The expression of Cav3.1 on T-type calcium channels of rats with subarachnoid hemorrhage

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1. Introduction

Subarachnoid hemorrhage (SAH) is a severe and destructive disease with a high mortality risk, which can seriously affect the whole body (Geraghty and Testai, 2017). The current view is that the pathogenesis of SAH is related to factors such as intracranial hypertension, inflammation and vasospasm (Aldakkan et al., 2017). These pathological factors can lead to different complications in different stages of the disease. Among many complications, cerebral vasospasm (CVS) after SAH is the most common complication. Furthermore, the CVS has been considered as the main cause of death or disability after SAH (Griessenauer et al., 2018; Palfi et al., 2018). In general, vasospasm is divided into acute cerebral vasospasm (ACVS) and delayed cerebral vasospasm (DCVS) (Lee et al., 2018a). It is generally supposed that the pathogenesis of DCVS may stem from complications such as inflammation, blood disintegration products, vascular endothelial injury as well as calcium. In addition, it may also be prompted by multiple factors. However, regardless of the mechanisms, generated calcium influx leads to vasospasm (Ma et al., 2018; Lee et al., 2018b). It is pointed out that after SAH, the sensitivity of contractile proteins to calcium ions would increase, which could further enhance the role of calcium ions in vasospasm. The T-type calcium channel is an important member of the calcium channel family, also known as the low voltage activated calcium channel. T-type calcium channel protein is composed of tetramers, and each monomer is α1 subunit, containing about 2,000 amino acids. Based on this structure, the opening and closing of the channel can be controlled when the membrane potential changes (Nagahama et al., 2018). The T-type calcium channel Cav3.1 is an important link in the cascade effect, which can generate action potentials and currents under quiescent condition, thereby activating a series of biological effects and widely participating in the initiation of various physiological
and pathological mechanisms. At present, the drugs for delayed vasospasm are mainly L-type calcium channel antagonist nimodipine and flunarizine hydrochloride (Al-Jehani et al., 2018). Nimodipine is one of the dihydropyridine calcium antagonists, which can reduce intracellular calcium concentration by blocking L-type calcium channels on vascular endothelial cells, consequently achieving vasodilation effects (Okada and Suzuki, 2017). Flunarizine hydrochloride is a calcium channel blocker that prevents cell damage caused by intracellular pathological calcium overload elicited by ischemia. It is suitable for cerebral circulation disorders caused by arachnoid, cerebral hemorrhage, etc. (Patel et al., 2017). In SAH, T-type calcium channel Cav3.1 may be involved in the occurrence and development of DCVS, which may lead to the development of DCVS after SAH. This possibility urges researchers to further understand the role of T-type calcium channel Cav3.1 in SAH, and provide feasible treatment for clinical practice.

2. Materials and methods

2.1. Animals

60 healthy male SD (Sprague Dawley) rats weighing 260–290 g and aged 7–8 weeks were purchased from Shanghai Yisen Biotechnology Co., Ltd., China. All the rats were housed with a 12-h light and dark cycle, and allowed free access to food and water as well. Besides, the temperature was controlled at 22 ± 2 °C and the humidity was 40–60%. The experimental rats were allowed 1 week to acclimate prior to the intervention. The protocol was approved by the ethics committee of Tongde Hospital of Zhejiang Province, and the experiment also followed the regulations of the State Science and Technology Commission on the management of laboratory animals.

2.2. Grouping

Sixty rats were randomly divided into 5 groups (12 rats in each group):

Group A: Sham operation group (n = 12) was divided into three groups: 2nd, 4th and 6th days, injecting saline into subarachnoid space.

Group B: Blood injection model group (n = 12) was divided into three groups: 2nd, 4th and 6th days, injecting autologous blood into subarachnoid space.

Group C: Nimodipine (Sigma Company, USA) group (n = 12) was divided into three groups: 2nd, 4th and 6th days. Three groups were given nimodipine orally (2 mg/kg/d) on the premise of blood injection;

Group D: Flunarizine hydrochloride (Sigma Company, USA) group (n = 12) was divided into three groups: 2nd, 4th and 6th days. Three groups were given flunarizine hydrochloride (1 mg/kg/d) by intragastric administration on the premise of blood injection;

Group E: Normal group (n = 12) was divided into 3 groups: 2nd, 4th and 6th days without any treatment.

2.3. Construction of blood injection models in rats

SD rats in Group B were anesthetized by intraperitoneal injection of 10% chloral hydrate. Rats were anesthetized with intraperitoneal injection of 1 mL/100 g of 10% chloral hydrate, and then placed in a prone position. Vital signs were monitored using a vital sign monitor. After anesthesia, the rats lied prone and the vital signs of the rats were continuously monitored. The head was fixed to keep it straightforward and routinely disinfected. Autologous blood was taken from the rat tail and transferred to syringe. In addition, skin along with fascia of the head were incised, and bone sutures were exposed by ophtalmic forceps. Furthermore, 1.2–1.8 mm before the intersection of intraocular canthus was marked. The skull was drilled at the mark with a miniature electric cranial drill (Thermo Company, USA), and the dura mater was picked with a small needle. After the clear cerebrospinal fluid was discharged, a small long needle syringe (RAININ Company, USA) was taken to extract the autologous blood of the rat. 0.3 mL autologous blood was slowly injected through a drill hole. Then, the hole was blocked with bone wax, and the wound was sutured. Since the rats classified in group C and group D were blood injection models, 12 + 12 * 2 = 36 rat models should be constructed. Animals in sham operation group A was operated the same way as group B, replacing autologous blood with saline of the same volume (a total of 12 rats).

2.4. Drug intervention

24 SD rats with successful hematopoietic injection were randomly divided into two groups. Group C (Nimodipine group, n = 12): Nimodipine was administered by gavage for 2 days, 4 days, and 6 days after modeling at 2 mg/kg/d every day, respectively. Group D (Flunarizine Hydrochloride group, n = 12): Each experimental rat was given 1 mg/kg/d flunarizine hydrochloride per day by gavage for 2 days, 4 days, and 6 days after modeling, respectively.

2.5. Weight and neurobehavioral observation

Body weight observation: The daily intake of food in each group was recorded, and the changes in body weight of rats in each group before and after surgery were recorded. The mean values of each group were compared.

Neurobehavioral observation: Scores were graded at 2nd, 4th, and 6th days after surgery, respectively. The degree of neurological impairment was negatively correlated with the score (full score, 18). The scoring criteria: Spontaneous activities are rated at four levels, 0–3. In details, no spontaneous activity, 0; moving position only, 1; activities but not touching the three sides of the cage wall, 2; activities and touching the cage wall above the three sides, 3. Spontaneous limb movement evaluation is divided for four levels, 0–3. No spontaneous physical movement score, 0; slight physical activity, 1; physical activities but slow, 2–3. Forelimb activity tailing evaluation: Forefoot movements when lifted, 0; forefoot movements with a slight extension, 1; forelimb movements with a forward extension but worse than normal, 2–3. Climbing wire cage walls are rated in three grades, 1–3: incapable of climbing, 1; climbing difficulties, 2; normal climbing, 3. The touch response to the rat whisker was rated in three grades, 1–3.

2.6. Brain tissue sampling

A group of 4 rats were killed at 2 days, 4 days, and 6 days, respectively, 20 were killed each day, and the cerebral vascular tissues were collected. After the rats were successfully anesthetized by intraperitoneal injection of 10 mL/kg of 10% chloral hydrate, the rats were placed in the supine position. The xiphoid was used as the surgical approach, and the thoracic cavity was opened. The cardiac vesicle was cut, the heart was exposed, and the abdominal aorta were clamped. The aorta was punctured, and fixed. The right atrium was cut open. The blood was rinsed out through aorta with phosphate buffer saline (PBS) solution (0.01 mol/L) (Genview Company, China). After the effluent was clear, lavage was paused, and the head was then immediately cut off with tissue scissors. The skull was cut and the brain tissues were removed. A portion of brain tissues were placed in 4% paraformaldehyde (Shanghai
of A260 to A260/A280 (1.71–1.99) was determined by an ultraviolet
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2.7. Brain tissue staining

Fixed brain tissues were put into a glass bottle, and the bottle-
neck was sealed with gauze. Then the rubber tube was inserted to
connect running water and the bottle was washed for 24 h. Then
the tissues were dehydrated, transparentized, fixed, embedded
with hard wax and beeswax (Sigma Company, American) and cut
into slices. The protein was mixed with neutral gum (Sigma Com-
pany, USA) and applied to two-thirds of the slices. Then the slices
were placed in a water bath (CRYSTAL Company, USA) for surface,
dried and put into xylene (Sigma Company, American) for dewater-
ing. Ethanol was used to elute paraffin and xylene. The slices were
stained with hematoxylin (Shanghai Rongbai Biotechnology Co.,
Ltd., China), separated by hydrochloric acid alcohol, and rinsed
with running water overnight. The tissues were sliced and soaked
in a solution of 0.5%-1% eosin (Shanghai Rongbai Biotechnology Co.,
Ltd. China), and the slices were taken out and put into 95% ethanol
to continue color separation and dehydration. They were dehy-
drated twice with anhydrous alcohol, and xylene was used for
transparency. Finally, they were sealed with a neutral gum.


2.8. Real-time fluorescence quantitative PCR (polymerase chain
reaction) detection

In order to extract total RNA from cerebrovascular tissues, rat
brain tissues were added with excessive liquid nitrogen, and the
blood vessels were removed under the anatomical microscope. A
small amount of liquid nitrogen was quickly poured into the brain
and ground until it turned into powder, which was put into a 5 mL
EP tube. Then, 1 mL Trizol (TakaRa, Japan) homogenate was added,
and placed in ice bath for 15 min. On the super-clean workbench, it
stood for 5 min and take the supernatant in the new EP
tube. Then, 1 mL Trizol (TakaRa, Japan) homogenate was added,
and placed in an ice bath for 15 min. On a clean bench, it stood for
5 min, and the supernatant was placed into a new EP tube. 5:1
chloroform (Sigma Company, USA) was added, mixed slowly, and
placed for 10 min. The supernatant was transferred to an EP tube
after it was centrifuged at 4 °C and 14,000 rpm for 15 min, and
the same volume of isopropanol (Sigma Company, USA) was added.
The solution stood for 10 min. The supernatant was discarded by
centrifugation at 14,000 rpm and 4 °C for 15 min, and 1 mL of
75% ethanol was added. The supernatant was discarded by cen-
trifugation at 7500 rpm for 4 min at 4 °C, and 10 μL of DEPC
enzyme-free water (Genview Company, China) were added to dis-
solve. The ratio of A260/A280 (1.71–1.99) was measured by an
ultraviolet spectrophotometer (Eppendorf Company, USA), rever-
sely transcribed by a reverse transcription kit (Thermo Scientific
Company), amplified, and stored at −20 °C until use.

After total RNA was extracted from the cerebral blood vessels,
the expression level of the related gene of Anti-CACNAI (Cruz
Company, USA) was examined. Primer Premier 5.0 software was
used to design the target gene primers according to the full-
length sequence of each target gene in RatBank. The amplified frag-
ment was 263 bp. Quantitative PCR amplification was performed
by the BR Green I fluorescent dye method. A 10 μL reaction system
was used according to the kit. Quantitative PCR amplification was
performed using the SYBR Green I fluorescent dye (Beijing TianGen
Company, China) method. A 10 μl reaction system was used
according to the kit. Pre-denaturation was carried out at 95 °C
for 30 s, 95 °C for 15 s, 60 °C for 30 s, and repeated for 40 times,
followed by the dissolution reaction phase. A single peak should
be displayed for each PCR product dissolution profile analysis.
The expression of CACNAI-related genes was analyzed based on
the internal reference and the ct value of the target gene.

2.9. Detection of protein by Western Blot (WB)

After grinding cerebrovascular samples, 500μL of cell lysate
(Shanghai Biyun Tian Biotechnology, China) was added, including
PMSF (Phenylmethanesulfonyl fluoride) 5μL. Freezing and thawing
were repeated for three times, 5 min each time. After a slight
shock, tissue homogenate was moved into a 1.5 mL EP tube. Then,
supernatant was centrifuged and afterward, the protein concentra-
tion was determined by bicinchoninic acid (BCA). 10% PAGE sepa-
rating gel and 5% PAGE concentrating gel were prepared and the
proteins in the homogenate were separated by electrophoresis.
The extracted protein was transferred to PVDF (Millipore Com-
pany, USA) membrane. Then, 5% skimmed milk was prepared by
tris buffered saline with tween (TBST) to reserve the membrane
for 2 h. Anti-CACNAI primary antibody was added and incubated
for 8–12 h at 4 °C. Phosphate buffered saline with tween (PBST)
was consumed three times along with the corresponding second
antibody labeled with peroxidase for 1 h. Finally, the expression
of protein was detected and analyzed by appropriate amount of
ECL (electrochemiluminescence) luminescent solution.

After the brain blood vessel samples were ground, 500 μL of cell
lysate buffer (Shanghai Biyun Tian Biotechnology Research) were
added, including 5 μL of PMSF (Millipore Company, USA). Freezing
was repeated and thawing was performed three times, 5 min for
each time. After a slight shock, the tissue homogenate was trans-
ferrred to a 1.5 mL EP tube and the supernatant was obtained by
centrifugation. The protein in the homogenate was separated by
electrophoresis. The protein extract was transferred to a PVDF
(polyvinylidene fluoride) membrane (Millipore Company, USA),
which was sealed in 5% skim milk prepared by TBST (Millipore
Company, USA) for 2 h. Anti-CACNAI primary antibody (Wuhan
Dr. De Company, China) was added for incubation for 8–12 h at
4 °C. The membrane was washed 3 times with PBST (mixture of
tris-buffered saline (TBS) and Tween 20) (Millipore Company,
USA), and the PVDF membrane was incubated for 1 h with the cor-
responding secondary antibody labeled with peroxidase (Shanghai Biyuntian Biotechnology Research). Finally, the expression of the protein was detected by an appropriate amount of ECL luminescent solution (Elabscience Company, China).

2.10. Statistical analysis

A database was established based on collected data and SPSS 19.0 (SPSS Inc., Chicago, IL, USA) was employed for analyzing the data. The data were expressed by mean ± standard deviation (Mean ± SD). First, the homogeneity of variance was tested, and the SNK method was utilized to compare the multiple groups of mean. When the variance was uneven, Kruskal WallisH method can be applied as a non-parametric test. P < 0.05 indicated significant difference.

3. Results

3.1. Changes of physiological parameters in model rats after drug intervention

On the 2nd, 4th, and 6th days of the experiments, the weight loss of the rats before operation was compared with that after operation. There was no significant difference between the sham operation group A and the normal group E, and the body weight was almost unchanged (it was hence not listed). The weight of rats in the blood injection model decreased. As can be seen in Fig. 1, the weight loss of rats in group B was the most obvious. After blood injection, the weight of rats in group C and group D also decreased significantly. On the 2nd day, the weight loss of rats in group C was less than that in group D, however, more than that in group D on the 4th and 6th days (Fig. 1).

In this study, the food intake of group E was used as the basis to demonstrate the decrease of the food intake after operation. There was no significant difference between the sham operation group A and the normal group E, and the food intake was almost unchanged (group A and group E were hence not listed). There were no significant differences in food intake between group E and group A on the 2nd, 4th, and 6th days (p > 0.05), so it was not confirmed as a significant factor in the list. As shown in Fig. 2, after blood injection, all the rats in group B showed a decrease in food intake, which was the most significant in the model group. The rats in group C ate more food on the 2nd day than those in group D; however, on the 4th and 6th days, the rats in group D ate more food than those in group C.

The neurobehavioral score of the normal group was 18 for the full score, and there was no significant difference between the sham operation group and the normal group on the 4th and 6th days. As can be seen in Fig. 3, the behavioral ability scores of the blood injection model group were lower than those of the normal group on the 2nd, 4th and 6th days after the operation, and the rats showed behavioral retardation and decreased reactivity after the operation. Moreover, the behavioral ability score of the blood injection model group was the lowest. The behavioral ability score of the nimodipine group was higher than that of the flunarizine hydrochloride group on the 2nd day. On the 4th and 6th days, the behavioral ability score of the flunarizine hydrochloride group was higher than in the nimodipine group (Fig. 3).

3.2. Cerebrovascular staining in rats

All rat brain tissues were sliced in groups on the 2nd day, the 4th day as well as the 6th day. Fig. 4 showed results of the staining. In sham operation group, cerebral vascular endothelial dilation, no vasospasm and no blood infiltration in cortex were observed at each time point. Vasospasm was observed at each time point in the blood injection model group, with the most obvious endothelial folds on the 2nd day, followed by the 6th and 4th days. The number of endothelial folds in nimodipine group and flunarizine hydrochloride group were more than that in sham operation group and less than that in model group.

The number of vascular endothelial spasm in nimodipine group on the 2nd day was less than that in flunarizine hydrochloride group. In addition, the number of vascular endothelial folds on the 6th day was greater than that in flunarizine hydrochloride group. Furthermore, the number of vascular endothelial spasm in flunarizine hydrochloride group on the 2nd day was greater than that in nimodipine group, and moreover, the number of vascular endothelial spasm on the 6th day was less than that in nimodipine group.
Fig. 4. Vascular slices (*400) (A: slices of brain tissues in rats of sham operation group in three different periods; B: slices of brain tissues in rats of blood injection model group in three different periods; C: slices of three periods of brain tissues in rats of nimodipine group in three different periods; D: slices of three periods of brain tissues in rats of flunarizine hydrochloride group in three different periods).

Fig. 5. Real-time fluorescent immuno-PCR for Cav3.1 mRNA content (*P < 0.05).
3.3. Real-time fluorescence quantitative PCR

Real-time fluorescent immuno-PCR showed that Cav3.1 gene was expressed in normal group, blood injection model group, nimodipine group along with flunarizine hydrochloride group. The amount of Cav3.1 gene in model group was higher than that in normal group. There was no significant difference between model group and nimodipine group and flunarizine hydrochloride group, as shown in Fig. 5.

3.4. WB results

As shown in Fig. 6, Cav3.1 was widely expressed in the cerebrovascular of model rats, nimodipine group and flunarizine...
hydrochloride group. The expression of Cav3.1 was significantly higher than that of sham operation group, which was related to the time of blood injection. In addition, there was no significant difference in the Cav3.1 expression between sham operation groups at different time periods. According to the obtained electrophoretic WB maps, the expression trend of Cav3.1 in rat cerebrovascular at different time points after operation could be obtained.

4. Discussion

Caudal vein blood sampling (Diringer and Zazulia, 2017) was adopted to construct the rat model, which reduced the possibility of blood loss or large wounds that affected the experimental results. In this experiment, the method of optic chiasma blood injection was adopted (Almufti et al., 2018; Hejcˇl et al., 2017), which can control the blood volume and speed, and control the mortality of rats within the normal range. Delayed vasospasm after subarachnoid hemorrhage is a complex disease caused by many factors (Aum et al., 2017). At the molecular level, the degree of vascular stenosis is generally improved by regulating the channel activity of calcium ions to cause smooth muscle contraction, which ultimately changes the degree of vasospasm (Da Silva et al., 2017; Hendrix et al., 2017). Therefore, the effect of T-type calcium channel antagonist nimodipine and flunarizine hydrochloride on the expression of T-type calcium channel Cav3.1 was investigated. It was found that the greater the degree of vasospasm in rats, the more obvious the decrease in food intake and body weight, and the expression of T-type calcium channel Cav3.1 was higher. Calcium channel antagonists nimodipine and flunarizine hydrochloride can increase the food intake and body weight of rats compared with the model group, reduce the number of endothelial folds, and relieve vascular endothelium spasticity. However, the change in the expression level of T-type calcium channel Cav3.1 was not obvious, indicating that the calcium channel antagonist nimodipine and flunarizine hydrochloride have a certain therapeutic effect on delayed vasospasm, but not through T-type calcium channels. Through the animal experiment, this study expands the application range of T-type calcium antagonists, changes the clinical dilemma for the treatment of delayed vasospasm to a certain extent, and improves the therapeutic effect, which is clinically significant.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Aldakkan, A., Mansouri, A., Jaja, B.N., Alotaihi, N.M., Macdonald, R.L., 2017. Subarachnoid Hemorrhage International Trialists Collaborators. Predictors of delayed cerebral ischemia in aneurysmal subarachnoid hemorrhage patients with asymptomatic angiographic vasospasm on admission. World Neurosurg. 97, 199–204.
Al-Jehani, H., Angle, M., Marcoux, J., Teitelbaum, J., 2018. Early abnormal transient hyperemic response test can predict delayed ischemic neurologic deficit in subarachnoid hemorrhage. Crit. Ultrasound J. 10 (1), 1.
Almufti, F., Amuluru, K., Damodara, N., et al., 2018. Novel management strategies for medically-refractory vasospasm following aneurysmal subarachnoid hemorrhage. J. Neurol. Sci. 390, 44–51.
Aum, D.J., Vellimana, A.K., Singh, I., Milner, E., Nelson, J.W., Han, B.H., Zipfel, G.J., 2017. A novel fluorescent imaging technique for assessment of cerebral vasospasm after experimental subarachnoid hemorrhage. Sci. Rep. 7 (1), 9126.
Da Silva, I.R.F., Gomes, J.A., Wachsman, A., de Freitas, C.R., Provencio, J.J., 2017. Hematologic counts as predictors of delayed cerebral ischemia after aneurysmal subarachnoid hemorrhage. J. Crit. Care 37, 126–129.
Diringer, M.N., Zazulia, A.R., 2017. Aneurysmal subarachnoid hemorrhage: strategies for preventing vasospasm in the intensive care unit. Semin. Respir. Crit. Care Med. 38 (6), 760–767.
Geraghty, J.R., Testai, F.D., 2017. Delayed cerebral ischemia after subarachnoid hemorrhage: beyond vasospasm and towards a multifactorial pathophysiology. Curr. Atheroscler. Rep. 19 (12), 50.
Griessenauer, C.J., Starke, R.M., Foreman, P.M., et al., 2018. Associations between endothelin polymorphisms and aneurysmal subarachnoid hemorrhage, clinical vasospasm, delayed cerebral ischemia, and functional outcome. J. Neurosurg. 128 (5), 1311–1317.
Hejcˇl, A., Cihlář, F., Smolka, V., et al., 2017. Chemical angioplasty with spasmyltics for vasospasm after subarachnoid hemorrhage. Acta Neurochir. (Wien) 159 (4), 189–197.
Hematologic counts as predictors of delayed cerebral ischemia after subarachnoid hemorrhage. World Neurosurg. 115, e558–e569.
Lee, H., Perry, J.J., English, S.W., et al., 2018b. Clinical prediction of delayed cerebral ischemia in aneurysmal subarachnoid hemorrhage. J. Neurosurg., 1–8.
Ma, Y., Qiao, G., Yin, Y., Zhang, Y., Yu, Y., Xu, X., 2018. Protective effects of Astragaloside IV on delayed cerebral vasospasm in an experimental rat model of subarachnoid hemorrhage. World Neurosurg. 118, e443–e448.
Nagahama, Y., Allan, L., Nakagawa, D., et al., 2018. Dual antiplatelet therapy in aneurysmal subarachnoid hemorrhage: association with reduced risk of clinical vasospasm and delayed cerebral ischemia. J. Neurosurg. 120 (3), 702–710.
Okada, T., Suzuki, H., 2017. Toll-like receptor 4 as a possible therapeutic target for subarachnoid hemorrhage. J. Clin. Med. 6 (4), 128 (5), 1311–1317.
Patel, A.S., Griessenauer, C.J., Gupta, R., et al., 2017. Safety and efficacy of noncompliant balloon angioplasty for the treatment of subarachnoid hemorrhage-induced vasospasm: a multicenter study. World Neurosurg. 98, 189–197.