

Effects of Peiyuan Tongnao Capsule on Working Memory and the Expression of Glutamic Acid and Receptor in Hippocampal Area in Rats with Cerebral Ischemia

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Abstract

Objective: To explore the effects of Peiyuan Tongnao (PYTN) Capsule on working memory, the content of glutamic acid, and the expression of NMDA receptor 2B (NR2B) in rats with cerebral ischemia. **Materials and Methods:** 52 Sprague-Dawley rats were randomly divided into sham group, model group, nimodipine group, and PYTN group. The bilateral common carotid artery occlusion (BCAO) was performed to establish rat model with cerebral ischemia in the model, nimodipine, and PYTN groups. Gastric lavage with some drug based on body weight conversion was performed daily for 4 weeks in the nimodipine and PYTN groups. The working memory of the rats was tested by Morris water maze. The expression of Nissl body in hippocampus tissue was observed by Nissl staining. The determination of Glu content in hippocampus was detected by high-performance liquid chromatography. The expression level of NR2B in hippocampus area was determined using Western blot. **Results:** Morris water maze test on working memory escape latency. In the model group, day 1 versus day 4, there was a statistical difference ($P < 0.05$). In the nimodipine group, day 1 vs. day 4 displayed a statistical difference ($P < 0.05$). In the PYTN group, day 1 versus day 3 and day 1 versus day 4 were in significant differences ($P < 0.05$). The Glu content in hippocampus of the sham group was significantly different from that of model group ($P < 0.05$). The Glu content in the PYTN group was significantly different from that of the model group ($P < 0.05$). With regard to the expression of NMDA 2B in hippocampus between the sham group and the model, nimodipine, and PYTN groups, all were displayed statistical significance ($P < 0.01$). As the same, the expression of NMDA 2B in the model group was significant from that of the nimodipine group ($P < 0.05$) and PYTN group ($P < 0.01$). **Conclusion:** PYTN capsule was beneficial for improving working memory and protect neural cells in rats of cerebral ischemia, which may be associated with upregulation of the expression of Glu and NMDAR2B in hippocampus.

Keywords: Cerebral ischemia, Glu, NMDA receptor 2B, Peiyuan Tongnao capsule, working memory

INTRODUCTION

The survival and function of brain tissue depends on the continuous flow of blood, which provides energy and oxygen and carries away metabolic waste.^[1,2] The cranial blood vessels are the channels through which blood flows from the brain tissue. Therefore, the blood itself and the cerebral and cervical vascular lesions can cause brain tissue injuries. Carotid artery stenosis can lead to partial blood supply insufficiency to the brain tissue and brain tissue atrophy or even necrosis due to blockage of substances exchange, giving rise to cognitive dysfunction.^[3,4] This not only reduces the quality of life of patients but also shortens the survival time of stroke patients and causes a heavy burden to both families and society. This

has become the second most common cause of age-related cognitive impairment and dementia.^[5]

In traditional Chinese medicine, it is believed if qi and blood are not sufficient, the brain marrow will lack of nourishment.

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Constant nourishment and enrichment of qi, blood, and body fluids can ensure the normal function of the brain. If the brain marrow lacks nourishment, actions of governing organs and skeleton will not be exerted.^[6] The kidney is the foundation of the storing essence. It stores essence, dominates the bone, and produces marrow which connects with the brain. The view of the kidney replenishing the brain believes that the kidney can produce the marrow and generate the sperm, and the brain can govern the marrow, and the marrow goes upward and replenishes the brain. The subtle substance of the kidney is enriched in the brain, and it is the material basis for the function of the brain. If the kidney lacks of essence, no essence replenishment will lead to emptiness of the brain, finally brain aging ensues.^[7]

The formula of Peiyuan Tongnao (PYTN) capsule adheres to the principle of natural law and takes analogies from various factors and complex steady-state systems. From the theory of kidney viscera, it is believed that the essential qi of the kidney is the foundation of the life activities in humans. It plays an extremely important role in all kinds of physiological activities of the body. Therefore, it is necessary to observe the deficiency and excess of the kidneys in the treatment of internal function of the body, to assist the healthy qi in the kidneys. Meanwhile, herbs with the actions of dispelling stasis, extinguishing wind, and unblocking the collaterals are added, which can reduce injury and also prevent first and even prevent transmission. We applied PYTN capsule with the actions of enriching the kidneys and replenishing the essence, extinguishing wind, and unblocking the collaterals to treat cerebral ischemia rats to observe its effects on cognitive function.

MATERIALS AND METHODS

Experimental animals and design

Fifty-two white healthy male Sprague-Dawley (SD) rats (weight, 250 g $\pm$ 10 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., (License No. (SCXK [Beijing] 2012-0006), Beijing, China), raised in the Key Laboratory of Traditional Chinese Internal Medicine, Ministry of Education, Dongzhimen Hospital, Beijing University of Chinese Medicine. The rats had free access to food and water, at a 12 h light/dark cycle. Except daily intragastric administration at regular mornings, specifically, the same dose of water was administered in the sham group, and the same dose of drugs were provided in the nimodipine group and PYTN group; the rats had free access to food and water during feeding. Random numbers were generated according to SPSS 24.0 (licensed materials property of IBM Corp.,) statistical software for grouping. These SD rats were randomly divided into sham operation group ($n = 13$), model group ($n = 13$), positive control group (nimodipine group) ($n = 13$), and PYTN group ($n = 13$).

Morris water maze positioning and navigation test was carried out until 8 weeks of feeding.^[8,9] Two days later, the working memory test was performed. After that, the glutamic

acid content was determined by high-performance liquid chromatography (HPLC) in the left hippocampus, and the NMDA2B receptor was determined by Western blot in the right hippocampus.

Surgical method

Anesthesia

10% chloral hydrate (3 mL/kg body weight) was intraperitoneally injected into the rats for anesthesia.

Bilateral common carotid artery occlusion

The rats after the observation of cerebral blood flow would be placed on the operating table. At the cervical midline incision, the muscles and fascia were separated along the medial edge of the sternocleidomastoid muscle for finding the carotid sheath. After gently stripping the right common carotid artery and the vagus nerve, two ends of the common carotid artery were ligated and cut using a 6-0 sterilized wire harness. The same operation process was performed on the left common carotid artery. After that, the neck incision was sutured. The same cervical vascular ligation was performed in the model, nimodipine, and PYTN groups, while in the sham operation group, only bilateral common carotid arteries were bluntly separated and the cervical incision was sutured without other operations. Immediately after the end of cervical surgery, cerebral blood flow was detected, and the postoperative cerebral perfusion was recorded. The head incision was sutured after cerebral blood flow observation. All the rats in each group were intraperitoneally injected with 1 mL penicillin injection to prevent postoperative infection.

Drugs

Ingredients of Peiyuan Tongnao capsules

429 g *Radix Polygoni Multiflori Praeparata cum Succo Glycines Sotae* (zhì hé shǒu wū), 286 g *Radix Rehmanniae Praeparata* (shǔ dì huáng), 286 g *Radix Asparagi* (tiān dōng), 46 g *Carapax et Plastrum Testudinis* (guī jiǎ, vinegar prepared), 23 g *Cornu Cervi Pantotrichum* (lǜ róng), 114 g *Herba Cistanches* (ròu cōng róng, alcohol-prepared), 24 g *Cortex Cinnamomi* (ròu guì), 49 g *Radix Paeoniae Rubra* (chì sháo), 48 g *Scorpio* (quán xiē), 96 g *Hirudo* (shuǐ zhì, scald), 49 g *Pheretima* (dì lóng), 142 g *Fructus Crataegi* (dry-fried), 48 g *Poria* (fú líng), and 29 g *Radix et Rhizoma Glycyrrhizae Praeparata cum Melle* (zhì gān cǎo). The above-mentioned herbs are made into the dry extract powder, and water is added to prepare a solution for gastric administration.

Drug administration

According to the body surface area conversion in *Chinese Medicine Pharmacology Experimental Methodology* edited by Qi Chen,^[10] the dose of nimodipine in rats was 8.1 mg/kg, and the dose of PYTN capsule was 0.486 g/kg.

Behavior test (Morris water maze)

Environmental adaptation

Twenty-four hours before the start of training, the rats were allowed to swim for 3 min in the water maze without the platform to adapt to the environment.

Training period

The rats with back to the pool were placed in the maze from the midpoint of each quadrant, and the position of the platform remained unchanged (quadrant 3). The rats stayed on the platform for 5 s after they found the platform within 60 s. If they did not find the platform within 60 s, they would be directed to the platform and stayed for 5 s. Rats were repeated with nonplatform quadrant water training daily for two blocks (each block includes 3 trials). The interval between each trial was 60 s, and the interval between the two blocks was 3 min. There were 3 days in total for training. The rats are taught to learn the general rules for finding the platform.

Working memory

With a reference to memory training performed 2 days later. In the reference to memory training stage, the platform was located at the North-East. On the 1st day, the platform was changed to the South-West. The platform quadrant was changed every day, and the training was performed three times a day, that is, the quadrants except the platform quadrant were in the water once. The sequence of the water quadrant: day 1 – South-West, day 2 – North-West, day 3 – South-East, and day 4 – North-East.

Nissl staining

Five rats in each group were randomly selected to fetch the whole brain, and the hippocampus was sliced in wax for Nissl staining to observe the expression of Nissl bodies in hippocampal CA1, CA3, and DG areas.

Bioassay

Determination of glutamate in hippocampus

Application of American Agilent 1200 HPLC, Agilent Chem Station, Chromatographic column: ZORBAX Eclipse AAA (4.6 mm × 150 mm, 5 μm), Detection wavelength: excitation wavelength_{lex} = 340 nm, emission wavelength_{lem} = 450 nm, column temperature 40°C, and injection volume 15 μl. Weight centrifugation of hippocampal and cortical samples, sampling supernatant, amino acid precolumn automatic derivation record.

Detection of NMDAR2B protein expression

Sample protein extraction: Grinding and crushing tissue samples into RIPA pyrolysis solution (protease inhibitors, phosphatase inhibitors) resuspended, extraction of Protein by Ice Cracking, boiling denaturation of protein, acrylamide gel electrophoresis. Transmembrane: PVDF membrane immersed in methanol, transfer to the electrolyte at a constant current of 300 mA, dilute, incubate, wash film with first antibody, incubate with second antibody, closed liquid dilution second antibody with (NMDAR2B antibody Abcam/ab65783), sheep anti-rabbit-HRP 1:5000 dilution, wash the film with TBST, the gray value of strip is read by Quantity One v. 4.6.2 software.

Statistical methods

Application of SPSS 24.0 statistical software to analyze and process data, the measurement data are expressed as $\bar{x} \pm s$, means were compared by one-way ANOVA, least significant

difference $-t$ -test was used for posterior comparisons, $P < 0.05$ was statistically significant.

RESULTS

Comparison of escape latency on day 1, 2, 3, 4 of water maze was seen in Table 1 and Figure 1.

Morris water maze: in the sham operation group, rats on day 1 versus day 2 and day 1 versus day 3 were not in significant differences ($P > 0.05$), while on day 1 versus day 4, it displayed a statistical difference ($P < 0.05$). In the model group, rats on day 1 versus day 2 and day 1 versus day 3 were not in great differences ($P > 0.05$), while on day 1 versus day 4, there was a statistical difference ($P < 0.05$). In the nimodipine group, rats on day 1 versus day 2 and day 1 versus day 3 were not in statistical differences ($P > 0.05$), while on day 1 versus day 4, there was a significant difference ($P < 0.05$). In the PYTN group, rats on day 1 versus day 2, there was no great difference ($P > 0.05$), while on day 1 versus day 3 and day 1 versus day 4, they were displayed significant differences ($P < 0.05$).

Each group of rats could complete the memory task for space learning after the adaptation training. However, the latency in finding the platform for completing the working memory task was significantly longer on the 2nd and 3rd days in the model group than PYTN group, while there was no great difference

Table 1: Comparison of the average escape latency of the first day of the platform replacement among each group with the average value of the daily latency ($\bar{X} \pm s$, $n=8$, S)

	Sham operation	Model	Nimodipine	PYTN
Day 1	17.26±0.375	31.29±2.69	30.04±4.51	30.49±2.62
Day 2	11.41±4.39*	29.42±0.50*	25.94±1.37*	26.35±3.52 [△]
Day 3	10.54±4.09*	27.32±4.38*	23.01±6.59*	22.31±3.73 [△]
Day 4	9.95±4.59 [△]	25.99±1.10 [△]	17.83±3.49 [△]	18.87±11.54 [▲]

* $P > 0.05$, [△] $P < 0.05$, [▲] $P < 0.01$. PYTN: *Peiyuan tongnao*

Table 2: Content of glutamic acid in hippocampus of each group ($\bar{X} \pm s$, $n=8$, mg/g)

Sham operation	Model	Nimodipine	PYTN
1.23±0.114	1.07±0.13*	1.20±0.101 [△]	1.21±0.176 [▲]

*Sham operation group versus model group, $P < 0.05$; [△]Sham operation group versus nimodipine group, model group versus nimodipine group; sham operation group versus PYTN group, nimodipine group versus PYTN group, $P > 0.05$; [▲]Model group versus PYTN, $P < 0.05$. PYTN: *Peiyuan tongnao*

Table 3: Comparison of relative gray value of NMDA 2B receptors in hippocampus of each group ($\bar{X} \pm s$)

<i>n</i>	Sham operation	Model	Nimodipine	PYTN
8	0.58±0.0394	0.29±0.123*	0.38±0.084 [△]	0.42±0.091 [▲]

*Model group versus sham group $P < 0.01$, [△]model group versus nimodipine group, $P < 0.05$, [▲]Model group versus PYTN group, $P < 0.01$

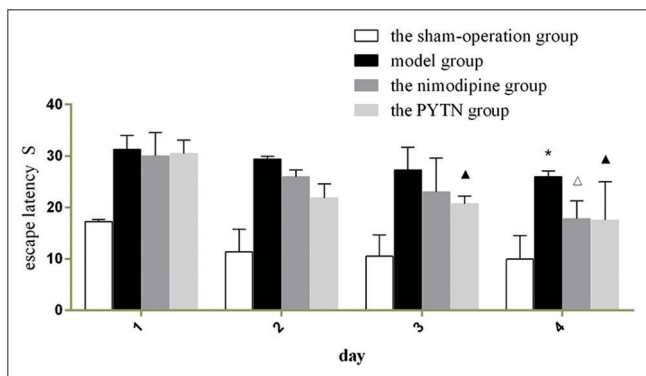


Figure 1: Comparison of escape latency after platform quadrant replacement in rats of each group

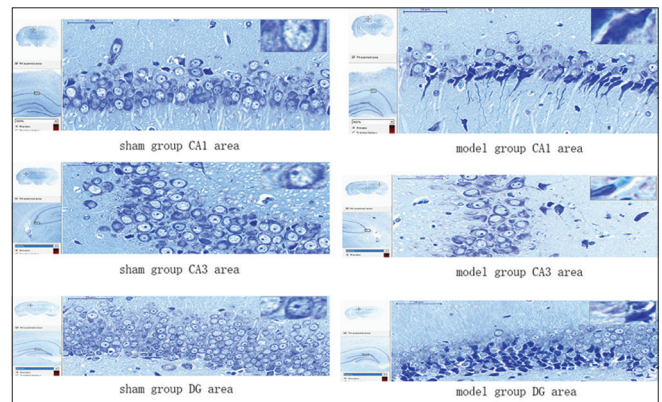


Figure 2: Nissl staining result in sham group and model group

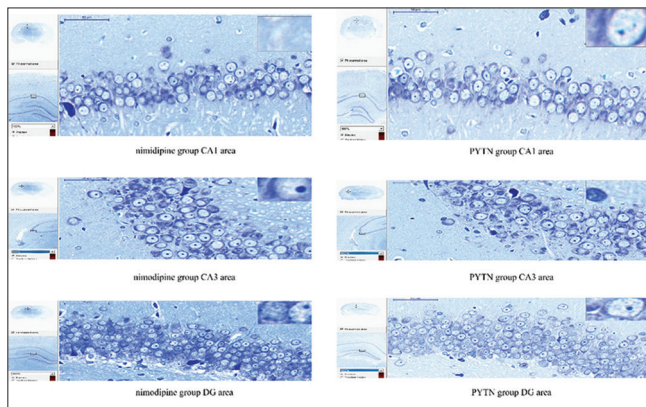


Figure 3: Nissl staining result in nimodipine group and PYTN group

in the latency of finding the platform on the 4th day between the model group and PYTN group ($P > 0.05$).

Nissl staining results

Nissl body expression

The number of Nissl bodies decreased or even disappeared in the model group pictures, cell shrinkage, and nuclear pyknosis. The nerve cells were loosely and stained lightly and indistinctly. Nissl bodies were more in Nimodipine group and PYTN group; the cells were arranged tightly and stained clearly [Figures 2 and 3].

Comparison of glutamic acid content in hippocampus of each group

With regard to the Glu content in the hippocampus of each group, the Glu content was higher in the sham operation group than in the model group with a statistical difference, but no great difference in the nimodipine group or the PYTN group. In the Glu content in the model group and nimodipine, there was no statistical difference ($P = 0.056$), but between the model group and PYTN group, there was a statistical difference ($P = 0.045$). There was no statistical difference in the Glu content between the nimodipine group and the PYTN group [Table 2 and Figure 4].

Comparison of relative gray value of NMDA 2B receptors in hippocampus of each group

On the expression of NMDA 2B receptor protein in hippocampus of each group, there was significant difference

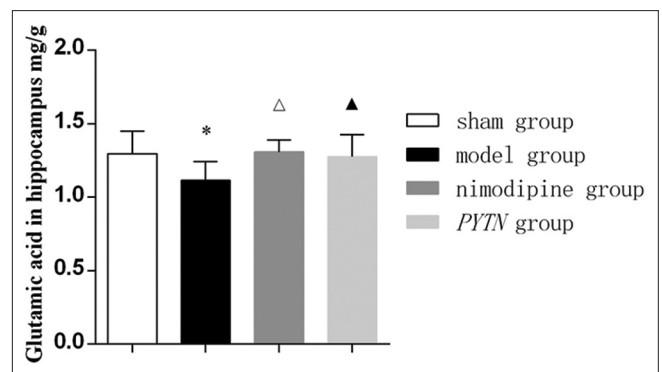


Figure 4: Comparison of glutamic acid content in hippocampus of each group

between the sham operation group and the model, nimodipine, and PYTN groups ($P < 0.01$). The expression of NMDA 2B receptor protein displayed statistical significance between the model group and the nimodipine group ($P = 0.036$), between the model group and PYTN group ($P = 0.005$). There was no great difference in the expression of NMDA 2B receptor protein between the nimodipine group and PYTN group ($P = 0.404$) [Table 3 and Figure 5].

DISCUSSION

Ischemic cerebrovascular disease is the leading cause of disability and death worldwide. Symptoms of cognitive impairment caused by high-risk nondisabling ischemic cerebrovascular events are concealed and not easy to be detected, which affects the patient's fine workability and social activities, leading to a decline in the quality of life of patients, which has been paid great attention in the medical field.^[11] We applied a rat model that completely blocked the carotid blood supply system^[12,13] and simulated forebrain ischemic injury.^[14]

The pathogenesis of this study bases the ischemia-induced toxins damaging the brain collaterals. The treatment principle mainly focuses on strengthening the healthy qi and protecting the brain. The main functions of brain refer to high-level neurological thinking activities including memory, cognition, and thinking, which all depend on the perfusion of qi and blood in the brain collaterals. Academician Wang *et al.*^[15,16]

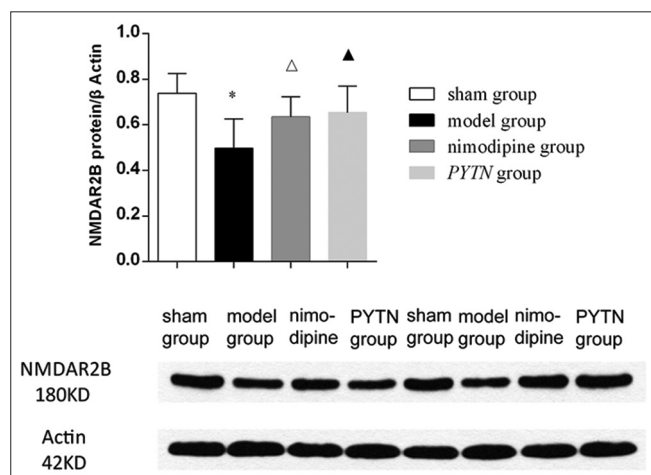


Figure 5: Comparison of relative gray value of NMDA 2B receptors in hippocampus of each group

proposed the concept of cerebral blood collateral and cerebral qi collateral. He believes that cerebral qi collaterals and cerebral blood collaterals fail to propel, regulate, and control the blood circulation, lack of nourishment on brain and toxin evils ensue. The kidney is the root of yin and yang of the whole body. If the kidney yang is insufficient, it cannot exert the effects of warming and steaming, involving the spleen, the latter cannot transform and transport, and dampness and turbidity produce. If the kidney yin is insufficient, essence and blood cannot be generated leading to empty marrow and vitality uselessness (*Neijing Jingyi, Yilin Gaicuo*). The PYTN capsule has the actions of enriching the kidney and replenishing essence and extinguishing wind and unblocking collaterals. In the formula, zhi hé shǒu wū supplements the liver and kidney, nourishes blood, and dispels wind. shù dì huáng enriches yin and nourishes blood, generates essence, and boosts marrow. The two herbs are selected as the chief. The deputy herbs of guī jiǎ and tiān dōng assist the chief herbs on nourishing yin, which are effective on extinguishing wind due to insufficiency of internal fluid. The other two deputy herbs of lù róng and ròu cōng róng assist the chief herbs on supplementing kidney qi. Quán xiē, dì long, chì sháo, and shuǐ zhī as assistant herbs can unblock the collaterals and dispel blood stasis. Ròu guī assists the lù róng and ròu cōng róng on invigorating yang and also returns fire to its source to avoid the upflaming of deficiency yang. Shān zhā and fú líng are used to prevent the tonics affecting the functions of the spleen and stomach. Zhì gān cǎo as an envoy herb harmonizes all the ingredients.^[17]

In this study, we observed the process of rats completing the working memory in the water maze and found that in the behavioral aspects, rats in each group were basically familiar with the maze environment after the completion of the basic training, but the escape latency of the model group was significantly prolonged. There was a statistical difference between day 4 and day 1. In the PYTN group, there was significant difference on day 2 compared with day 1. In the nimodipine group, the escape latency was in a significant

difference between day 3 and day 1. On days 2, 3, and 4, the recall of memory depends on the first test of that day. Working memory is a special short-term memory with the characteristics of short-term storage, real-time refresh, and limited capacity. It is the basis of advanced cognitive functions including animal planning and organizational behavior, language, thinking, and decision-making.^[18] In present study, we found that the rats adapted to the platform changes quickly in the PYTN group; so, the rats in this group can store the platform position markers for a short time after the first discovery of the platform in the first trial on day 2 and choose a shorter path to arrive at the platform with a short escape latency. However, in the model group, on day 4, the overall escape latency is improved. The kidney dominates agility and the improvement of the working memory of the water maze reflected the actions of PYTN on boosting the kidney and replenishing essence.

In the expression of Nissl bodies, in the model group, it showed that the number of Nissl bodies reduced or even disappeared, the cells shrunk, and the nerve cells were loosely arranged. While in the sham operation, nimodipine, and PYTN groups, there were many Nissl bodies in which nerve cells synthesize proteins. The treatment on improving ischemia can protect the Nissl body and realize its function providing necessary proteins for neural activity.

The highly complex organization of the brain is due to the large number of interacting cells that can encode and transmit information at a certain distance. The movement of this information is within the nervous system, where synapse is a key structure.^[19] Different neurotransmitters have different roles in synaptic transmission of information. Recently, many reports are about the Glu and its receptors in morphological or functional studies.^[20] The Glu NMDA receptor excitation can enhance the efficiency of synaptic transmission, known as long-term potentiation. The excitatory synapse transmission of hippocampus is mediated by NMDA receptors.^[21] Water maze inventor, Richard *et al.* observed that rat could not remember the platform position in water after injection with NMDA receptor blockers, which provided evidence for the role of NMDA receptor-dependent processes in memory. Functional NMDA receptors need to form a tetramer from NR2 subunit and NR1. The various protein kinase phosphorylation sites of NMDA receptor 2B (NR2B) at the C-terminal serve as the basis of phosphorylation and dephosphorylation of NR2B. The reversible protein phosphorylation is a common mechanism in cellular signal transduction.^[22] Therefore, we selected the Glu content and NR2B as the observation indices.

With regard to the Glu content in the hippocampus of each group, the Glu content of the sham operation group was higher than that of the model group with a statistical difference, but no great difference in the nimodipine group or PYTN group. There was no significant difference in the Glu content between the model group and the nimodipine group but displayed a significant difference between the model group and the PYTN group. There was no great difference in the Glu content between

the nimodipine group and the PYTN group. On the expression of the NMDA 2B receptor protein, there was statistical significance between the sham operation group and the model, nimodipine, and PYTN group. Statistical differences in the expression of the NMDA 2B receptor protein were displayed between the model group and the nimodipine group ($P < 0.05$) and between the model group and the PYTN group ($P < 0.01$). There was no great difference in the expression of the NMDA 2B receptor protein between the nimodipine group and the PYTN group. The Glu as an excitatory neurotransmitter, after PYTN application, the content in the hippocampus increased compared with that in the model group. The Glu content after nimodipine treatment was not in great change but did not differ statistically compared with the PYTN group. The expression of NR2B in the nimodipine and PYTN groups was superior to that in the model group, but no great difference between the nimodipine group and PYTN group.

Therefore, PYTN capsule can achieve the function of improving the metabolism of neurons by herbal combination following the principle of chief, deputy, assistance, and envoy. Its action of extinguishing wind and unblocking collateral can improve the blood flow so that the fine substances can be transported to the brain, and the cerebral collaterals can be improved to smoothly discharge the circulating wastes. The possible drug mechanism may be that improvement of performance of ischemic rat in Morris water maze is through the upregulation of the Glu content in hippocampal neurons of rats with cerebral ischemia and the protein expression of NR2B, which embodies the thought of boosting kidney and replenishing essence; once the kidney qi is robust, the tendons and bone will become strong, and the movements will be agile. In our observation, we find PYTN capsule has effects on working memory, neurotransmitters, and receptors in cerebral ischemia rats.

Shortcomings

In the present study, we found that there was no significant difference in indices between the PYTN group and the nimodipine group. First, we considered that the two drugs have similar effects on improving the microcirculation and protecting the neurons. Second, the observational sample size is not sufficient and the observation period is short due to limited experimental conditions; the effect of the drugs may not be fully exerted. We will overcome the above shortcomings in our further study.

CONCLUSION

PYTN capsule was beneficial for improving working memory and protect neural cells in rats of cerebral ischemia, which may be associated with upregulation of the expression of Glu and NMDAR2B in hippocampus.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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