15] have found that brain tissue cells around ICH lesions are in hypoxic-ischemic state, which can produce a series of damage factors, such as hypoxia-inducible factor-1α (HIF-1α) and other stress factors. These stress factors are the main reason for damaging neurological function via causing the activation of related genes and the initiation of apoptosis. Apoptosis is a gene-controlled process of active death of cells[16]. Pytel et al[17] found that Bax and Bcl-2 are closely related to apoptosis. Expression levels of Bax and Bcl-2 are directly related to apoptosis control due to their expression features in the central nervous system. The ratio of Bax/Bcl-2 protein may determine cell survival after receiving apoptosis stimulation signal[18]. FVII is a serine protease that is produced by the liver and is a vitamin K-dependent clotting factor, which is essential for the body to maintain its normal clotting function[19]. RFVIIa may be able to quickly correct coagulation abnormalities in acute intracranial hemorrhage, which is very important for the development of less intracranial hematoma[20].

Conclusions

We showed that apoptotic cells were found at 6h after ICH, which were increased gradually at 24-72 h, peaked at 72 h and then decreased. The data suggested that apoptosis exists in the process of brain tissue injury around the hemorrhage and can cause neuron loss after ICH. Compared with that in ICH control group, the number of apoptotic cells around the hematoma was significantly decreased in ICH rats receiving rFVIIa treatment. The results indicated that rFVIIa may inhibit apoptosis and reduce brain damage on brain injury after hemorrhage. Apoptosis mechanism may be involved in secondary brain injury after ICH. RFVIIa may play an important protective role in neuronal injury after ICH by stimulating Bcl-2 expression and inhibiting Bax protein expression.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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