

Study on the expression and mechanism of inflammatory factors in the brain of rats with cerebral vasospasm

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Abstract. – **OBJECTIVE:** We investigated the significance of IL-1 and ICAM-1 in rat's subarachnoid hemorrhage (SAH) cerebral vasospasm (CVS) model.

MATERIALS AND METHODS: A total of 30 Sprague-Dawley (SD) rats were randomly divided into the SAH group and the Sham group. Cisterna magna auto blood injection was used to prepare the CVS models. We studied and compared changes in the basilar arteries diameters before and after SAH. We measured the cerebrovascular inner diameter before and after SAH modeling using the ultrasound. ELISA method was used to measure the expression of IL-1 and ICAM-1 in peripheral blood. The expression of MAPK and P38 in the brain was tested using Western blot. Brain cells apoptosis was studied using TUNEL method.

RESULTS: Cerebrovascular inner diameter reduced significantly in the SAH group as compared to the control group. The expression of IL-1 and ICAM-1 increased significantly after 48 hours. Compared to the Sham group, p-38 and p-MAPK expression levels in the SAH group increased significantly after 48 hours. Results showed that 48 hours after the operation, the level of apoptosis was significantly higher in the SAH group.

IL-1 and ICAM-1 expression levels were associated with a P38-MAPK signal pathway in the brain. p38 and MAPK activation were closely related to apoptosis in the cortex.

CONCLUSIONS: We suggest that the cerebral basilar vasospasm was occurred in rats 48 hours after ASH onset, with an increase in IL-1 and ICAM-1 expression and brain cells apoptosis.

Key Words:

Subarachnoid hemorrhage (SAH), Cerebral vasospasm (CVS), IL-1, ICAM-1.

Introduction

Subarachnoid hemorrhage (SAH) is a clinical syndrome resulting from blood direct-

ly flowing into the subarachnoid space due to pathological vascular rupture in the bottom and surface of the brain. SAH, which is also known as primary ASH, accounts for 10% of all acute strokes. It is a very serious disease and, according to World Health Organization, the incidence rate of SAH is about 2 in 0.1 million in China^{1,2}. However, there are reports suggesting a higher incidence rate (6-20 in 0.1 million). If blood breaks through the brain and flows into the subarachnoid space due to brain parenchyma and ventricular bleeding, or epidural or subdural vascular rupture, it is called the secondary ASH. A large number of inflammatory cells and massive inflammation can be seen after SAH onset due to ischemia and cerebral vasospasm (CVS). Among various types of inflammatory factors, IL-1 is generated by fibroblasts, monocytes/macrophages, T lymphocytes, B lymphocytes, epithelial cells, keratinocytes and various kinds of oncocytes³⁻⁵. Several factors including TNF- α monocytes/macrophages cAMP can induce normal cells to generate interleukin-1 (IL-1)⁶⁻⁸.

Intercellular cell adhesion molecule-1 (ICAM-1) also known as CD54, is a member of the immune globulin superfamily (IGSF). ICAM-1 is an important adhesion molecule with low expression level in resting vascular endothelial cells (VEC). ICAM1 plays an important role in stabilizing the intercellular interaction and promoting the migration of white blood cells (WBC) and endothelial cells^{9,10}. Previous studies showed that the ICAM-1 expression could be mediated by the P38-MAPK signal pathway. It plays a role in VEC and cerebrovascular endothelial cells, various histiocytes and also plays an important role in the ischemic diseases of renal tissues, heart tissue, and brain tissue¹¹. Previous studies also reported that ICAM-1 expression was significantly increased in SAH rabbit secondary brain damage models, but the

mechanism of CVS after SAH remains unclear. We, in this study, investigated the significance of IL-1 and ICAM-1 in rat's SAH CVS model.

Materials and Methods

Laboratory Reagents

0.9% sterile saline solution (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), rat-anti-human Egr-1 antibody (Wuhan Boster Biological Technology Co., Ltd. Wuhan, China), rat-anti-human PTEN antibody (Wuhan Boster Biological Technology Co., Ltd. Wuhan, China), immunohistochemistry SP900 staining kits (ZSGB-BIO, Beijing, China), DAB concentration staining kit (ZSGB-BIO, Beijing, China), Mayer hematoxylin (ZSGB-BIO, Beijing, China), fresh xylene (The Third Chemical Regent Factory of Tianjin, Tianjin, China), 35% ethanol (The Third Chemical Regent Factory of Tianjin, Tianjin, China), $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (The Third Chemical Regent Factory of Tianjin, Tianjin, China), $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (The Third Chemical Regent Factory of Tianjin, Tianjin, China), citric acid (The Third Chemical Regent Factory of Tianjin, Tianjin, China) and sodium citrate (The Third Chemical Regent Factory of Tianjin, Tianjin, China).

Experimental Instruments

Micropipettor (Eppendorf, Hamburg, Germany) Haier ice maker, 4°C constant temperature refrigerator (Thermo, Shanghai, China), 10 ml syringe, 5 ml syringe (HANACO, Tianjin, China), pathological section (Leica, Hamburg, Germany), EP tube (Eppendorff, Germany), water bath (Beijing Medical Device Factory, Beijing, China), microscope camera (Olympus, Tokyo, Japan) and optical microscopy (Olympus, Tokyo, Japan).

Laboratory Animals

Adult male SD rats with a weight range of 270 to 330 g were purchased from Animals Center of Zhejiang University of TCM (Hangzhou, China). Rats were placed in a feeding room with air filtration equipment and fed with sufficient water and food before the operation. The temperature of the feeding room and operating room was about 25°C. Number of cases suffering different grades of pain in the two groups.

Cerebral vasospasm (CVS) Modeling

A total of 30 Sprague-Dawley (SD) rats were randomly divided into the SAH group and the Sham group. SAH group was set up with subarachnoid

hemorrhage (SAH). After giving 25% urethane of abdominal anesthesia, rats were prone and fixed on a stereotactic instrument to expose the atlanto-occipital fascia. A No. 4.5 syringe needle was used to puncture the cisterna magna and draw out 0.1 ml of cerebrospinal fluid, then 0.2 ml of autologous arterial blood or 0.9% normal saline (NS) was slowly injected. The wound was stitched after using a medical adhesive to close the puncture opening. The head and tail were kept high. Changes of basilar arteries diameter were examined.

To investigate the dynamic changes in ICAM-1 and IL-1 expression in the wall of arteries, immunohistochemical staining was conducted.

Immunohistochemical Streptavidin Peroxidase (SP) Method

Within a designed sampling time, the basilar arteries were taken out under the craniotomy after rats being sacrificed. Samples were fixed in 2% glutaraldehyde paraformaldehyde for 8 hours and washed with 0.1 mol/L PBS. Gradient dehydration and Epon812 embedding were conducted. 3% H_2O_2 was added, and samples were washed after 15 min with phosphate-buffered saline (PBS) 3 times (5 min each time). 10% normal goat serum was added and samples were incubated for 30 min at 37 °C. The primary antibodies against IL-1 (1:200) and ICAM-1 (1:150) were added and samples were incubated at 4 °C. Biotinylated goat-anti-ribbit IgG (1:200) was added followed by 30 minutes incubation at 37 °C. It was washed with PBS 3 times (5 min each time). DAB color developing solution was added for 10 minutes. We then carried out hematoxylin counterstaining and neutral gum mounting. Samples were observed under the microscope. PBS was used as negative control to replace the primary antibodies.

Western Blot

Proteins from brain tissue were extracted and protein concentration was measured using Bio-Rad protein kits (Bio-Rad, Hercules, CA, USA). Proteins samples were then subjected to SDS-PAGE for separation followed by a transfer to a membrane. Primary antibody was added to the membrane followed by overnight incubation at 37°C. The secondary antibody was added next followed by visualization enhanced chemiluminescence (VEC). The primary antibodies included anti-ICAM-1, anti-IL-1 (1:500; Biolegend, San Diego, CA, USA) and anti-P-actin (1:5000, Invitrogen, Carlsbad, CA, USA). ImagePro+6.0 imaging software was used to perform the quantitative analysis.

Statistical Analysis

SPSS 20.0 statistical software package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All data were expressed as $\bar{x}\pm s$. For time-dose effect we used ANOVA. For comparison among groups and comparison of multiple time-point data, we used one-way ANOVA. LSD method was used for pairwise comparison between groups. For data with the heterogeneity of variance, Tamhane's analysis was used. For variables with a related tendency, the Pearson correlation analysis was used: $\alpha = 0.05$ (two-tailed) was acted as the test criteria. $p < 0.05$ meant that the difference was statistically significant.

Results

Comparison of Baseline Data in Rats

We compared the baseline data for rats in each group, including BW, HR, SP, DP, MAP, stimulus response time and CPR duration. The difference was not statistically significant ($p > 0.05$) (Table I).

Changes in Inner Vascular Diameter

We measured the inner cerebrovascular diameter before and after SAH modeling using the ultrasound. Results showed that the cerebrovascular inner diameter reduced significantly in the model group (1.20 ± 0.22 mm) as compared to the control group (3.27 ± 0.49 mm). The difference was statistically significant ($p < 0.05$) (Table II).

IL-1 and ICAM-1 Expression

ELISA was used to measure the expression levels of IL-1 and ICAM-1 in peripheral blood. Results showed that the expression levels of IL-1 and ICAM-1 increased significantly after 48 hours. The difference was statistically significant ($p < 0.05$). IL-1 and ICAM-1 expression levels in basilar arteries were tested using IHC, 48 hours after treatment. IL-1 and ICAM-1 expression levels increased significantly in the SAH group as compared to the Sham group. The difference was statistically significant ($p < 0.05$) (Table III and Figure 1).

MAPK-P38 Single pathway

The expression of MAPK and P38 in the brain was studied using Western blot. Results showed that, compared to the sham group, p-38 and p-MAPK expression levels in the SAH group increased significantly after 48 hours as, and continuously increased within 72 hours. The difference was statistically significant ($p < 0.05$) (Figure 2).

Table I. Comparison of baseline data in rats.

Group	N	Body weight BW (g)	Heart rate HR (bpm)	Systolic pressure SP (mmHg)	Diastolic pressure DP (mmHg)	Mean artery pressure MAP (mmHg)	Stimulus Response Time	Cardiopulmonary resuscitation CPR duration Time
Sham	10	355.7±46.21	414.6±33.96	111.3±10.27	92.3±7.61	96.2±7.39	-	-
SAH	10	363.6±47.14	418.58±39.46	111.8±10.66	91.8±9.96	97.5±9.66	82.5±10.20	117.4±48.77
F-value	-	0.72	0.44	0.71	0.49	0.51	0.93	0.43
p-value	-	0.34	0.52	0.19	0.57	0.62	0.11	0.57

Table II. Comparison of inner diameter of basilar arteries in rats ($\bar{x}\pm s$).

Group	N	12h	24h	48h	F-value	p-value
Sham	10	3.17±0.22	3.33±0.18	3.27±0.49	1.29	0.02
SAH	10	1.43±0.19	1.19±0.18	1.20±0.22	2.27	0.01
F-value	-	0.72	0.44	2.71	0.49	0.51
p-value	-	0.34	0.52	0.02	0.57	0.62

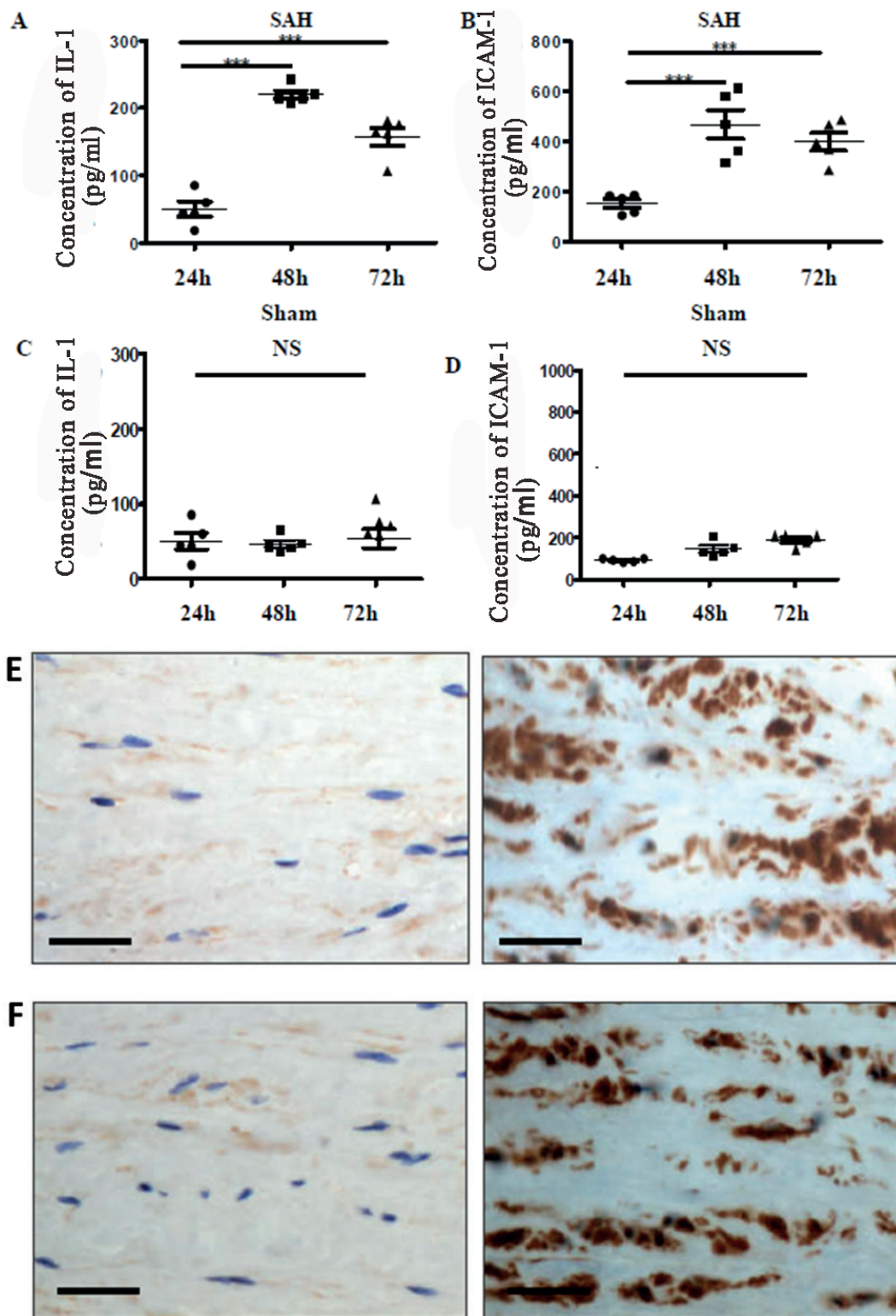


Figure 1. A-D, ELISA result in the SAH and Sham group. Expression of IL-1 and ICAM in the peripheral blood 48 hours after treatment in the SAH group was significantly higher than that of pretreatment. The difference was statistically significant ($***p<0.05$). Dynamic changes were not found in the sham group. E, The difference in the expression of IL-1 in the basilar arteries was statistically significant ($p<0.05$) as compared to the sham group. F, The difference of expression of ICAM-1 in the basilar arteries.

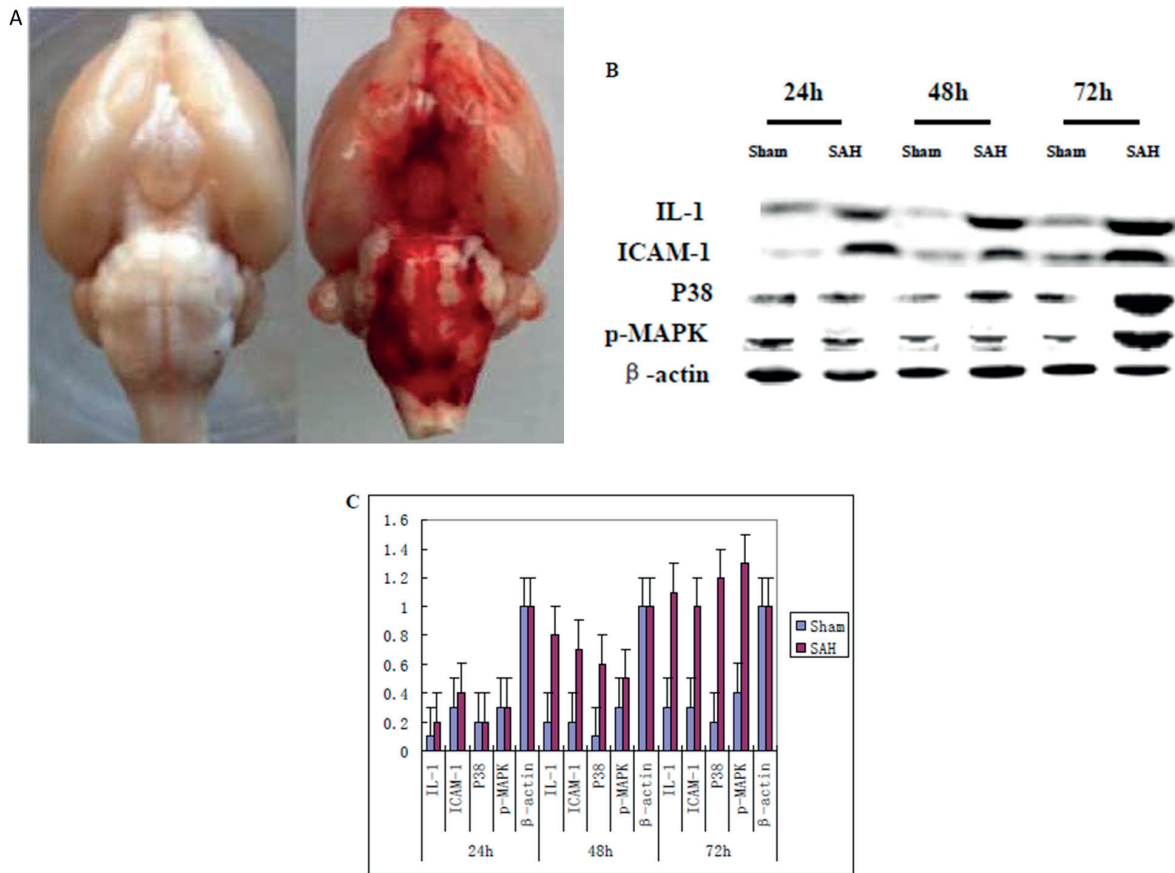


Figure 2. *A*, Brain tissue in the sham group and the SAH group. *B*, Western blot results show that the levels of p-38 and p-MAPK in SAH group significantly increased as compared to Sham group. *C*, The difference was statistically significant ($p < 0.05$).

Apoptosis Results by TUNEL Staining in Rats

Brain cells apoptosis was tested using TUNEL method. Results showed that 48 hours after the operation, compared to the sham group, the level of apoptosis was significantly higher in the SAH group. The difference was statistically significant ($p < 0.05$) (Figure 3).

Discussion

Subarachnoid hemorrhage (SAH) is usually caused by aneurysm rupture in 2% to 9% of stroke victims¹². CVS is deemed to be an important cause of poor prognosis of SAH. A great number of studies have been conducted on the secondary brain injury induced by CVS^{13,14}. Some large-scale clinical trials have shown that in patients with SAH, we cannot improve the prognosis by inhibiting CVS^{15,16}. There are evidences sug-

gesting that early brain injury (EBI) can happen within the first 72 hours after SAH onset. It plays an important role in SAH prognosis. Prior studies showed that the level of neuronal apoptosis was closely related to SAH patient's life quality¹⁷⁻²⁰.

Our results revealed that basilar arteries' walls were thickened and the lumen was significantly narrowed 48 hours after SAH onset. Our results suggested that IL-1 and ICAM-1 expression levels in peripheral blood increased significantly in the SAH group and was apparently higher than those in the sham group. IL-1 and ICAM-1 expression levels in brain tissue were boosted 48 hours after SAH onset. Our results indicated that the inflammatory factors were released in the course of cerebral vasospasm. Results obtained from prior studies²¹ showed that IL-1 polymorphism was closely related to the prognosis of the stroke. IL-1 played an important role in both acute and chronic inflammation that

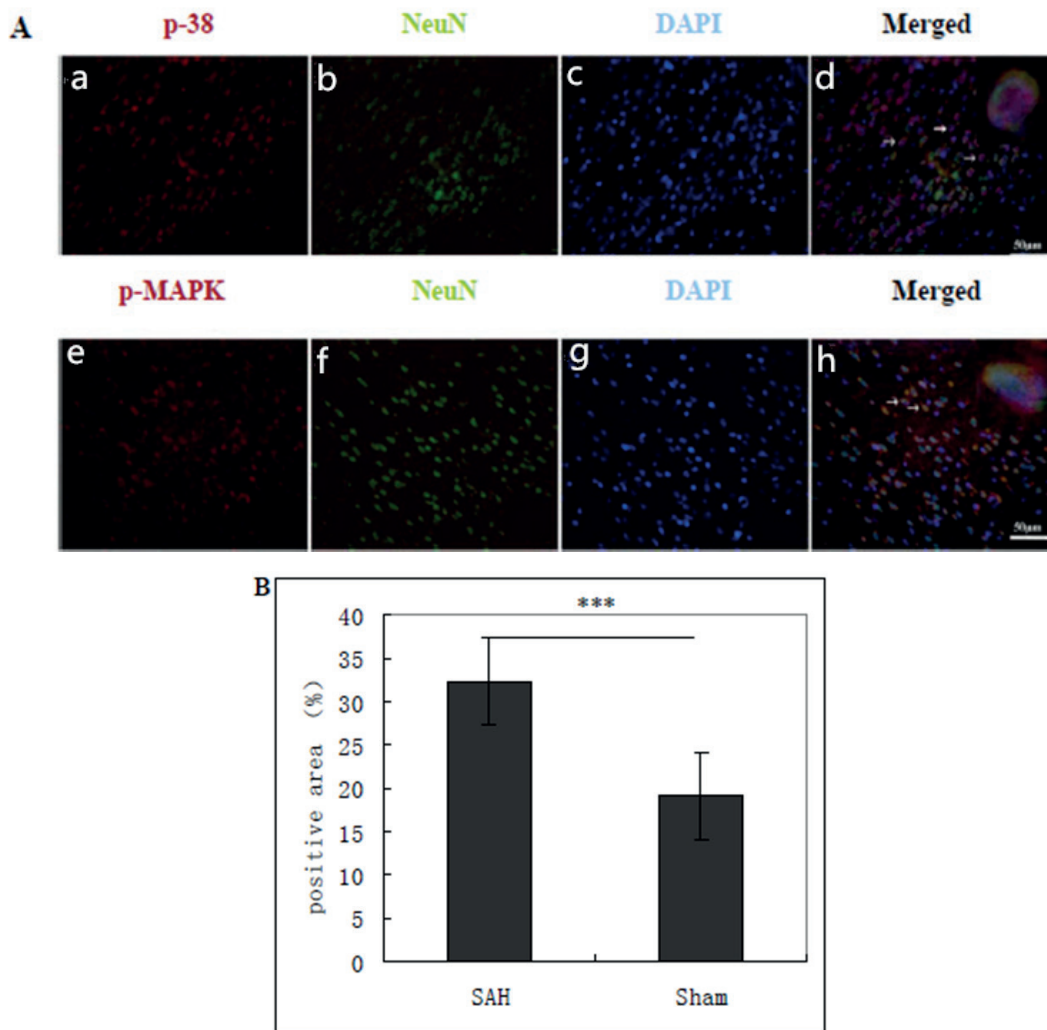


Figure 3. 100 X under the fluorescence microscope. **A**, TUNEL staining results (*green fluorescence*): *Left*: SAH group; *Right*: Sham group. **B**, Compared with Sham group, the percentage of positive areas is significantly increased in the SAH group. The difference is statistically significant (** $p < 0.05$).

was induced by various factors such as antigens, endotoxin, bacteria, viruses and ischemia²². It was also bound up with the pathological process of diabetes, rheumatoid arthritis, and periodontitis. It played a role in patients with past history of ischaemic heart disease²³ and SAH to some extent. There are evidences suggesting that IL-1 receptor antagonist may antagonize the levels of inflammatory factors in the cerebrospinal fluid during SAH²⁴. It demonstrates certain protective effects on brain injury. Moreover, *in vivo* and *in vitro* experimental results showed that IL-1P gene polymorphisms had some impact²⁵ on ischemic stroke in young people. ICAM-1 is an important adhesion molecule that mediates

adhesive response. It plays a role in biological activities via binding to those specific receptors on VES surface. Chang et al²⁶ showed that in the CVS rat model, the valproic acid suppressed the action of ICAM-1 and E-selectin via chemotactic factor ligand 5 independent mechanism. Our results showed that IL-1 and ICAM-1 expression levels peaked at 48 hours in the SAH group. We also discovered that IL-1 and ICAM-1 expression levels were associated with a P38-MAPK signal pathway in the brain. Additionally, p38 and MAPK activation were closely related to apoptosis in the cortex. However, these changes may only be seen 48 hours after the operation. The difference between expression levels was

Table III. Comparison of expression of IL-1 and ICAM-1 in peripheral blood (pg/ml).

Item	Group	N	12h	24h	48h	72h	F-value	p-value
IL-1	SAH	5	117±22.4	133.4±19.2	327.2±49.5	319.4±12.7	1.29	0.02
	Sham	5	103 ±19.5	109.2±18.3	98.3 ±22.4	99.4±10.9	0.27	0.69
	F-value	-	0.72	0.44	2.71	8.2	-	-
	p-value	-	0.34	0.52	0.02	0.008	-	-
ICAM-1	Sham	5	152.7±18.9	143.4±21.8	128.4±25.4	139.8±19.7	0.43	0.57
	SAH	5	141.3±21.4	149.4±13.2	420.4±18.7	401.6±28.7	2.89	0.02
	F-value	-	0.22	0.97	3.24	2.81	-	-
	p-value	-	0.31	0.08	0.01	0.02	-	-

not detected in the early and immediate-early stages of SAH.

There are some shortcomings associated with this study. For example, the expression levels of inflammatory factors in the immediate-early stage of SAH still need to be further studied.

Conclusions

We concluded that the cerebral basilar vasospasm was occurred in rats 48 hours after ASH onset, with an increase in IL-1 and ICAM-1 expression and brain cells apoptosis.

Conflict of interest

The authors declare no conflicts of interest.

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