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Effect of miR-130a on neuronal injury in rats with intracranial hemorrhage through PTEN/PI3K/AKT signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of micro-ribonucleic acid (miR)-130a on neuronal injury in rats with intracerebral hemorrhage (ICH) through the phosphatase and tensin homolog deleted on chromosome ten/phosphatidylinositol 3-hydroxy kinase/protein kinase B (PTEN/PI3K/AKT) signaling pathway.

MATERIALS AND METHODS: A total of 30 healthy male rats were randomly divided into three groups, including the blank control group, ICH model group (ICH group) and ICH model + miR-130a treatment group (miR-130a treatment group). The differences in neurological injury, the number of apoptotic cells in brain tissues, the activity of Caspase-9 and protein expressions of PTEN/PI3K/AKT were analyzed among the three groups, respectively.

RESULTS: Neurological function was normal without injury in the control group. However, the neurological injury was severe in the ICH group and mild in the miR-130a treatment group. There were statistically significant differences in neurological function in the control group relative to those of the ICH group and miR-130a treatment group ($p<0.05$). Meanwhile, the neurological injury was markedly milder in the miR-130a treatment group than that of the ICH group, showing a statistically significant difference ($p<0.05$). The number of apoptotic cells was remarkably smaller in the control group when compared with the ICH group and miR-130a treatment group. However, it was markedly larger in the ICH group than that of the miR-130a treatment group, showing significant differences ($p<0.05$). The activity of Caspase-9 was significantly lower in the control group than ICH group and miR-130a treatment group ($p<0.05$). However, it increased remarkably in the ICH group compared with that of the miR-130a treatment group ($p<0.05$). Moreover, the protein level of PTEN in the ICH group was significantly higher than control group and miR-130a treatment group, displaying statistically significant differences ($p<0.05$). However, no marked difference in the protein level of PTEN was observed between the control group and miR-130a treatment group ($p>0.05$). The protein levels of the phosphorylated 3-hydroxy kinase (p-PI3K) and phosphorylated protein kinase B (p-AKT) were remarkably lower in the ICH group than those of the control group and miR-130a treatment group, displaying statistically significant differences ($p<0.05$). However, they were remarkably higher in the miR-130a treatment group than that of the control group ($p<0.05$).

CONCLUSIONS: MiR-130a promotes neuronal growth in brain tissues in ICH rats and alleviates neuronal injury after ICH through the PTEN/PI3K/AKT signaling pathway. Our findings suggest that miR-130a exerts important clinical significance in the treatment of ICH.

Key Words:
MiR-130a, PTEN/PI3K/AKT, Intracranial hemorrhage, Neuronal injury.

Introduction

Currently, the major risk factor for intracranial hemorrhage (ICH) is hypertension. If not effectively controlled, it will cause the rupture of cerebral vessels, eventually leading to intraparenchymal blood leakage. At the same time, the risk of ICH gradually rises with the increase of global aging, making it the main cause of sudden cerebral death[1,2]. Alafaci et al[3] have demon-

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strated that neuroinflammatory response, neuronal apoptosis and cytotoxic reaction can result in neurological injury after ICH. Suffering from neurological deficits, about 79% of rehabilitation patients are unable to move independently within 6 months after ICH. In addition to cognitive disorder, motor dysfunction after ICH is the most intractable feature[4]. Therefore, it is urgent to search for effective treatment means for ICH. At present, promoting neuroprotection is an effective therapeutic regimen for ICH. However, its efficacy is far from satisfactory. Moreover, studies have found that micro-ribonucleic acid (miR)-130a is able to promote the differentiation and self-renewal of nerve cells. In addition, it can be involved in regulating the expressions of apoptosis-related proteins[5].

The phosphatidylinositol 3-hydroxy kinase/protein kinase B (PI3K/AKT) signal is a transduction protein in the intracellular phosphorylation process. Kilic et al[6] have shown that the PI3K/AKT signaling pathway can regulate cellular biological behaviors. It is known as one of the major signaling pathways for cellular transcription, translation, metabolism, proliferation, migration and survival[6,7]. Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a tumor suppressor gene that plays an important role in cell metabolism after removing lipid phosphate[8]. At present, few studies in the literature have explored whether the effect of miR-130a on neurons after ICH is achieved by regulating the PTEN/PI3K/AKT signaling pathway.

In this paper, the ICH model was first successfully established in rats. Furthermore, the effects of miR-130a on nerve cells, the activity of Caspase-9 and expressions of related proteins in ICH rats by regulating the PTEN/PI3K/AKT signaling pathway were observed.

Materials and Methods

Laboratory Animals and Grouping

Rats used in this experiment were purchased from the Laboratory Animal Center of Qingdao Institute of Drug Control. This study was approved by the Animal Ethics Committee of Jiamusi University Animal Center. A total of 30 healthy male rats aged about 2 months and weighing (0.22 ± 0.015) kg were fed adaptively for 2 days under the temperature of (23 ± 1.2)°C, humidity of 45% and a 12/12 h light/dark cycle. All rats were randomly divided into three groups, including the blank control group (n=10), ICH model group (ICH group, n=10) and ICH model + miR-130a treatment group (miR-130a treatment group, n=10).

Laboratory Instruments and Reagents

Instruments and reagents used in this experiment included: PTEN reagent (Shanghai Ruiqi Biotechnology Co., Ltd., Shanghai, China), polyclonal primary antibodies of p-PI3K, p-AKT and ACTB (Santa Cruz Biotechnology, Santa Cruz, CA, USA), alkaline phosphatase-labeled goat anti-rabbit IgG secondary antibody (Beijing Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China), stereometer (Shanghai Yuyan Scientific Instrument Co., Ltd., Shanghai, China), chloral hydrate (Qingdao yulong seaweed co. LTD., Qingdao, China), normal saline (Tangshan Jixiang Pharmaceutical, Tangshan, China), paraformaldehyde (Shandong Aldehyde Chemical Industry Co., Ltd., Shandong, China), 37°C constant temperature water bath (Jiangsu Jintan Medical Instrument Factory, Changzhou, China), microscope (Olympus, Tokyo, Japan), microplate reader (Shenzhen Highcreation Co., Ltd., Shenzhen, China), paraffin slicer (Leica, Wetzlar, Germany), apoptosis assay kit and Caspase-9 activity assay kit (Beyotime, Shanghai, China), ABI 7500 Real Time-fluorescence quantitative Polymerase Chain Reaction (RT-PCR) instrument (Applied Biosystems, Foster City, CA, USA), and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) apparatus and membrane transfer instrument (Bio-Rad, Hercules, CA, USA).

Establishment of ICH Model in Rats

In the ICH group and miR-130a treatment group, the rats were fixed on a stereometer after fasting for 8 h. Subsequently, the rats were anesthetized through an intraperitoneal injection of 1% pentobarbital sodium (0.1 g/kg). The periosteum was then peeled off, and 60 μL of fresh blood was injected into the caudate nucleus of rats. After 10 min, the incision was sutured, and the modeling was finished. In the miR-130a treatment group, miR-130a was intraperitoneally injected (3000 UI/kg) in rats at 5 min after the operation.

Evaluation Criteria for Neurological Deficit

After the rats were fed for 1 week after the operation, the conditions of limbs were

observed. Neurological deficit was evaluated using the Longa scoring criteria (Table I): grade 0: no neurological deficit, grade 1-2: mild neurological deficit, and grade 3-4: severe neurological deficit. The cumulative grade 1 and above indicated the successful establishment of the ICH model in rats.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Apoptosis Assay

Rats in the three groups were anesthetized through an intraperitoneal injection of 1% pentobarbital sodium (0.1 g/kg). Subsequently, the heart was perfused with 200 mL of normal saline and fixed with 200 mL of 4% paraformaldehyde (Beyotime, Shanghai, China). Brain tissues were collected, embedded in paraffin and serially sliced into three 5 μm-thick sections. TUNEL (Roche, Basel, Switzerland) staining was performed to detect the apoptosis of brain cells in each group according to the corresponding instructions. Five uncrossed non-repeated fields were randomly selected in each pathological section, followed by observation under an optical microscope. Brown yellow cells were apoptotic cells. The apoptotic rate of brain cells = the number of apoptotic cells/the total number of cells × 100%.

Detection of Caspase-9 Activity

Brain tissues were taken, centrifuged and lysed at -5°C for use. 5 μL of 4 mM deoxyribonucleic acid (DNA) substrate, 50 μL of protein diluted with buffer, and reaction buffer containing 10 mM dl-dithiothreitol (DTT) were added into 96-well plates (Corning, Lowell, MA, USA), followed by incubation at 37°C for 1 h. The absorbance of Caspase-9 protein (Beyotime, Shanghai, China) at 405 nm was measured using a microplate reader.

Table I. Longa scoring criteria for neurological deficit.

Grade	Evaluation criteria
0	No evident neurological symptoms
1	Unable to fully stretch the left forelimb
2	Rotate to the left
3	Lean to the left while walking
4	Able to walk independently, with conscious disturbance

Hematoxylin-Eosin Staining (HE) Staining

Hematoxylin-eosin staining (HE; Boster, Wuhan, China) was performed for 4 μm-thick coronal sections to observe brain cells and Caspase-9 activity after ICH. HE staining images in each group were observed under a microscope (400×).

Detection of Protein Expressions of PTEN, PI3K and AKT via Western Blotting

Brain tissues were first taken, lysed at -5°C for 2 h and centrifuged. The concentration of extracted protein was detected using the bicinchoninic acid (BCA) method (Pierce, Waltham, MA, USA). The concentration of protein was adjusted to 30 μg/μL. Subsequently, protein samples were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibodies of PI3K, AKT and PTEN (1:1000; Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. After rinsing with a Tris-Buffered Saline and Tween solution (TBST; Sigma-Aldrich, St. Louis, MO, USA), the membranes were incubated with the corresponding secondary antibody. Immunoreactive bands were developed with enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) developer, and the gray value was scanned to calculate the expression of the corresponding proteins.

Detection of mRNA Expressions of PTEN, PI3K and AKT via Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The mRNA expressions of PTEN, PI3K and AKT were detected *via* qRT-PCR. Briefly, total RNA was extracted from tissues and cells using the TRIzol kit (Invitrogen, Carlsbad, CA, USA). The concentration of extracted RNA was measured by an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). Subsequently, RNA was reverse transcribed into complementary Deoxyribose Nucleic Acid (cDNA) according to the instructions of the SYBR Premix Ex Taq™ II kit (TaKaRa, Otsu, Shiga, Japan). Specific PCR conditions were as follows: 98°C for 6 min, 98°C for 28 s, 75°C for 30 s, and 80°C for 4 min, for a total of 55 cycles. The relative expression level of the target gene was expressed by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were shown in Table II.

Table II. Primer sequences.

Gene	Primer sequence
PI3K	F: 5'-GACTCCAAGATGAAGAAGATGTG-3'
	R: 5'-GAGCATTCGCAGGTVCAAGCC-3'
AKT	F: 5'-CGAGGCCCAACACCTTCATC-3'
	R: 5'-CCGGAAGTCCATCGTCTCCT-3'
PTEN	F: 5'-ACACCGCCAAATTAACTGC-3'
	R: 5'-TACACCAGTCCGTCCCTTTC-3'
GAPDH	F: 5'-CAACGGGAAAGCCATCACCA-3'
	R: 5'-ACGCCAGTAGACTCCACGACAT-3'
	R: 5'-CCGGAAGTCCATCGTCTCCT-3'
PTEN	F: 5'-ACACCGCCAAATTAACTGC-3'
	R: 5'-TACACCAGTCCGTCCCTTTC-3'
GAPDH	F: 5'-CAACGGGAAAGCCATCACCA-3'
	R: 5'-ACGCCAGTAGACTCCACGACAT-3'

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Neurological injury, apoptosis, the activity of Caspase-9 and protein expression levels of PTEN, PI3K and AKT were compared using the *t*-test among the control group, ICH group and miR-130a treatment group. One-way analysis of variance (ANOVA) was applied to compare the differences among different groups, followed by Post-Hoc Test (Least Significant Difference). Enumeration data were expressed as a percentage (%) and ($\bar{x} \pm s$). $p<0.05$ was considered statistically significant.

Results

Evaluation of Neurological Injury

According to the evaluation results of neurological injury, neurological function was normal without injury in the control group (grade 0). However, neurological injury was severe in the ICH group (grade 2.3) and mild in the miR-130a treatment group (grade 1.1), respectively. Statistically significant differences were observed in the neurological function between the control group with the ICH group and miR-130a treatment group ($p<0.05$). Meanwhile, the neurological injury was remarkably milder in the miR-130a treatment group than that of the ICH group ($p<0.05$). The above results indicated that miR-130a had a neuroprotective effect and could markedly improve neuronal injury after ICH in rats (Table III and Figure 1).

Comparison of the Number of Apoptotic Cells in Brain Tissues

The number of apoptotic cells was significantly smaller in the control group (43.81 ± 10.05) than the ICH group (191.01 ± 14.63) and miR-130a treatment group (130.12 ± 14.89) ($p<0.05$). However, it was markedly larger in the ICH group than that of the miR-130a treatment group, showing statistically significant differences ($p<0.05$; Figure 2 and 3).

Comparison of Caspase-9 Activity in Brain Tissues

The activity of Caspase-9 in the control group [(0.92 ± 0.06) U] was remarkably lower than those of the ICH group [(2.28 ± 0.24) U] and miR-130a treatment group [(1.63 ± 0.12) U] ($p<0.05$). However, it increased significantly in the ICH group when compared with the miR-130a treatment group ($p<0.05$; Figure 4 and 5).

Protein Expression Levels of PTEN, Phosphorylated PI3K (p-PI3K) and p-AKT in Brain Tissues

The protein level of PTEN was markedly higher in the ICH group (1.51 ± 0.18) than the control group (0.95 ± 0.11) and miR-130a treatment group (1.02 ± 0.15), displaying statistically significant differences ($p<0.05$). However, no statistically significant difference in PTEN expression was

Table III. Evaluation results of neurological injury.

Group	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Control group	10	0	0	0	0
ICH group	1	2	2	3	2
MiR-130a treatment group	3	4	2	1	0
ICH group	1	2	2	3	2
MiR-130a treatment group	3	4	2	1	0

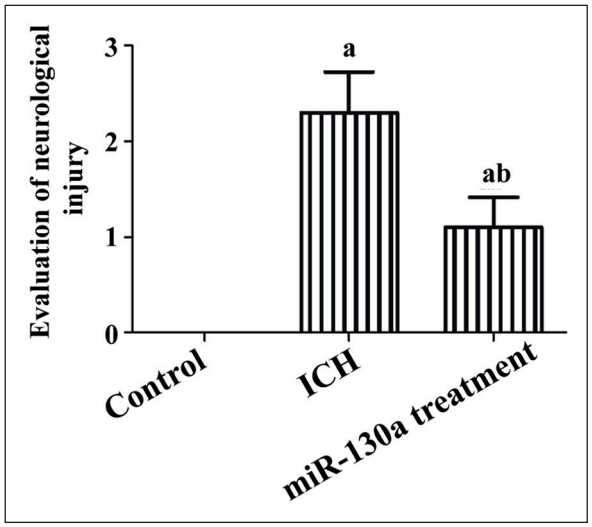

Figure 1. Evaluation of neurological injury. Note: a: $p<0.05$ *vs.* control group, b: $p<0.05$ *vs.* ICH group.

observed between the control group and the miR-130a treatment group ($p>0.05$). The protein levels of p-PI3K and p-AKT were remarkably lower in the ICH group [(0.48 ± 0.07) & (0.61 ± 0.1)] than those of the control group [(0.91 ± 0.09) & (0.96 ± 0.12)] and miR-130a treatment group [(1.52 ± 0.14) & (1.76 ± 0.16)] ($p<0.05$). However, they were markedly higher in the miR-130a treatment group than the control group, displaying statistically significant differences ($p<0.05$). The above findings suggested that PTEN was negatively correlated with p-PI3K and p-AKT. In other words, an increased protein level of PTEN corresponded to the decreased protein levels of p-PI3K and p-AKT (Figure 6 and 7).

Discussion

With the increase of global aging, ICH accounts for 20-30% of sudden cerebral death. Hypertension accounts for 60% of deaths in patients with cerebrovascular disease, which is an important cause of ICH and patient's death[9,10]. Due to the reason that the surrounding brain tissues are damaged in different degrees after hemorrhage, apoptosis occurs rapidly in normal brain tissue cells after ICH. This may eventually lead to neurological injury. Therefore, effectively relieving neuronal apoptosis in brain tissues is the key point in the treatment of ICH[11,12]. According to the study of Kim et al[13], neuronal injury and apoptosis can be observed in the brain after ICH. In this work, neurological injury was severe in the ICH group and mild in the miR-130a treatment group, respectively. The number of apoptotic cells was significantly smaller in the control group than ICH group and miR-130a treatment group ($p<0.05$). However, it was significantly larger in the ICH group than that of the miR-130a treatment group, showing statistically significant differences ($p<0.05$). Slattery et al[14] have found that miR-130a can increase the viability of bovine aortic smooth muscle cells. Kachko et al[4] have also shown that up-regulated miR-130a after ICH suppresses the expression of CAV-1 in brain microvascular endothelial cells, thereby breaking the integrity of the blood-brain barrier. This was similar to the results obtained in this paper. Therefore, we quite speculated that miR-130a had a neuroprotective effect and could significantly ameliorate neurological injury after ICH in rats.

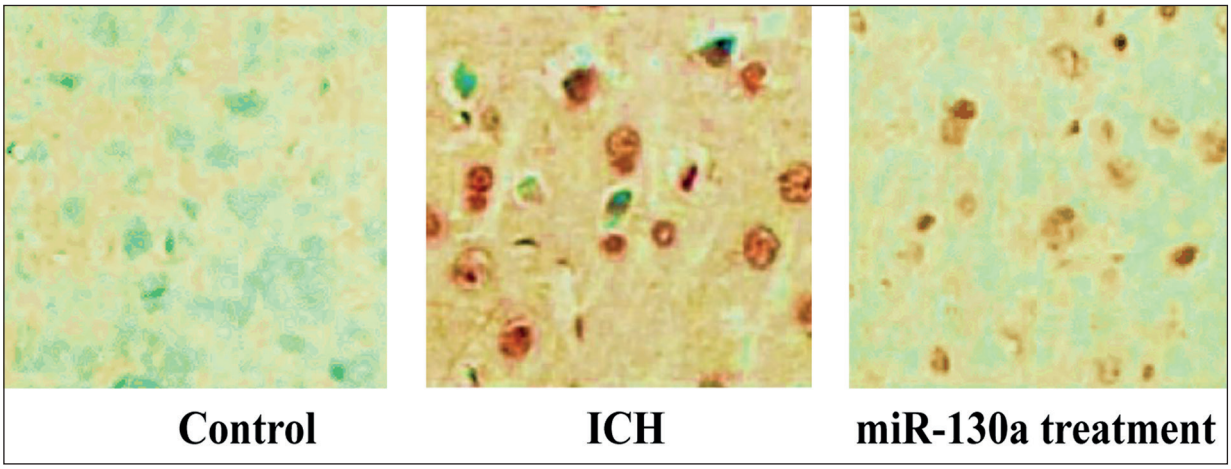

Figure 2. Number of apoptotic cells in brain tissues in each group (×400).

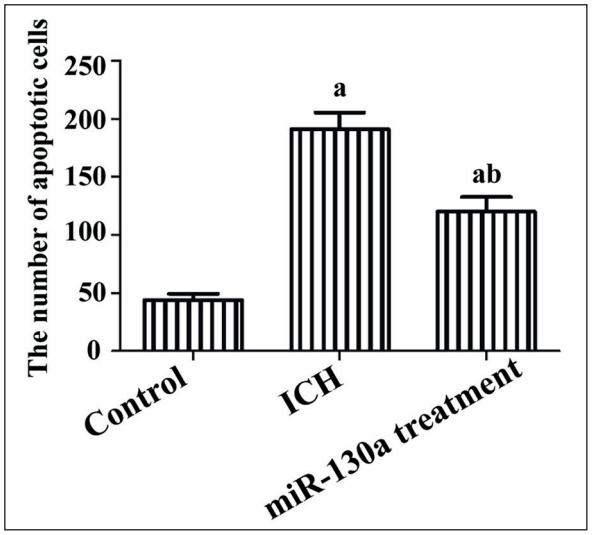

Figure 3. Comparison of the number of apoptotic cells. Note: a: $p<0.05$ *vs*. control group, b: $p<0.05$ *vs*. ICH group.

In this study, the activity of Caspase-9 in the control group [(0.92 ± 0.06) U] was significantly lower than the ICH group [(2.28 ± 0.24) U] and miR-130a treatment group [(1.63 ± 0.12) U] ($p<0.05$). However, it increased markedly in the ICH group when compared with that of the miR-130a treatment group, showing statistically significant differences ($p<0.05$). According to the study of Slattery et al[14], the expression of Caspase-9 is significantly up-regulated in the ischemic lesion in ICH when compared with that in the control group, consistent with the results in this work. Caspase has been observed to inhibit nerve cells in ICH by promoting the apoptosis level of brain tissues[15]. MiR-130a can phosphorylate AKT gene through PI3K, inhibiting Caspase from exerting its biological effects. This may also facilitate the survival, proliferation and division of brain tissue cells[16,17]. In addition, enhanced expression of Caspase-9, as a promoter of Caspase, can interact with its downstream apoptotic target genes, thereby damaging the nervous system[18].

In the present study, the protein level of PTEN was significantly higher in the ICH group than the control group and miR-130a treatment group, displaying statistically significant differences ($p<0.05$). Meanwhile, the protein levels of p-PI3K and p-AKT were remarkably lower in the ICH group than those of the control group and miR-130a treatment group ($p<0.05$). However, they were remarkably higher in the miR-130a treatment group than that of the control group, displaying statistically significant differences ($p<0.05$). Iorio et al[19] have demonstrated that the imbalance of the PTEN/PI3K/AKT signaling system seriously affects learning, memory and neurological functions. Lee et al[20] have indicated that the protein levels of p-PI3K and p-AKT in brain tissues of rats with cerebral infarction are markedly lower than those in the control group. The PI3K/AKT signaling pathway is an important link in the central nervous system; it is involved in regulating cell metabolism, neuronal development and neurotransmitter signal transduction. Moreover, it plays an important role in cell proliferation, differentiation and apoptosis[21]. PTEN is an important tumor suppressor gene with phosphatase activity discovered currently. The expression of PTEN can be inhibited by miR-130a. In addition, PTEN is an important negative

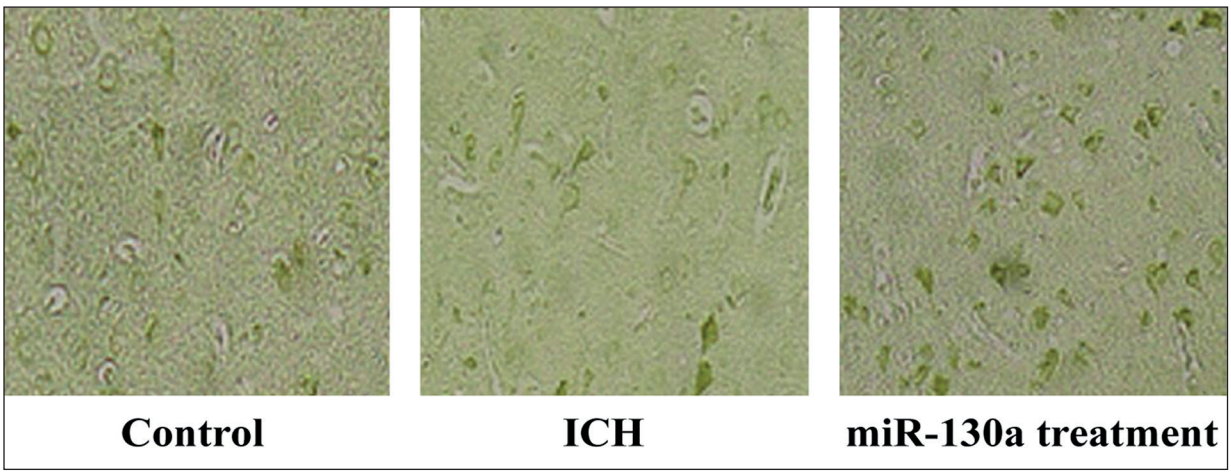

Figure 4. Caspase-9 activity in brain tissues (×400).

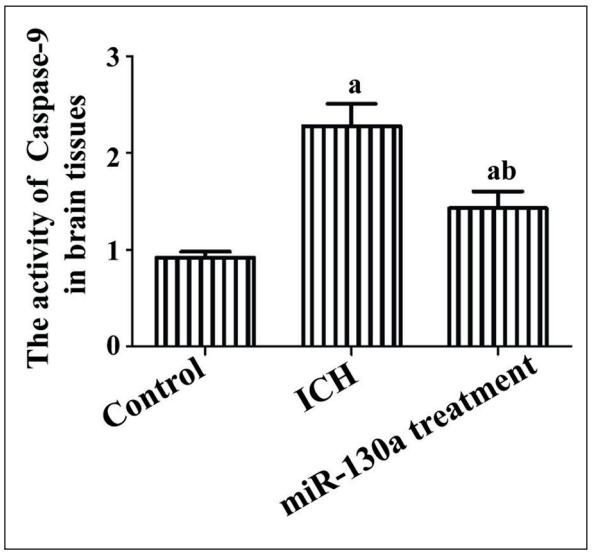

Figure 5. Comparison of Caspase-9 activity in brain tissues. Note: a: $p<0.05$ *vs.* control group, b: $p<0.05$ *vs.* ICH group.

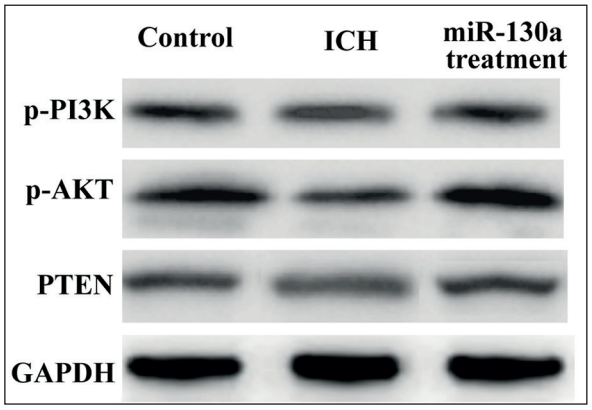

Figure 6. Protein expression levels of PTEN, p-PI3K and p-AKT in brain tissues.

regulator of the PI3K/AKT signaling pathway. The decline in PTEN level can promote the transformation from PIP2 to PIPS, thereby activating the PI3K/AKT signaling pathway[22]. Ultimately, this can significantly inhibit the apoptosis of brain tissues, reduce neuronal injury and alleviate sequelae after ICH in rats, such as cognitive disorder and motor disorder[23].

Conclusions

We proved that miR-130a could significantly improve neurological function and apoptosis in ICH rats, promote neuronal growth in brain tissues in ICH rats and alleviate neuronal injury after ICH through the PTEN/PI3K/AKT signaling pathway. In addition, our findings showed important clinical significance in the treatment of ICH.

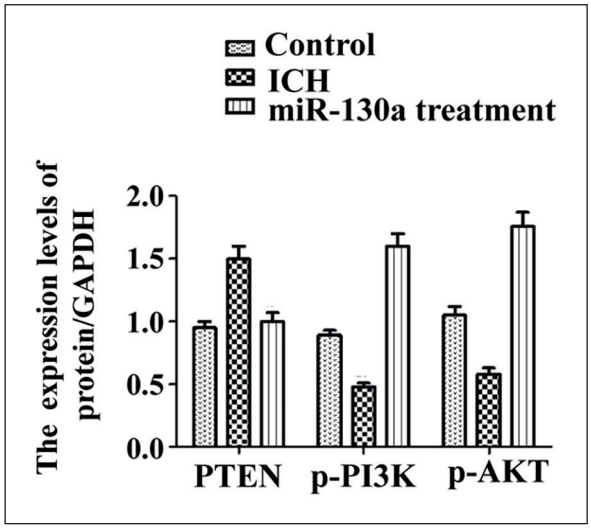

Figure 7. Comparison of protein expression levels of PTEN, p-PI3K and p-AKT in brain tissues among the three groups. Note: a: $p<0.05$ *vs.* control group, b: $p<0.05$ *vs.* ICH group.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

Plasmid pVAX1-Mediated Adrenomedullin Gene Therapy for Cerebral Vasospasm Following Subarachnoid Hemorrhage in Rats (2016-KYYWF-0579).

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