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IL-10 inhibits apoptosis in brain tissue around the hematoma after ICH by inhibiting proNGF

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Abstract. – OBJECTIVE: To explore the roles of interleukin-10 (IL-10), proNGF and p75NTR in apoptosis of brain tissues induced by intracerebral hemorrhage (ICH).

PATIENTS AND METHODS: According to the time of sample collection after ICH, brain tissue samples were divided into < 6 h group, 6-24 h group (including 24 h), 24-72 h group (including 72 h) and > 72 h group. Meanwhile, 10 tissues that dropped from the beginning at the cortical stoma (distal part of the hematoma) were harvested as controls. AI in brain tissues around the hematoma after ICH was calculated based on TUNEL staining. Expression levels of IL-10, proNGF and p75NTR in brain tissues were determined by quantitative Real-time polymerase chain reaction (qRT-PCR) and Western blot, respectively. Protein expressions of Bcl-2 and Bax were detected by Western blot. Rat cortical astrocytes were harvested and cultured *in vitro*. After transfection of IL-10 overexpression plasmid, expression levels of IL-10, proNGF and p75NTR were detected by Western blot.

RESULTS: AI increased in 6-24 h group, 24-72 h group and > 72 h group compared with < 6 h group and control group, which achieved the peak at 24-72 h. However, no significant difference in AI was observed between < 6 h group and control group. With the prolongation of ICH, IL-10 level gradually decreased and achieved the lowest level at 24-72 h. After 72 h, IL-10 level began to increase. Additionally, mRNA and protein levels of proNGF and p75NTR started to upregulate within 6 h of ICH, achieveing the peak at 24-72 h. Bcl-2 level gradually decreased after 6 h of ICH, while Bax level increased. We did not found significant difference in mRNA and protein levels of IL-10 in brain tissues around hematoma between < 6 h group and control group. With the prolongation of ICH, IL-10 level gradually decreased and achieved the lowest level at 24-72 h. After 72 h, IL-10 level began to increase. Transfection with IL-10 overexpression plasmid in rat astrocytes markedly downregulated protein levels of proNGF and p75NTR compared with those of controls.

CONCLUSIONS: IL-10 expression is downregulated in brain tissues around the hematoma after ICH. IL-10 alleviates inflammation and apoptosis by inhibiting levels of proNGF, p75NTR and Bax/Bcl-2, thus protecting brain tissue after ICH.

Key Words
ICH, Interleukin-10 (IL-10), p75NTR.

Introduction

Intracerebral hemorrhage (ICH) has become one of the major diseases threatening human health and life quality due to its high morbidity, mortality and disability. Apoptosis exerts an important role in the secondary injury of ICH[1-4]. Neurotrophin receptor p75 (p75NTR), also known as TNFRSF16, is a member of the tumor necrosis factor receptor superfamily (TNFRSF). It could promote apoptosis by binding to the nerve growth factor precursor (proNGF), which requires the involvement of synergistic receptor sortilin to form a proNGF/sortilin/p75NTR apoptotic signal complex[5,6]. Inflammatory response is a crucial pathogenic factor of ICH. It is reported that inflammatory response after ICH leads to worse damage than ICH itself. Expression levels of pro-inflammatory factors and anti-inflammatory factors are correlated to the degree of inflammatory response and its secondary injury. Drugs that target on upregulating anti-inflammatory factors or inhibit pro-inflammatory factors are one of the most common therapies for ICH[7,8]. Researches[9] have shown that apoptosis is involved in the brain tissue damage around the hematoma. As an inflammation inhibitor, IL-10 not only reduces

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inflammatory response by inhibiting neutrophil infiltration and inflammatory mediators, but also provides a suitable microenvironment for nerve damage repair[10]. IL-10 also exerts a neuroprotective effect by regulating neuronal apoptosis[11]. However, the expression patterns of proNGF and p75NTR at different stages after ICH and whether IL-10 can regulate brain cell apoptosis have not been reported. We detected apoptosis in the brain tissue around the hematoma at different stages after ICH. We also determined expression levels of IL-10, proNGF, p75NTR, Bcl-2 and Bax after ICH. Thus, we aim to elucidate the mechanism of neuronal apoptosis after ICH.

Patients and Methods

Sample Collection

40 ICH patients who underwent craniotomy through temporal lobe approach for removal of hematoma in Neurosurgery Department, Liaoyang City Central Hospital from January 2013 to December 2017, were selected. Enrolled patients were pathologically diagnosed with ICH by CT or MRI. According to the time of sample collection after ICH, samples harvested from enrolled ICH patients were divided into < 6 h group, 6-24 h group (including 24 h), 24-72 h group (including 72 h) and > 72 h group. In < 6 h group, there were 6 males and 4 females, with the age of 50-73 years (mean age of 62.24 ± 10.78 years) and haemorrhagia amount of 40-82 mL (mean amount of 58.32 ± 11.96 mL). 5 males and 5 females were enrolled in 6-24 h group, with the age of 52-76 years (mean age of 64.18 ± 11.26 years) and haemorrhagia amount of 45-80 mL (mean amount of 60.47 ± 12.18 mL). 4 males and 6 females were enrolled in 24-72 h group, with the age of 54-78 years (mean age of 63.19 ±12.73 years) and haemorrhagia amount of 42-82 mL (mean amount of 59.67 ± 10.34 mL). 7 males and 3 females were enrolled in > 72 h group, with the age of 53-80 years (mean age of 65.26 ± 14.01 years) and haemorrhagia amount of 43-75 mL (mean amount of 61.08 ± 11.45 mL). Brain tissues at 1 cm away from the hematoma in each case were harvested as experimental samples. Meanwhile, tissues that dropped from the beginning at the cortical stoma (distal part of the hematoma) were harvested as controls. A total of 10 cases were enrolled in the control group, including 5 males and 5 females, with the age of 52-80 years (mean age of 63.76 ±13.18 years) and haemorrhagia amount of 42-82 mL (mean amount of 60. 79 ± 11. 83 mL). No significant differences in sex, age and haemorrhagia amount were found among the five groups ($p>0.05$). This study was approved by the Ethic Committee of Liaoyang City Central Hospital. Informed consent was obtained prior to the experiment.

Reagents

TUNEL determination kit was obtained from Boster (Wuhan, China); TRIzol and polyvinylidene difluoride (PVDF) membrane were obtained from Invitrogen (Carlsbad, CA, USA); Reverse transcription kit was obtained from Promega (Madison, WI, USA); Quantitative Real-time polymerase chain reaction (qRT-PCR) determination kit was obtained from Roche (Basel, Switzerland); IL-10, proNGF, p75NTR, Bcl-2 and Bax antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

TUNEL

Cell apoptosis in brain tissue was determined according to the instructions of TUNEL determination kit. Apoptotic cells were manifested as yellow stain in cell nucleus. Apoptotic index (AI) = the amount of apoptotic cells / the amount of total cells × 100%.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA in brain tissues was extracted with TRIzol and dissolved in RNase. RNAs with A260/A280 of 1.8-2.0 were selected and reversely transcribed for complementary Deoxyribose Nucleic Acid (cDNA). QRT-PCR was performed as predenaturation at 94°C for 3 min, denaturation at 94°C for 45 s, annealing at 62°C for 30 s and extension at 72°C for 60 s, for a total of 35 cycles. β-actin was used as the loading control. Primers used in the study were as follows: IL-10, forward: CGGCGCTGTCATCGATTTCT, reverse: ATAGAGTCGCCACCCTGATG; proNGF, forward: CTTCTACGTTCCAAGATCCTTA, reverse: CCGCACTAGGTTTGCCGAGTAGT; p75NTR, forward: AACAAGACCTCATAGCCAGCA, reverse: CAGGATGGAGCAATAGACAGG; β-actin, forward: GTGGACATCCGCAAAGAC, reverse: TCAACGCAATGTGGGAAAG.

In Vitro Culture of Rat Cortical Astrocytes

Ten neonatal Wistar rats in Specific-Pathogen-Free (SPF) level were obtained from Department of Laboratory Animal Science, University of South China. Rat brain was harvested for iso-

lating the cortex. Rat cortex was cut and digested in 1.25 g/L trypsin for preparing cell suspension. Cell density was adjusted to 3-5 $\times 10^8$/L and culture medium was replaced every 3-4 days.

IL-10 Overexpression

Astrocytes were seeded in 24-well plate coated with polylysine and transfected with IL-10 overexpression plasmid. Briefly, 5 μL Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or 5 μg plasmid were added into 150 μL Opti-MEM, and maintained for 5 min. The two solutions were mixed and maintained for another 20 min. Astrocytes were maintained in the mixture for 48 h. Astrocytes were treated with culture medium, pcDNA-4TO Vector or IL-10 overexpression plasmid, respectively for 24 h.

Western Blot

Total protein was extracted and loaded in equal amounts. After being separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred to membrane, which was then blocked with 5% skim milk for 1 hour. The specific primary antibody was used to incubate with the membrane overnight at 4°C. After being washed with 1×Tris-buffered saline and Tween 20 (TBST) for 5 times, the secondary antibody was used to incubate the membrane for 2 h at room temperature. After washing with 1×TBST for 1 min, the chemiluminescent substrate kit was used for exposure the protein band.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 (SPSS Inc., Chicago, IL, USA) was utilized for data analyzed. Measurement data were expressed as mean ± standard deviation ($\bar{x}\pm s$). Differences between the two groups were analyzed by *t*-test. $p<0.05$ was considered statistically significant.

Results

Cell Apoptosis in Brain Tissues Around Hematoma After ICH

TUNEL assay showed abundant apoptotic cells, manifesting as yellow stain in the nucleus. The majority of apoptotic cells were astrocytes with a little number of neurons (Figure 1A and 1B). Significant difference in AI was observed among groups, except for that between < 6 h group and control group. Compared with control group and < 6 h group, AI markedly increased in 6-24 h group (including 24 h), 24-72 h group (including 72 h) and > 72 h group ($p<0.05$ and $p<0.01$). Compared with 6-24 h group, AI increased in 24-72 h group (including 72 h) and > 72 h group ($p<0.01$ and $p<0.05$). In addition, AI was higher in 24-72 h than that of > 72 h group ($p<0.05$, Figure 1C).

Expression Levels of IL-10, proNGF and p75NTR in Brain Tissues Around Hematoma After ICH

We did not find significant differences in mRNA and protein levels of IL-10 in brain tissues around hematoma between < 6 h group and control group ($p>0.05$). With the prolongation of ICH, IL-10 level gradually decreased and achieved the lowest level at 24-72 h. After 72 h, IL-10 level began to increase ($p<0.01$). Additionally, mRNA and protein levels of proNGF and p75NTR started to upregulate within 6 h of ICH, which achieved the peak at 24-72 h. Expressions of proNGF and p75NTR still remained high after 72 h of ICH ($p<0.01$, Figure 2).

Expression Levels of Bcl-2 and Bax in Brain Tissues Around Hematoma After ICH

Significant differences in protein levels of Bcl-2 and Bax in brain tissues around hematoma were found among the five groups as Western blot indicated (F=26.41 and 29.54; $p<0.01$). Among them, we did not find significant differences in protein levels of Bcl-2 and Bax between < 6 h group and control group. Bcl-2 level gradually decreased after 6 h of ICH, while Bax level increased. In particular, Bcl-2 level was lower in 24-72 h group (including 72 h) and > 72 h group compared with 6-24 h group (including 24 h). Bax level showed the opposite trend to that of Bcl-2 level. Moreover, Bcl-2 level was lower in 24-72 h group than > 72 h group, whereas Bax level was higher in 24-72 h group than > 72 h group (Figure 3).

Transfection of IL-10 Overexpression Plasmid Downregulated its Level in Rat Astrocytes

Rat cortical astrocytes were transfected with IL-10 overexpression plasmid. The transfection efficiency was determined by Western blot. As shown in Figure 4A, protein level of IL-10 after transfection in astrocytes was 8.3 times higher than controls.

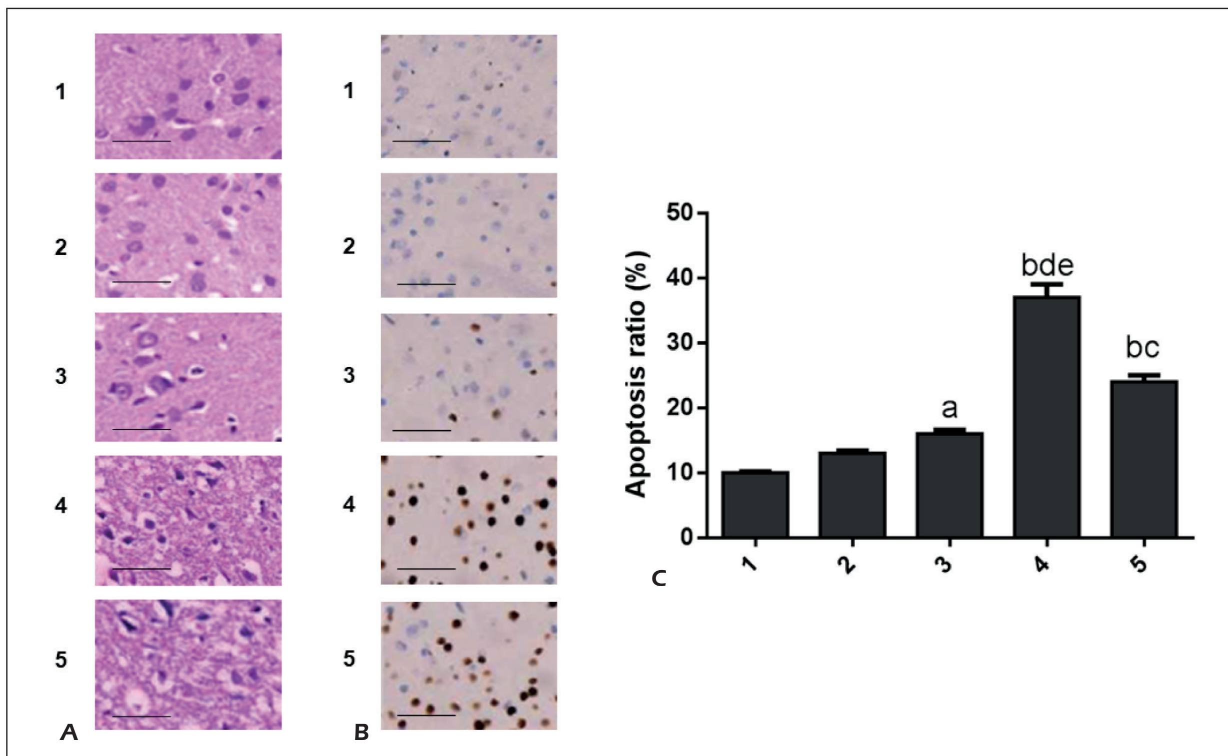

Figure 1. Cell apoptosis in brain tissues around hematoma after ICH. **A**, Structure of neurons in the cortex by HE staining (magnification ×400, scale bar = 50 μm). **B**, Cell apoptosis in the cortex by TUNEL staining (magnification ×400, scale bar = 50 μm). **C**, Comparison of AI in each group. 1: control group; 2: < 6 h group; 3: 6-24 h group (including 24 h); 4: 24-72 h group (including 72 h); 5: > 72 h group. $^{a}p<0.05$, $^{b}p<0.01$ compared with 1 or 2; $^{c}p<0.05$ compared with 3, $^{d}p<0.01$ compared with 4; $^{e}p<0.05$ compared with 5.

IL-10 Overexpression Downregulated Protein Levels of proNGF, Sortilin and p75NTR in Rat Astrocytes

Transfection with IL-10 overexpression plasmid in rat astrocytes markedly downregulated protein levels of proNGF and p75NTR compared with those of controls ($p<0.01$, Figure 4B, 4C).

Discussion

Secondary injury is one of the major causes of sequelae of ICH. Apoptosis is the main way of cell death in secondary brain injury[12,13]. In the present study, we did not observe significant change in AI within 6 h around ICH-induced hematoma tissue. However, AI markedly increased in 6-24 h group (including 24 h), 24-72 h group (including 72 h) and > 72 h group, which reached the peak at 24-72 h. Our results demonstrated abundant apoptotic cells around hematoma tissue in the brain after ICH. Cell apoptosis could be closely related to secondary injury after ICH.

Nerve growth factor (NGF) is an important neurotrophic factor. As a precursor of NGF, proNGF forms NGF when it is cleaved by protease[14-16]. The biological effects of NGF and proNGF are diametrically opposite, depending on their receptors. NGF maintains cell survival and axonal growth by binding to tyrosine kinase A (TrkA), whereas proNGF induces cell apoptosis by binding to p75NTR receptor[17, 18]. Sortilin is a member of the Vps10p-D receptor family and it is highly expressed in the central nervous system[19]. ProNGF/p75NTR-mediated apoptosis requires the involvement of sortilin to form a proNGF/sortilin/p75NTR complex. Sortilin is capable of inhibiting proNGF degradation, which may serve as a molecular switch in this process. Subsequently, NGF stimulates the formation of trimers and inhibits TrkA activation, thereafter promoting cell survival[20]. Jansen et al[21] reported that the expression levels of proNGF and p75NTR are significantly upregulated after nerve injury. However, sortilin knockdown inhibited the death of impaired corticospinal motor neurons. Systemic anti-inflammatory response after ICH trig-

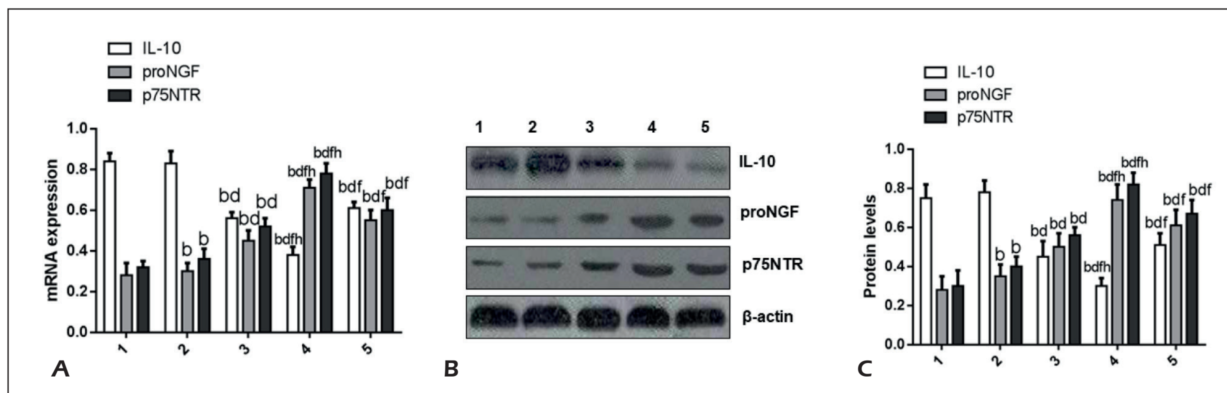

Figure 2. Expression levels of IL-10, proNGF and p75NTR in brain tissues around hematoma after ICH. **A**, The mRNA levels of IL-10, proNGF and p75NTR. **B**, The protein levels of IL-10, proNGF and p75NTR. **C**, Quantification of protein levels of IL-10, proNGF and p75NTR. 1: control group; 2: < 6 h group; 3: 6-24 h group (including 24 h); 4: 24-72 h group (including 72 h); 5: > 72 h group. $^{a}p<0.\ 05$, $^{b}p<0.01$ compared with 1 or 2; $^{c}p<0.05$ compared with 3, $^{d}p<0.01$ compared with 4; $^{e}p<0.05$ compared with 5.

gers release of monocyte-derived cytokines (IL-6 and IL-10), but stress-produced IL-10 is insufficient to inhibit secondary inflammatory response and apoptosis after ICH[22]. We first established ICH model in rats and intervened with exogenous IL-10. We explored the role of IL-10 in protecting brain tissue after ICH. With the prolongation of ICH, mRNA and protein levels of proNGF and p75NTR gradually increased, which peaked at 24-72 h and then decreased. IL-10 level did not remarkably change within the first 6 h, whereas it gradually decreased until 72 h and then increased. Our results showed that expression levels of proNGF and p75NTR present the opposite changing trend to IL-10 level in hematoma tissue after ICH. To verify the regulatory effect of IL-10 on proNGF/sortilin/p75NTR complex, rat cortical astrocytes were transfected with IL-10 overexpression plasmid. The results showed that IL-10 overexpression downregulates expression levels of proNGF and p75NTR in cortical astrocytes. It is suggested that IL-10-induced downregulation of these genes may exert key roles in inhibiting neuronal apoptosis. Bcl-2 and Bax are considered as important factors in the regulation of apoptosis. Bcl-2 is capable of inhibiting cell apoptosis, whereas Bax promotes apoptosis. Previous study has pointed out that p75NTR triggers apoptosis of melanoma cells by downregulating Bcl-2 level and upregulating Bax level[23]. In our work, we detected protein expressions of Bcl-2 and Bax in

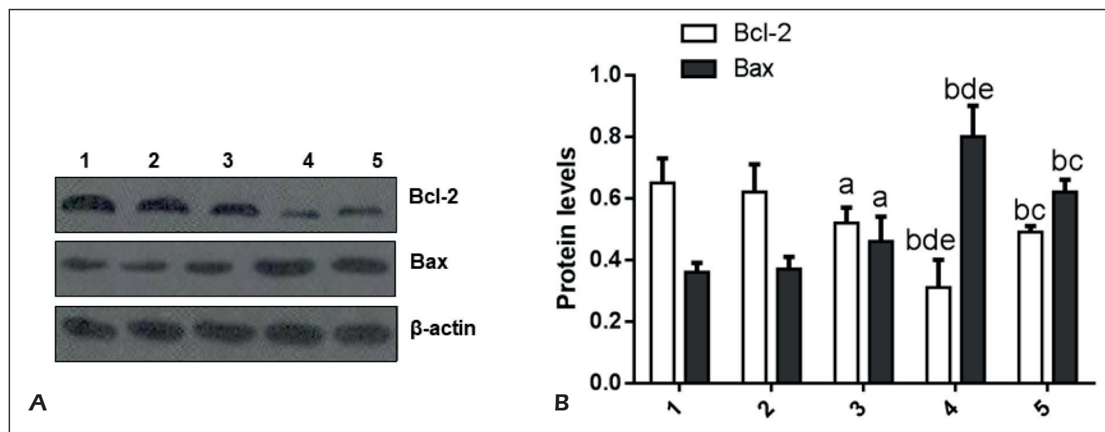

Figure 3. Expression levels of Bcl-2 and Bax in brain tissues around hematoma after ICH. **A**, The protein levels of Bcl-2 and Bax. **B**, Quantification of protein levels of Bcl-2 and Bax. 1: control group; 2: < 6 h group; 3: 6-24 h group (including 24 h); 4: 24-72 h group (including 72 h); 5: > 72 h group. $^{a}p<0.05$, $^{b}p<0.\ 01$ compared with 1 or 2; $^{c}p<0.05$ compared with 3, $^{d}p<0.01$ compared with 4; ep<0.05 compared with 5.

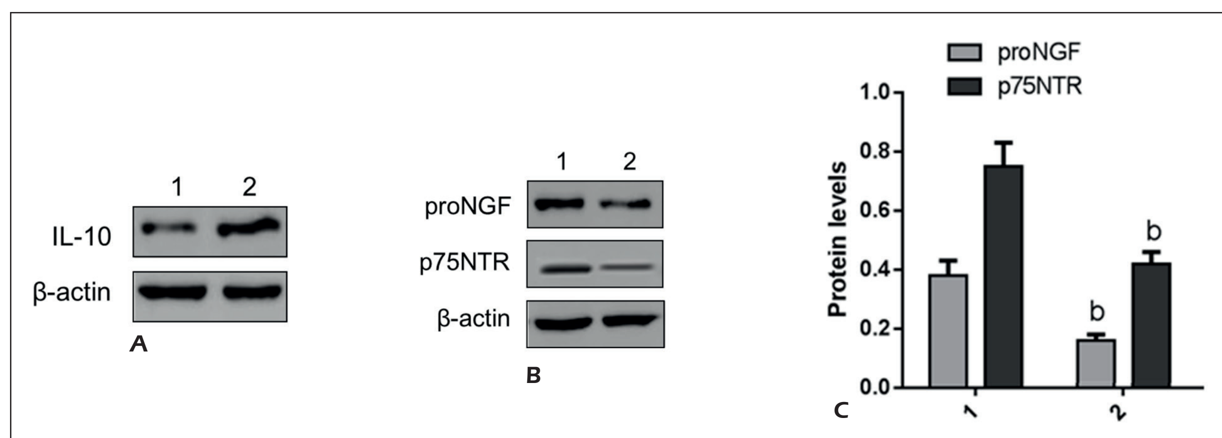

Figure 4. IL-10 overexpression downregulated protein levels of proNGF, sortilin and p75NTR in rat astrocytes. **A**, Protein level of IL-10 after transfection of IL-10 overexpression plasmid. **B**, Protein levels of proNGF and p75NTR. **C**, Quantification of protein levels of proNGF and p75NTR. 1: control group; 2: scramble group; 3. Overexpression group. $^{b}p<0.01$ compared with 1 or 2.

brain tissues around hematoma at different time points after ICH by Western blot. It was shown that Bcl-2 level is lower in 6-24 h group (including 24 h), 24-72 h group (including 72 h) and > 72 h group compared with that of control group. Bax level showed the opposite trend to that of Bcl-2 level, which reached the peak at 24-72 h. The changing trend of Bax/Bcl-2 was similar to that of AI. We suggested that IL-10 regulates apoptosis in brain tissues around hematoma after ICH by upregulating Bcl-2 level and downregulating Bax level.

Conclusions

We found that IL-10 expression is downregulated in brain tissues around the hematoma after ICH. IL-10 alleviates inflammation and apoptosis by inhibiting levels of proNGF, p75NTR and Bax/Bcl-2, thus protecting brain tissue after ICH. IL-10 upregulation or proNGF/p75NTR inhibition may contribute to improve the prognosis of ICH patients and alleviate secondary injury after ICH.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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