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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 detected 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-MAPK expression 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 vasospasm was occurred in rats 48 hours after ASH onset, with an increase 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 directly flowing into the subarachnoid space due to pathological vascular rupture in the bottom and surface of brain. SAH, which is also known as primary SAH, accounts for 10% of all acute strokes. It is 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 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 oncocytes[3-5]. 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

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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), $NaH_2PO_42H_2O$ (The Third Chemical Regent Factory of Tianjin, Tianjin, China), $Na_2HPO_412H_2O$ (The Third Chemical Regent Factory of Tianjin, Tianjin, China), citric acid (The Third Chemical Regent Factory of Tianjin, Ti... China) and sodium citrate (The Third C... Regent Factory of Tianjin, Tianjin, China).

Experimental Instruments

Micropipettor (Eppendorf, H... Germa... ny) Haier ice maker, 4°C c... perature refrigerator (Thermo, Shan..., China... 0 ml syringe, 5 ml syringe (HA...O, Ti...), pathological section ...ica..., G... ny), EP tube (Eppe...ff, Ger...) water bath (Beijing Medical ...e Factory, ..., China), microscope ca... ...pus, Tokyo, ...pan) and optical micros...py (O..., Tokyo, Japan).

Labor... ...y Animals

A... ...male S... ...ats with a weight range of 270 to ... were ...chased from Animals Center of Zheji... ...rsity c... ...M (Hangzhou, China). ... wer... ...ed in ...eding room with air filtra... ...quipm... ...ed with sufficient water and foo... ...efore the ...eration. The temperature of the fe... ...m and operating room was about 25°C. ... ...es suffering different grades of pain ... the two groups.

C... ...ral 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 ...d to puncture the cisterna magna and draw... of cerebrospinal fluid, then 0.2 ml of ...logous ar... terial blood or 0.9% normal saline ... was slowly injected. The wound was stitche... using a medical adhesive to close th... unctu... ning. The head and tail were ken... n. Change... lar arteries diameter we... amined...

To investigate the d... ...ic ch... ...s in ICAM-1 and IL-1 expressio... in ... ...arteri... ...amunohistochemical ... ling ... nduct...

Immunoh... ...mical Strep... ...din Peroxid... (SP... ...thod

Within a designed ... ...pling time, the basilar arte... ...e taken out t... ...the craniotomy after ra... ...eing sacrificed. Samp...es were fixed in 2% ... ...raldehyde pa... ...formaldehyde for 8 hours and ... ...ed with 0.1 ...L PBS. Gradient dehydration a... ...on812 em... ...ding were conducted. 3% H_2O_2 was... ...d, an... ...mples were washed after 15 min with p... ...buffered saline (PBS) 3 times (5 ...in each time). 10% normal goat serum was added ... ...les were incubated for 30 min at 37 °C. The ... ...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 x±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: a = 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 cerebrovascula eter before and after SAH modeling using t l- trasound. Results showed that the cerebrovas inner diameter reduced significantly in the mo group (1.20±0.22 mm) as compa he contr group (3.27 ±0.49 mm). The d ene s statis- tically significant ($p<0.05$) ble II).

IL-1 and ICAM-1 Exp sio

ELISA was used easure pression lev- els of IL-1 and I 1 in periph lood. Results showed th ession level L-1 and ICAM-1 incre d sign ly after 48 hours. The difference s statistical ificant ($p<0.05$). IL-1 a AM-1 expressio ls in basilar arteries were teste ing IHC, 48 hours after treatment. IL-1 an AM-1 expression levels increased signi the SA oup as compared to the n gr The d ence was statistically significant ($p <$ ble III and Figure 1).

M 38 Single pathway

The sion of MAPK and P38 in the brain was studied using Western blot. Results showed compared to the sham group, p-38 and p PK 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 (bmp)	Systolic pressure SP (mmHg)	Dias pres DP (g)	artery sure mm g) MA	Stimulus Response Time	Cardiopulmonary resuscitation CPR duration Time
Sham	10	355.7±46.21	414.6±33.96	111.3±10.27	92.	96.2±7.39	-	-
SAH	10	363.6±47.14	418.58±39.46	111.8±10.66	91.8±	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	0.6	.11	0.57

Table II. Comparison of inner diameter of basilar arteries in rats (x̄±s).

Group	N	12h	24h	48h	F	p-value
Sham	10	3.17±0.22	3.33±0.18	3.27±0.49	1.29	2
SAH	10	1.43±0.19	1.19±0.18	1.20±0.22	2.27	
F-value	-	0.72	0.44	2.71	0.4	
p-value	-	0.34	0.52	0.02		

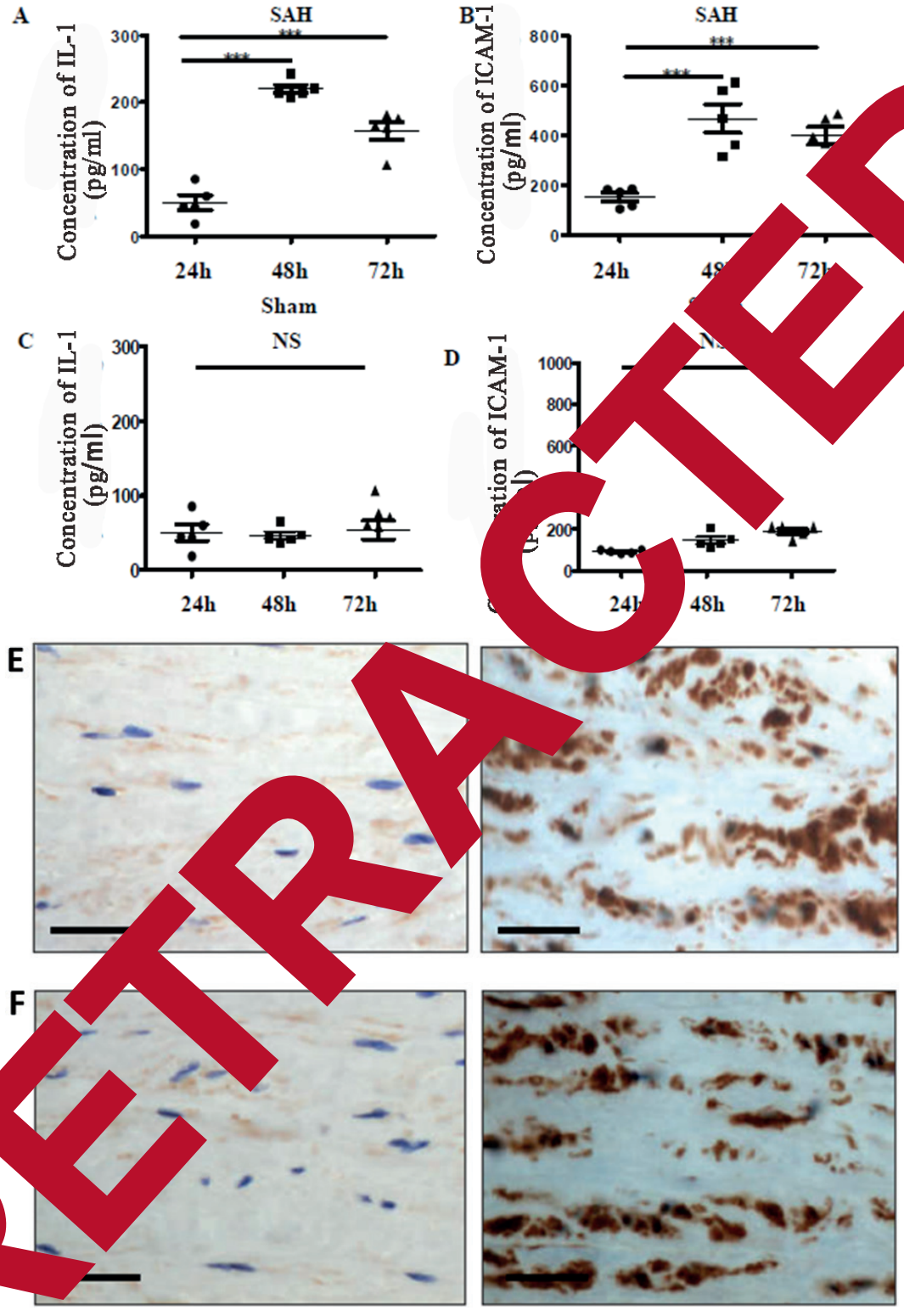

Figure 1. ***A-D,*** ELISA result in the SAH and Sham group. Expression of IL-1 and ICAM in the peripheral blood 48 hours after treatment in the SAH group was significantly higher than that of pretreatment. The difference was statistically significant (***p<0.05). Dynamic changes were not found in the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

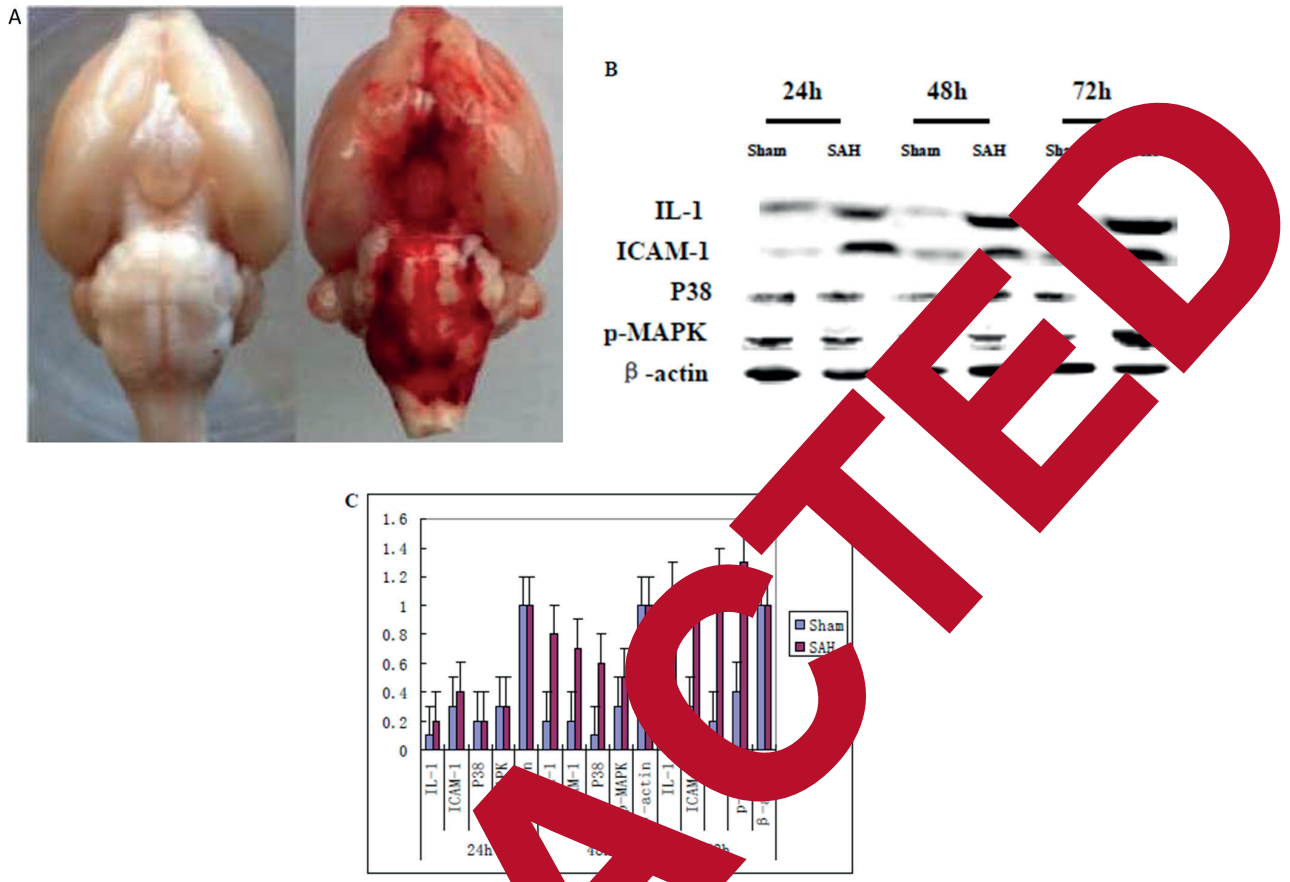

Figure 2. ***A,*** Brain tissue in the sham group and the SAH ... Western blot results show that the levels of p-38 and p-MAPK in SAH group significantly increased a... to Sham ... ***C,*** The difference was statistically significant ($p<0.05$).

Apoptosis Results b... NEL St... in Rats

Brain cells ap... was teste... TUNEL method. Resul... that 48 ho... after the operation, co...ared t... ham group, the level of apopto...as significa... higher in the SAH group. ... difference was st...cally significant ($p <0$... (Figu...).

D...ussion

S...arachno...emorrhage (SAH) is usually ...d by aneurysm rupture in 2% to 9% of ...[12]. CVS is deemed to be an important cause of poor prognosis of SAH. A great ...ber of studies have been conducted on the s...ary 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 suggesting 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 quality[17-20].

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 studies[21] 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

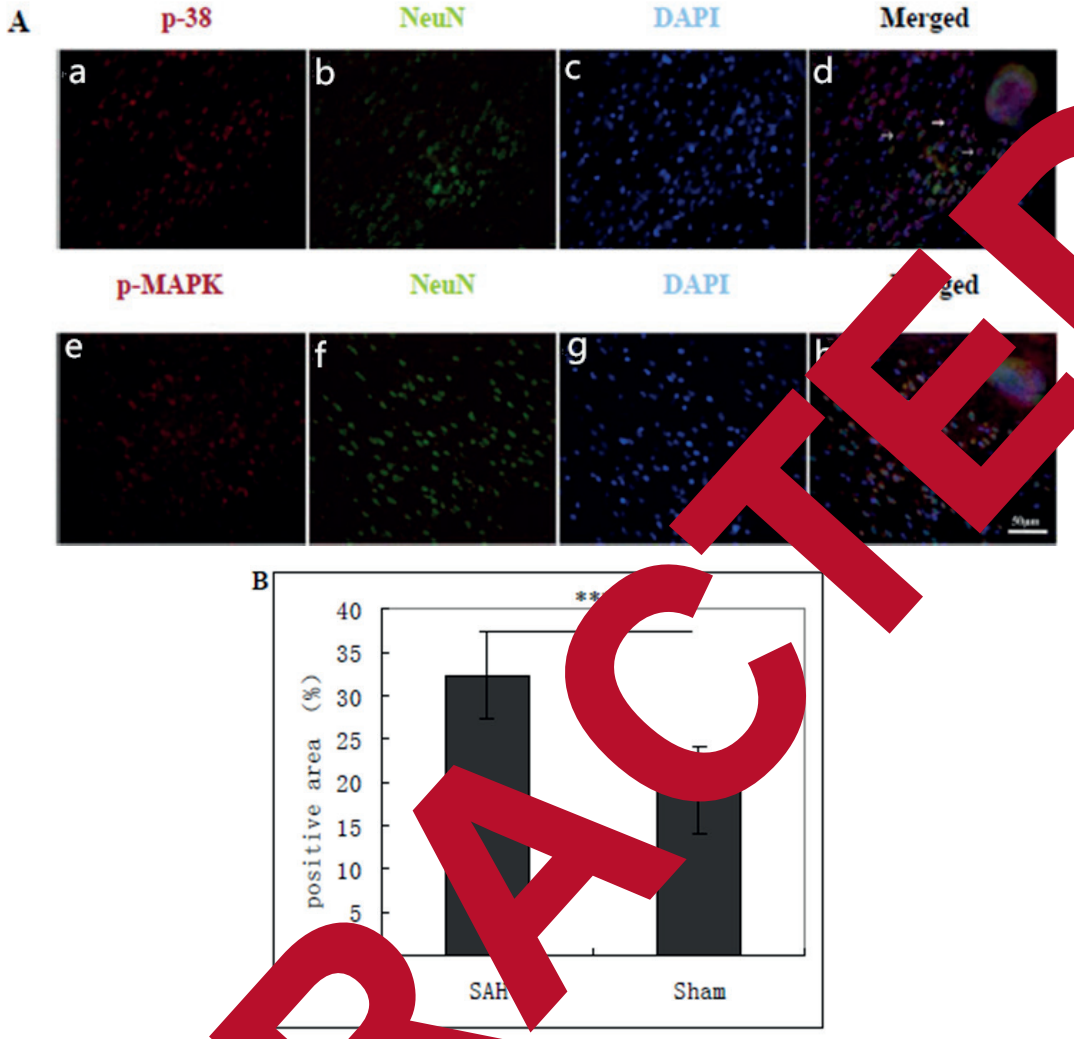

Figure 3. 100 X under th fl ce UNEL staining results (*green fluorescence*): *Left:* SAH group; *Right:* Sham group. ***B,*** C red group, the percentage of positive areas is significantly increased in the SAH group. The difference i stically s nt (***p<0.05).

was in d by various facte uch as antigens, endo n, bac a, viruses and ischemia[22]. It wa bou p with the pathological process of dia eumato rthritis, and periodon- It p a rol patients with past history haem isease[23] and SAH to some ext There a vidences suggesting that IL-1 re tagonist may antagonize the levels of factors in the cerebrospinal fluid ring SAH[24]. It demonstrates certain protec- effects on brain injury. Moreover, *in vivo* a *vitro* experimental results showed that IL-1P gene polymorphisms had some impact[25] 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 al[26] 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	[illegible]	24h	48h	72h	F-value	p-value
IL-1	SAH	5	117±[illegible]	133.4±19.2	327.2±49.5	319.4±12.7	1.29	0.02
	Sham	[illegible]	10[illegible]	109.2±18.3	98.3 ±22.4	99.4±10.9	0.27	0.69
	F-value	-	[illegible]	0.44	2.71	8.2	-	-
	p-value	-	[illegible].34	0.52	0.02	0.008	-	-
ICAM-1	Sham	5	[illegible]2.7±18.9	[illegible]±21.8	128.4±25.4	139.8±19.7	0.43	0.57
	SAH	5	141.3±21.4	[illegible]3.2	420.4±18.7	401.6±28.7	2.89	0.02
	F-value	-	0.22	[illegible]	3.24	2.81	-	-
	p-value	-	0.31	[illegible]	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 [illegible] of inflammatory factors in the imme[illegible] stage of SAH still need to be further [illegible]ied.

Conclusi[illegible]

We concluded that th[illegible]erebral basilar [illegible] spasm was occurred [illegible]ts 48 [illegible]s after ASH onset, with an inc[illegible]se[illegible] [illegible]d ICA[illegible] ex- pression and bra[illegible]lls ap[illegible]s.

Conflict [illegible]ter[illegible]

The authors declare no [illegible]icts of interest.

[illegible]eferences

1) [illegible]okolov AA, [illegible]ain S, Sztajzel R, Croquelois A, [illegible]nus JA, [illegible]er D, Stadler C, Hungerbuhler H, [illegible] GJ, Michel P. Fatal subarachnoid hem[illegible]ge following ischemia in vertebrobasilar dolichoectasia. Medicine (Baltimore) 2016; 95: [illegible]920.

2) [illegible]nglong Z, Guangwei Z, Xuenong H, Xiaohui X, Xiaochuan S, Yanfeng X. The role of platelet-derived growth factor receptor in early brain injury following subarachnoid hemorrhage. J Stroke Cerebrovasc Dis 2016; 25: 2203-2208.

3) Roth C, Ferbert A. Subarachnoid Hemorrhage and isolated Brainstem Death. Fortschr Neurol Psychiatr 2016; 84: 377-384.

4) Albanna W, Conzen C, Weiss M, Clusmann H, Fuest M, Mueller M, Brockmann MA, Vilser W, Schmidt-Trucksass A, Hoellig A, Seiz M, Thome C, Kotliar K, Schubert GA. Retinal vessel analysis (RVA) in the context of subarachnoid hemorrhage--a proof of concept study. PLoS One 2016; 11: e158781.

5) Tangen K, Narasimhan NS, Sierzega K, Preden T, Alaraj A, Linninger AA. Clearance of subarachnoid hemorrhage from the cerebrospinal fluid in computational and in vitro models. Ann Biomed Eng 2016; 44: 3478-3494.

6) Kim JH, Yi HJ, Ko Y, Kim YS, Kim DW, Kim JM. Effectiveness of papaverine cisternal irrigation for cerebral vasospasm after aneurysmal subarachnoid hemorrhage and measurement of biomarkers. Neurol Sci 2014; 35: 715-722.

7) Xie X, Wu X, Cui J, Li H, Yan X. Increase ICAM-1 and LFA-1 expression by cerebrospinal fluid of subarachnoid hemorrhage patients: involvement of TNF-α. Brain Res 2013; 1512: 89-96.

8) Wang Z, Zuo G, Shi XY, Zhang J, Fang Q, Chen G. Progesterone administration modulates cortical

TLR4/NF-κB signaling pathway after subarachnoid hemorrhage in male rats. Mediators Inflamm 2011; 2011: 848309.

9) Lad SP, Hegen H, Gupta G, Deisenhammer F, Steinberg GK. Proteomic biomarker discovery in cerebrospinal fluid for cerebral vasospasm following subarachnoid hemorrhage. J Stroke Cerebrovasc Dis 2012; 21: 30-41.

10) Zhou ML, Zhu L, Wang J, Hang CH, Shi JX. The inflammation in the gut after experimental subarachnoid hemorrhage. J Surg Res 2007; 137: 103-108.

11) Rothoerl RD, Schebesch KM, Kubitza M, Woertgen C, Brawanski A, Pina AL. ICAM-1 and VCAM-1 expression following aneurysmal subarachnoid hemorrhage and their possible role in the pathophysiology of subsequent ischemic deficits. Cerebrovasc Dis 2006; 22: 143-149.

12) Sudlow CL, Warlow CP. Comparable studies of the incidence of stroke and its pathological types: results from an international collaboration. International Stroke Incidence Collaboration. Stroke 1997; 28: 491-499.

13) Velat GJ, Kimball MM, Mocco JD, Hoh BL. Vasospasm after aneurysmal subarachnoid hemorrhage: review of randomized controlled trials and meta-analyses in the literature. World Neurosurg 2011; 76: 446-454.

14) Golan E, Vasquez DN, Ferguson ND, Adhikari NK, Scales DC. Prophylactic magnesium for im... neurologic outcome after aneurysmal su... noid hemorrhage: systematic review and m... analysis. J Crit Care 2013; 28: 173-181.

15) Ma J, Huang S, Ma L, Liu Y, Li H, You C. Endot... lin-receptor antagonists for a... al subar... chnoid hemorrhage: an upd... nalysis o... randomized controlled tri... rit Ca... 2012; 16: R198.

16) Wang X, Li YM, Li W... CG... Effect of clazosent... pa... aneurysmal subarachnoid h... rrhage: ... a-analysis of randomized c... ed trials. PL... e 2012; 7: e47778.

17) Hasegawa ... uzuki ... T, Altay O, Zhang JH. Apopt... mechanisms ... uronal cells in early brai... ry after subarac... hemorrhage. Acta N... chir Su... l 2011; 110: 4... 48.

18) Friedrich V, Flores R, Sehba FA. Cell death starts early after subarachnoid hemorrhage. Neurosci Lett 2012; 512: 6-11.

19) Ayer RE, Zhang JH. Oxidative stress in subarachnoid haemorrhage: significance in a... injury and vasospasm. Acta Neu... 2008; 104: 33-41.

20) Yuksel S, Tosun YB, Cahill J, Solar... Early brain injury following aneurysmal suba... d hemorrhage: emphasis on cellula... poptos... Neurosurg 2012; 22: 529-53...

21) Ding W, Gao N, Li MX... G LJ, L... FF, Mou... nical evaluation of t... icacy o... combina... of aneurysm embo... n an... ebrospi... al fluid replacement in... e t... aneur... l subarachnoid h... rhage... ev Me... rmacol Sci 2015; 1... 2-405.

22) Rezk NA... HS. Influe... interleukin-1 gene... er p... rphisms on t... e susceptibility and outcomes o... stroke in Egyptian patients... Biochem Bio... 2015; 71: 637-647.

23) ...B, Heidt T, Hulsm... M, Dutta P, Courties G, ...ebas M, Wojtkiewicz GR, Tricot B, Iwamoto Y, Sun Y, Weissleder ... ibby P, Swirski FK, Nahrendorf M. Targeting int... kin-1P reduces leukocyte production after ... e myocardial infarction. Circula... 2015; 1... 880-1890.

24) Si... kins SJ, Hulme S, Galea JP, Hoadley M, Vail A, Hutchinson PJ, Grainger S, Rothwell NJ, King AT, Tyrrell PJ. The effect of intravenous interleukin-1 ... receptor antagonist on inflammatory mediators in cerebrospinal fluid after subarachnoid haemorrhage: A phase II randomised controlled trial. J Neuroinflammation 2014; 11: 1.

25) Yang B, Zhao H, X B, Wang YB, Zhang J, Cao YK, Wu Q, Cao F. Influence of interleukin-1 beta gene polymorphisms on the risk of myocardial infarction and ischemic stroke at young age in vivo and in vitro. Int J Clin Exp Pathol 2015; 8: 13806-13813.

26) Chang CZ, Wu SC, Lin CL, Kwan AL. Valproic acid attenuates intercellular adhesion molecule-1 and E-selectin through a chemokine ligand 5 dependent mechanism and subarachnoid hemorrhage induced vasospasm in a rat model. J Inflamm (Lond) 2015; 12: 27.