European Review for Medical and Pharmacological Sciences 2017; 21: 3452-3458

The expression and mechanism of BDNF and NGB in perihematomal tissue in rats with intracerebral hemorrhage

Y.-C. GUO[1], X.-K. SONG[2], Y.-F. XU[1], J.-B. MA[1], J.-J. ZHANG[1], P.-J. HAN[1]

[1]Department of Neurosurgery, The Second People's Hospital of Liaocheng, Linqing City, Shandong Province, China
[2]Department of Neurosurgery, The Qinghe Central Hospital, Qinghe City, Hebei Province, China

Yuchen Guo and *Xiangkong Song* contributed equally

Abstract. – OBJECTIVE: To analyze how changes in the levels of brain-derived neurotrophic factor (BDNF) and neuroglobin (NGB) affect learning and memory in rats with intracerebral hemorrhage.

MATERIALS AND METHODS: Thirty male Sprague-Dawley rats were randomly divided into the control group, sham operation group and model group with 10 rats each. The rats in the control group were untreated, while those in the sham operation group were treated with sterile saline instead of type VII collagenase injection in the globus pallidus. The model of cerebral hemorrhage was established according to the methods described by Rosenberg. The expression of perihematomal BDNF mRNA was measured by Real-time quantitative PCR (RT-PCR) for 7 days consecutively. Perihematomal NGB-positive cells were detected by immunohistochemistry. The Morris water maze was used to test the spatial learning and memory of rats.

RESULTS: Compared with the control group and sham operation group, the expression of BDNF mRNA and number of NGB-positive cells in the model group were significantly higher. Furthermore, the escape latency was significantly prolonged ($p < 0.05$). The NGB and BDNF mRNA levels and escape latency were positively correlated. The correlation coefficients were as follows: rs (NGB) = 1.1838 ($p = 0.008$); rs (BDNF) = 0.5948 ($p = 0.012$).

CONCLUSIONS: Cerebral hemorrhage significantly inhibited the spatial learning and memory ability of rats. The mechanism may be related to decreased cerebral expression of BDNF and NGB.

Key Words:
Brain hemorrhage, BDNF, NGB

Introduction

In recent years, the incidence of cerebral hemorrhage has been increasing. Presently, the structure and function of mature brain tissues are considered to have a certain degree of plasticity. Promoting neural regeneration after cerebral hemorrhage has become a form of treatment for neuronal injury, and the secretion of neuroprotective factors after brain injury has become an area of intense research in the field of cerebrovascular disease. Brain-derived neurotrophic factor (BDNF), a protein widely distributed in the central nervous system, plays an important role in the growth, differentiation, and survival of neurons. Additionally, it can prevent nerve cells from death, and promote cell regeneration and differentiation[1,2]. Neuroglobin (NGB) is an oxygen-carrying protein expressed primarily in brain tissue. Under ischemia and hypoxia, NGB has been shown to be elevated, and has a very important function in protecting the nervous system[3,4], although its specific mechanism is not clear. Therefore, we used a rat model of collagenase-induced cerebral hemorrhage, and observed the expression of NGB and BDNF to explore their specific roles in spatial learning and memory.

Materials and Methods

Experimental Animals and Grouping

Thirty healthy male Sprague-Dawley (SD) rats weighing 220 ± 20 g were provided by the Second People's Hospital of Liaocheng City, Shandong Province, and randomly divided into the control group, sham operation group, and model group with 10 rats in each group. This study was approved by the Ethics Committee of the Second People's Hospital of Liaocheng.

Reagents and Equipment

Type VII collagenase (Sigma-Aldrich, St. Louis, MO, USA); Stoelting TL-2 mouse stereotaxic

Corresponding Author: Peijun Han, MD; e-mail: lc-hpj @163.com

apparatus (Biolegend, San Diego, CA, USA); Morris water maze and Ethovision system (Noldus, Wageningen, Netherlands); Trizol (Invitrogen, Carlsbad, CA, USA); PCR reagents (Biyun Tian Biotechnology Research Institute, Shanghai, China); Fluorescence Quantitative PCR machine (Model 7700) (ABI, Foster City, CA, USA); Rabbit anti-rat NGB polyclonal antibody (Xin Bo Sheng Biotechnology Company, Beijing, China).

Establishment of the Experimental Model of Intracerebral Hemorrhage

The method described by Rosenberg et al (5) was used to induce intracerebral hemorrhage in rats in the model group. Rats in the control group were untreated.

Rats in the sham-operated group were treated with sterile physiological saline instead of type VII collagenase, while the other operations were the same as those in the model group.

Observational Indexes

Neurological deficits were assessed as described by Longa et al[6] after awakening in rats; 0 points: no symptoms of neurological deficits; 1 point: subjects cannot fully extend the contralateral forelimb of the bleeding side; 2 points: circling rear-end to the side contralateral to the site of bleeding; 3 points: falling to the side contralateral to the site of bleeding side; 4 points: disturbance of consciousness and inability to spontaneously walk. Rats with scores between 1 and 3 were categorized as subjects for further analysis. The remaining animals were excluded, with corresponding replacement to ensure 10 rats in each group.

Determination of Perihematomal BDNF mRNA Expression Levels

Seven days after treatment, five rats in each group were euthanized with 10% chloral hydrate, and brains were quickly dissected. Some perihematomal tissues were separated. Total RNA was extracted according to the instructions of the Trizol kit. The concentration and purity of RNA were determined with an ultraviolet spectrophotometer. RNA was reverse transcribed into cDNA. The housekeeping gene, GADPH, was used as the internal reference to standardize gene expression levels. The gray values of the obtained bands were analyzed according to the instructions of the kit. The BDNF forward primer sequence: 5'-GTCCCTTCTACACTTACCTCTTG-3'; the BDNF reverse primer sequence: 5'-CTTTGTTTCACCCTTTCCACTCCT-3'. The GAPDH forward primer sequence: 5'-CACGGCAAGTTCAACGGCACAG-3'; the GAPDH reverse primer sequence: 5'-ACGCCAGTAGACTCCACGACAT-3'. PCR reaction conditions: pre-denaturation at 94°C for 5-10 min, denaturation at 94°C for 30 s-2 min, annealing for 30 s-2 min at 72°C, extension for 1-5 min at 72°C, and extension for 5-10 min. The three middle steps were repeated 30 times.

Immunohistochemical Detection of the Expression of NGB-positive Cells Around the Hematoma

Seven days after treatment, five rats in each group were euthanized with 10% chloral hydrate, and tissues were perfusion-fixed with 4% paraformaldehyde solution. When the bodies of rats hardened, they were decapitated and the brains were dissected. The brains were fixed in the same 4% paraformaldehyde solution, and then successively placed in 20% and 30% sucrose solutions. After the tissues sank to the bottom, they were sectioned in the cryostat and stored. Normal goat serum was added dropwise to tissue sections and incubated at room temperature for 30 min. A total of 50 µl primary antibody (rabbit-anti-mouse NGB) was added and placed at 4°C for 24 h. The corresponding biotin-labeled secondary antibody was added and incubated at room temperature for 30 min. SABC (Strept Avidin Biotin Complex) reagent was added and incubated at 30°C for 30 min. Freshly made 3,3'-diaminobenzidine (DAB) was added and the time of color development was controlled by microscopic examination. The sections were dehydrated, mounted, and sealed with neutral balsam. Next, they were observed, photographed, and analyzed.

Determination of Learning and Memory Ability

The Morris water maze was used to test spatial learning and memory capacity (7). Water maze device parameters: diameter of 120 cm; a circular escape plane of 10 cm diameter and 40 cm height. The device was wrapped with a black plastic bag and placed in the middle of the third quadrant, with the platform 2 cm below the water surface. The pool temperature was controlled at 25 ± 2°C. The water was blackened with ink while subjects around the pool for reference remained unchanged. The rats were placed in the pool from the four quadrants, respectively, and they were observed until they found the target platform.

Table I. The expression of BDNF mRNA in perihematomal tissue of rats ($\bar{x} \pm s$).

Group	BDNF mRNA expression level					*F* value	*p* value
	0 d	1 d	3 d	5 d	7 d		
Control	1.032 ± 0.328	1.047 ± 0.328	1.026 ± 0.282	1.082 ± 0.372	1.093 ± 0.378	0.873	0.128
Sham	1.073 ± 0.382	1.098 ± 0.326	1.067 ± 0.483	1.128 ± 0.372	1.298 ± 0.753	0.138	0.867
Model	1.037 ± 0.473	5.483 ± 0.327	8.372 ± 0.483	10.287 ± 0.483	18.287 ± 0.873	18.493	0.002
F value	0.632	14.387	12.873	9.389	10.278	–	–
p-value	0.387	0.003	0.008	0.013	0.012	–	–

If rats found the target platform within 60 s and stayed there for 15 s, they were removed from the maze. The time to move them from the starting points before finding the target platform was recorded and defined as the escape latency (EL). If rats could not find the target platform within 60 s, a rod was used to guide them to the platform. They were removed from the maze after staying on the platform for 15 s to guide their learning and memory. The EL was recorded as 60 s. This method was used for 5 consecutive days of training. On the 6th day, the mean time was recorded and used as an observant indicator to determine learning ability. The parameters of the entire experiment were recorded with a computer.

Statistical Analysis

SPSS13.0 (SPSS, Inc., Chicago, IL, USA) was used for data analysis. All quantitative data are presented as $\bar{x} \pm s$. Differences between groups were analyzed using ANOVA. The differences between two groups were analyzed by the LSD method. The number of BDNF- and NGB-positive cells in each group was analyzed by ANOVA. $p < 0.05$ suggested that the difference was statistically significant.

Results

The Expression of BDNF mRNA in Perihematomal Tissue of Rats

At day 0, there were no significant differences in the levels of perihematomal BDNF mRNA between the three groups. On days 1-7, the expression of BDNF mRNA in the control group was not significantly different from that in the sham operation group ($p > 0.05$). On days 1-7, the expression of BDNF mRNA in the model group was significantly higher than those in the control group and sham group ($p < 0.01$, respectively). There were differences between the expression of BDNF mRNA on day 0 and days 1-7 in the model group, but no differences were observed in the control group and sham group at the same time points ($p > 0.05$). See Table I and Figure 1.

The Expression of Perihematomal NGB-Positive Cells in Rats

On day 0, there were no significant differences in the number of NGB-positive cells between the three groups. However, a small number of NGB-positive cells were observed in

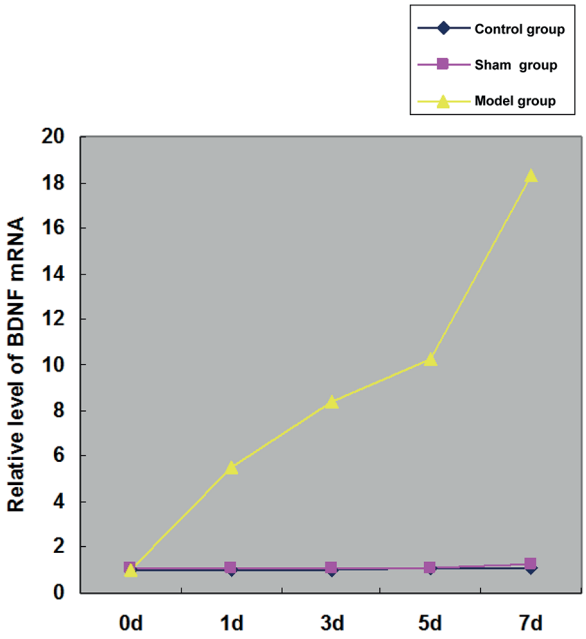

Figure 1. Comparison of the levels of BDNF mRNA in each group. According to RT-PCR, on days 1-7, the expression of BDNF mRNA in the control group was not significantly different from that in the sham group ($p > 0.05$). On days 1-7, the expression of BDNF mRNA in the model group was significantly higher than those in the control group and sham group ($p < 0.01$, respectively). There were differences in expression in the model group between day 0 and days 1-7, but no differences were observed in the control group and sham operation group at the same time points ($p > 0.05$).

Table II. The expression of perihematomal NGB-positive cells in rats.

Group	Number of NGB-positive cells					*F* value	*p* value
	0 d	1 d	3 d	5 d	7 d		
Control	20.382 ± 0.382	19.983 ± 0.493	19.327 ± 0.329	20.487 ± 1.298	20.323 ± 0.439	0.618	0.398
Sham	20.387 ± 1.298	19.989 ± 1.243	20.378 ± 2.123	21.843 ± 1.287	21.372 ± 0.483	0.672	0.329
Model	20.989 ± 1.478	26.483 ± 0.873	31.387 ± 1.987	48.325 ± 2.387	51.277 ± 0.483	21.879	0.001
F value	0.387	9.328	12.187	13.287	19.487	–	–
p-value	0.621	0.011	0.008	0.006	0.002	–	–

the brain tissues of rats in the control group and sham-operated group. On days 1-7, the number of NGB-positive cells was significantly increased in the model group compared with the control group and sham-operated group ($p < 0.01$). The number of NGB-positive cells in the model group was significantly different on days 1-7 compared with day 0. However, no significant differences were observed in the control group and sham group at the same time points. See Table II, Figure 2 and Figure 3.

Detection of Learning and Memory Ability in Rats

There was no significant difference in EL between the control group and sham-operated group ($p > 0.05$). On day 0, there were no significant differences between the three groups in EL. Afterward, the EL in the model group increased significantly compared with those in the control group and sham operation group. There was a significant difference in EL between day 0 and days 1-7 in the model group, but not in the control and sham operation group ($p > 0.05$). See Table III and Figure 4.

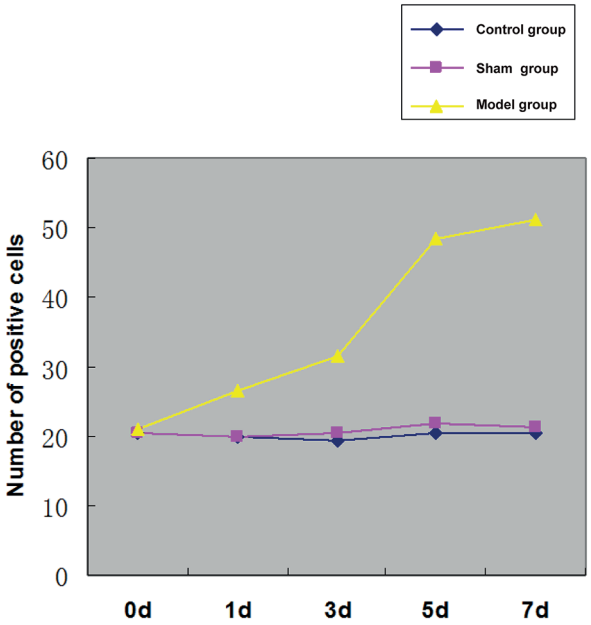

Figure 2. On day 0, there were no significant differences in the number of NGB-positive cells between the three groups. On days 1-7, the number of NGB-positive cells in the model group was significantly increased compared with the control group and sham-operated group ($p < 0.01$). The number of NGB-positive cells in the model group on days 1-7 was significantly different compared with day 0. However, no significant differences were observed in the control group and sham operation group at the same time points ($p > 0.05$).

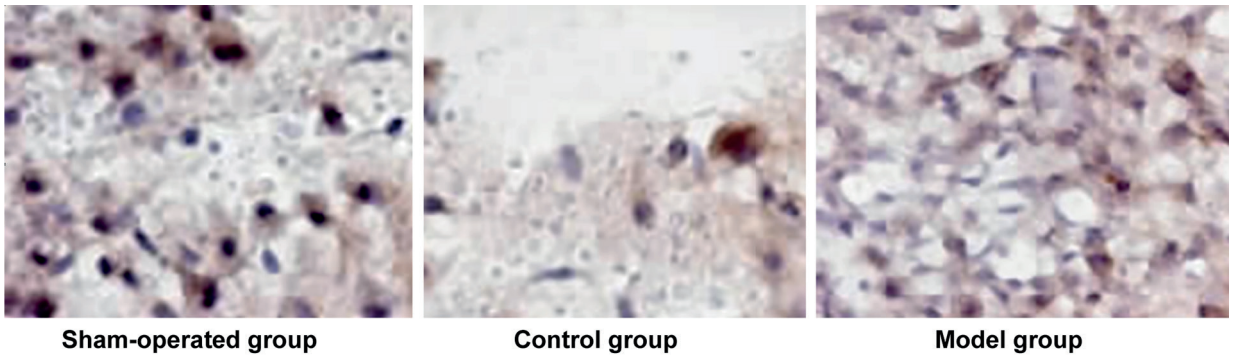

Figure 3. Immunohistochemical staining of NGB in different groups. Compared with the sham-operated group and control group, the number of NGB-positive cells in the model group was significantly increased ($p < 0.05$). (Magnification×40).

Table III. Comparison of escape latency between the three groups.

Group	EL (s)					*F* value	*p* value
	0 d	1 d	3 d	5 d	7 d		
Control	12.898 ± 0.232	13.029 ± 0.389	12.938 ± 0.382	13.287 ± 0.372	13.287 ± 0.839	0.598	0.382
Sham	12.327 ± 0.372	13.287 ± 0.323	13.287 ± 1.232	14.323 ± 0.382	14.378 ± 1.398	0.672	0.298
Model	12.894 ± 0.372	21.376 ± 3.372	28.387 ± 8.387	30.483 ± 3.211	39.483 ± 3.243	12.823	0.008
F value	0.682	8.324	17.323	19.382	21.897	–	–
p-value	0.372	0.012	0.003	0.002	0.001	–	–

The Correlation Analysis of NGB and BDNF mRNA Levels and Escape Latency

By analyzing the mRNA levels of NGB and BDNF and EL, we found there was a positive correlation between BDNF mRNA level and NGB-positive cell number and EL with correlation coefficients rs (NGB) = 1.1838 ($p = 0.008$) and rs (BDNF) = 0.5948 ($p = 0.012$). See Figure 5A-B.

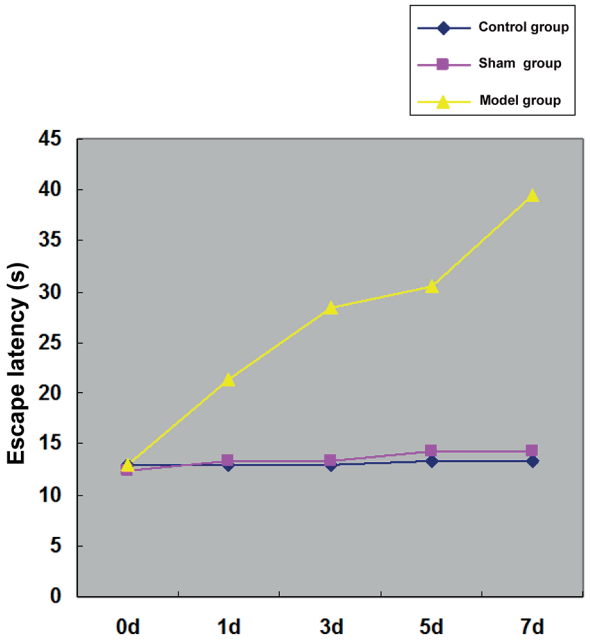

Figure 4. Comparison of escape latency between the three groups. Morris water maze test showed that there was no significant difference in escape latency between the control group and sham-operated group on days 1-7 ($p > 0.05$). On days 1-7, the escape latency in the model group was significantly higher than in the control group and sham operation group ($p < 0.01$, respectively). There was a significant difference between day 0 and days 1-7 in the model group ($p < 0.05$). In the control group and sham operation group, there were no significant differences in escape latency at each time point ($p > 0.05$).

Discussion

Cerebral hemorrhage is a common disease of the nervous system. Its rates of mortality and morbidity are high, accounting for 17.2-56% of cerebrovascular diseases. Axons cannot effectively regenerate because of nervous system damage caused by cerebral hemorrhage, often resulting in irreversible nervous system function loss and sequelae. Among the common sequelae are learning and memory dysfunction. They seriously affect the quality of life of patients and induce a heavy financial burden on patients, their families, and society[8-10]. Therefore, exploring effective treatments and promoting the recovery of nerve function have become focuses of current neurological research. The Morris water maze has been widely used in the study of memory behavior in experimental animals. The locating navigation experiment is used to record the animal's EL on each attempt to reflect their memory ability regarding the target platform's spatial position. This allows for evaluation of the animal's spatial learning and memory ability. The EL is inversely proportional to the spatial learning and memory capacity of animals[9]. The results suggested that EL was significantly longer after the occurrence of intracerebral hemorrhage in rats. Furthermore, the expression of NGB and BDNF increased significantly in the brain tissues of rats with cerebral edema, suggesting that they may be related to the decline of learning ability and memory[10]. NGB is a recently discovered and important oxygen-carrying protein that belongs to the same family as hemoglobin and myoglobin. Several studies[11,12] have shown that NGB mRNA expression increases significantly under ischemic and hypoxic conditions, indicating that NGB has an important protective effect on neurons in cerebral ischemic and hypoxic injury. Similar to myoglobin, NGB can promote the diffusion

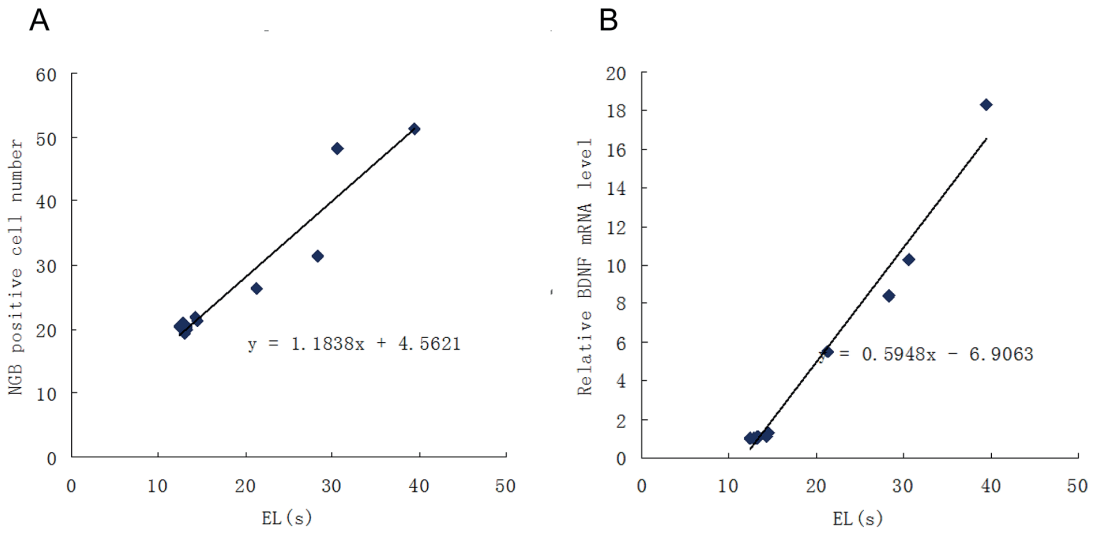

Figure 5. ***A.*** NGB-positive cell number shows a positive correlation with EL with rs (NGB) = 1.1838 ($p = 0.008$), Y = 1.1838X + 4.6621. ***B.*** The relative BDNF mRNA level shows a positive correlation with EL, with rs (BDNF) = 0.5948 ($p = 0.012$), Y = 0.5948X - 6.9063.

of oxygen to mitochondria in the hypoxic state, thereby enhancing the utilization of oxygen[12]. It was suggested that NGB may protect neurons from ischemia and hypoxia through the following mechanisms: (1) by attenuating the toxic effects of active oxygen free radicals and nitrogen free radicals[13]; (2) by helping oxygen from cells spread to mitochondria by increasing oxygen metabolism of nerve cells; (3) by regulating a G protein-coupled signal transduction pathway to reduce mitochondrial dysfunction and apoptosis, thereby improving cell viability[14-16]. In this study, the number of perihematomal NGB-positive cells in rats with intracerebral hemorrhage was significantly higher than in the control group and sham operation group ($p < 0.01$). These results suggest that after cerebral hemorrhage, as a compensatory response, damaged perihematomal neurons may further promote the release of NGB and affect the learning and memory ability of rats. Additionally, we found that the expression of BDNF mRNA was significantly higher in the model group compared with the other two groups ($p < 0.01$). In brain tissue, BDNF is primarily released following its synthesis in nerve cells and glial cells, playing an important role in central nervous system growth and development. It promotes nerve cell growth and differentiation, and plays an important role in nervous system development and the generation and maintenance of its functions[17-19]. By stabilizing the calcium concentration after injury of brain tissue, BDNF can enhance the activity of antioxidant enzymes, reduce the damage from free radicals, reduce apoptosis, and promote the regeneration of neurons to stimulate the recovery of functions of injured nerves. Pathological states such as ischemia, hypoxia, and other injuries can induce a significant increase of BDNF mRNA and protein expression in brain tissue[20,21], which is consistent with the results of the present study.

Conclusions

We suggest intracerebral hemorrhage can significantly inhibit the spatial learning and memory ability of rats, and the mechanism may be related to decreased cerebral expression of BDNF and NGB.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Wang XM, Zhang YG, Li AL, Long ZH, Wang D, Li XX, Xia JH, Luo SY, Shan YH. Expressions of serum inflammatory cytokines and their relationship with cerebral edema in patients with acute basal ganglia hemorrhage. Eur Rev Med Pharmacol Sci 2016; 20: 2868-2871.

2) Ozer AB, Demirel I, Erhan OL, Firdolas F, Ustundag B. Effect of different anesthesia techniques on the serum brain-derived neurotrophic factor (BDNF) levels. Eur Rev Med Pharmacol Sci 2015; 19: 3886-3894.

3) Jiang Y, Wang XF, Niu YL, Xia JH. The effects of MCA-MAO on cAMP pathway in rats with cerebral hemorrhage. Eur Rev Med Pharmacol Sci 2016; 20: 1834-1838.

4) Schabitz WR, Berger C, Kollmar R, Seitz M, Tanay E, Kiessling M, Schwab S, Sommer C. Effect of brain-derived neurotrophic factor treatment and forced arm use on functional motor recovery after small cortical ischemia. Stroke 2004; 35: 992-997.

5) Rosenberg GA, Mun-Bryce S, Wesley M, Kornfeld M. Collagenase-induced intracerebral hemorrhage in rats. Stroke 1990; 21: 801-807.

6) Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 1989; 20: 84-91.

7) Nawa H, Takei N. BDNF as an anterophin; a novel neurotrophic relationship between brain neurons. Trends Neurosci 2001; 24: 683-684.

8) Cheng Y, Gidday JM, Yan Q, Shah AR, Holtzman DM. Marked age-dependent neuroprotection by brain-derived neurotrophic factor against neonatal hypoxic-ischemic brain injury. Ann Neurol 1997; 41: 521-529.

9) Jin Y, Fischer I, Tessler A, Houle JD. Transplants of fibroblasts genetically modified to express BDNF promote axonal regeneration from supraspinal neurons following chronic spinal cord injury. Exp Neurol 2002; 177: 265-275.

10) Ren J, Zhou X, Wang J, Zhao J, Zhang P. Poxue Huayu and Tianjing Busui Decoction for cerebral hemorrhage (upregulation of neurotrophic factor expression): upregulation of neurotrophic factor expression. Neural Regen Res 2013; 8: 2039-2049.

11) Guan J, Zhang B, Zhang J, Ding W, Xiao Z, Zhu Z, Han Q, Wu C, Sun Y, Tong W, Dai J, Wang R. Nerve regeneration and functional recovery by collagen-binding brain-derived neurotrophic factor in an intracerebral hemorrhage model. Tissue Eng Part A 2015; 21: 62-74.

12) Solev IN, Balabanyan VY, Volchek IA, Elizarova OS, Litvinova SA, Garibova TL, Voronina TA. Involvement of BDNF and NGF in the mechanism of neuroprotective effect of human recombinant erythropoietin nanoforms. Bull Exp Biol Med 2013; 155: 242-244.

13) Hwang L, Choi IY, Kim SE, Ko IG, Shin MS, Kim CJ, Kim SH, Jin JJ, Chung JY, Yi JW. Dexmedetomidine ameliorates intracerebral hemorrhage-induced memory impairment by inhibiting apoptosis and enhancing brain-derived neurotrophic factor expression in the rat hippocampus. Int J Mol Med 2013; 31: 1047-1056.

14) Chen SJ, Tsai JC, Lin TY, Chang CK, Tseng TH, Chien CL. Brain-derived neurotrophic factor-transfected and nontransfected 3T3 fibroblasts enhance migratory neuroblasts and functional restoration in mice with intracerebral hemorrhage. J Neuropathol Exp Neurol 2012; 71: 1123-1136.

15) Han QQ, Jin W, Xiao ZF, Huang JC, Ni HB, Kong J, Wu J, Chen B, Liang WB, Dai JW. The promotion of neurological recovery in an intracerebral hemorrhage model using fibrin-binding brain derived neurotrophic factor. Biomaterials 2011; 32: 3244-3252.

16) Amri F, Ghouili I, Amri M, Carrier A, Masmoudi-Kouki O. Neuroglobin protects astroglial cells from hydrogen peroxide-induced oxidative stress and apoptotic cell death. J Neurochem 2017; 140: 151-169.

17) Lin CC, Lee CT, Lo YT, Huang TL. Brain-derived neurotrophic factor protein and mRNA levels in patients with bipolar mania - a preliminary study. Biomed J 2016; 39: 272-276.

18) Yoshikawa H, Yamada Y, Kurose M, Yamamura K, Maeda T, Seo K. Peripheral brain-derived neurotrophic factor modulates regeneration following inferior alveolar nerve injury in rats. J Oral Facial Pain Headache 2016; 30: 346-354.

19) Zhang MD, Barde S, Yang T, Lei B, Eriksson LI, Mathew JP, Andreska T, Akassoglou K, Harkany T, Hokfelt TG, Terrando N. Orthopedic surgery modulates neuropeptides and BDNF expression at the spinal and hippocampal levels. Proc Natl Acad Sci USA 2016; 113: E6686-E6695.

20) Camerino C, Conte E, Cannone M, Caloiero R, Fonzino A, Tricarico D. Nerve growth factor, brain-derived neurotrophic factor and osteocalcin gene relationship in energy regulation, bone homeostasis and reproductive organs analyzed by mRNA quantitative evaluation and linear correlation analysis. Front Physiol 2016; 7: 456.

21) Ives SJ, Norton C, Miller V, Minicucci O, Robinson J, O'Brien G, Escudero D, Paul M, Sheridan C, Curran K, Rose K, Robinson N, He F, Arciero PJ. Multi-modal exercise training and protein-pacing enhances physical performance adaptations independent of growth hormone and BDNF but may be dependent on IGF-1 in exercise-trained men. Growth Horm IGF Res 2017; 32: 60-70.