OXIRACETAM EXERTS NEUROPROTECTIVE EFFECT BY REGULATING NEURONAL APOPTOSIS AND AUTOPHAGY

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ABSTRACT

Objective: *To explore the neuroprotective effect of oxiracetam by regulating neuronal apoptosis and autophagy.*

Methods: *Forty-five clean grade healthy male SD rats were randomly divided into a sham operation group, a model group and an oxiracetam group, with 15 rats in each group. The rats in the sham operation group and the model group were given the same dose of normal saline by intraperitoneal gavage. The changes in escape latency, ratio of time spent in the original platform quadrant to the total time and pathological changes to the hippocampus were compared. The levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1 β (IL-1 β), phosphorylated protein kinase B (p-Akt), and phosphorylated rapamycin in the hippocampus were determined. The expression levels of p-mTOR, Bax, Bcl-2, Beclin 1, LC3 II/I, and p62 were changed.*

Results: *Compared with the sham operation group, the escape latency of the model group was significantly prolonged (P<0.05); compared with the model group, the escape latency of the oxiracetam group was shortened on the first day, but there was no significant difference (P>0.05); from the second day, the escape latency of the oxiracetam group was significantly shortened (P<0.05). Compared with the sham operation group, the ratio of residence time in the original platform quadrant to the total time in the model group was significantly decreased, and the expression levels of inflammatory factors, p-Akt, p-mTOR, Beclin 1, LC3 II/I, and Bcl-2 in the hippocampus were significantly increased. Meanwhile, the expression levels of Bax and p62 were significantly decreased (P<0.05). In comparison with the model group, the ratio of residence time in the original platform quadrant to the total time in the oxiracetam group was significantly increased; the expression levels of inflammatory factors, p-Akt, p-mTOR, Bcl-2/Bax, and p62 in the hippocampus were significantly increased; and the expression levels of Beclin 1 and LC3 II/I were significantly decreased (P<0.05). In the sham operation group, the hippocampal cells were arranged in an orderly fashion, the nuclear membrane was intact, the nucleolus was clear, and the staining was uniform; conversely, in the model group, the cells were arranged in a disorderly fashion, the number was significantly reduced, and the nucleolus disappeared; and in the oxiracetam group, the number of hippocampal cells was significantly increased and the pathological changes significantly improved in comparison with the model group.*

Conclusion: *Oxiracetam may activate the PI3K/Akt/mTOR signaling pathway and promote hippocampal neuronal apoptosis and autophagy, inhibiting the inflammatory response of the hippocampal tissue in rats with vascular dementia, improving the pathological changes in the hippocampal tissue, and thus playing a neuroprotective role.*

Keywords: *Oxiracetam, apoptosis, autophagy, neuroprotective effect.*

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Introduction

Vascular dementia is a central nervous system injury and disease and a type of Alzheimer's, which mainly manifests as defects in memory, behavior, and cognitive functions. Determining the pathogenesis of vascular dementia caused by stroke remains an important task, as that is still unclear. Presently, China has the highest incidence rate of stroke. This rate increases significantly as social pressure and unhealthy lifestyle practices increase, seriously affecting people's quality of life[(1)]. It is believed that the pathogenesis of vascular dementia may be closely related to chronic cerebral hypoperfusion, calcium overload, inflammatory response, and the activation of apoptotic pathways. Among these, chronic cerebral hypoperfusion is thought to be the main pathogenesis of vascular dementia[(2)]. Studies

have found that when the hippocampus is in a state of chronic avascular necrosis, the neurons in this part will have delayed cognitive dysfunction, which manifests as memory loss and mood or personality changes[3]. Oxiracetam, also known as "brain rejuvenation," is a novel type of pyrrolidone nootropic drug that can promote synaptic plasticity and the production of long-term brain enhancement, as well as improve learning and memory functions. It is less toxic and has fewer side effects than other similar drugs. However, the role of oxiracetam in improving vascular dementia has not yet been defined[4]. Aiming to explore the neuroprotective effect and related mechanisms of oxiracetam, this group of researchers conducted experiments with SD rats, using chronic cerebral hypoperfusion to establish a rat model of vascular dementia and oxiracetam for intervention.

Materials and methods

Subjects for the experiment

A total of 45 clean-grade healthy male SD rats were randomly selected (Shandong University Laboratory Animal Center, production license number: SCXK(Lu) 2019-0001, License number: SYXK(Lu) 2019 0005), with the body weight of 212±15g, and the age of eight weeks. All were given adapted feedings for seven days in an environment with a temperature of 23±2°C, humidity of 50±15% and a 1:1 ratio of day to night.

Main instruments and reagents

- Paraffin microtome (Shanghai Zhixin Instrument Co., Ltd., model: KD-2258S-VI);
- Biological microscope (Beijing Zhuoyueweiye Photoelectric Instrument Co., Ltd., model: XW880);
- Electronic balance (Shanghai Sunny Hengping Scientific Instrument Co., Ltd., model: JA12002);
- Low-temperature high-speed centrifuge (Shanghai Luxiangyi Centrifuge Instrument Co., Ltd., model: TGL-17M);
- Morris water maze (Shanghai TeoLinc Optoelectronics Co., Ltd.);
- Oxiracetam (Harbin Sanlian Pharmaceutical Co., Ltd., production number: 20190070, specification: 5mL: 1g);
- Rabbit anti-human Akt monoclonal antibody (Shanghai Ziqi Biotechnology Co., Ltd.);
- Rabbit anti-human mTO monoclonal antibody (Shanghai Yixin Biotechnology Co., Ltd.);
- Rabbit anti-human Bcl-2 monoclonal antibody (Beijing Taize Jiaye Technology Development Co., Ltd.);
- Rabbit anti-Bax monoclonal antibody (Beijing Shengke Boyuan Biotechnology Co., Ltd.);
- Rabbit anti-human Beclin 1 polyclonal antibody (Shanghai Future Industrial Co., Ltd.);
- Rabbit anti-LC3C polyclonal antibody (Amylet Scientific Technology Co., Ltd.).

Establishing a rat model of vascular dementia

The rats were randomly divided into three groups: the sham operation group, the model group, and the oxiracetam group, with 15 rats in each group. Oxiracetam rats received 150 mg/kg oxiracetam intraperitoneally. Rats in the sham operation group and model group received the same dose of normal saline, also intraperitoneally. Gavage was given once a day for two months in total.

The rats were anesthetized and fixed in a supine position. Skin was prepared on their necks. An incision was made along the middle of the neck, the surrounding tissues were separated, the common carotid artery was exposed and permanently ligated, and the incision was sutured layer by layer. Damage to the vagus nerve was avoided during the process. Rats in the sham operation group were not ligated.

Observation indicators

Morris water maze experiment

A cylindrical swimming pool of constant temperature, with a diameter of 1.6m and a height of 1.3m, was made. It was divided into four parts with two imaginary vertical lines passing through the center of the circle, and a platform with a diameter of about 11cm was fixed in the second quadrant. The platform was approximately 1.5cm below the surface of the water. On the second day after the last administration, a quadrant was randomly selected and the rats were placed in the water for a limited time of 120s for five days, three times a day, and the changes in the escape latency of the rats were observed and recorded.

On the sixth day, the original platform was removed, and the rats were placed in the water through a fixed entry point. The ratios of the staying time of rats in each group in the quadrant of the original platform to the total time were then compared. After the above experiment, five rats were selected from each group. Hippocampus tissue was taken from between the optic chiasm and papilla, fixed with formalin solution and dehydrated with alcohol and

transparent xylene; 4μm-thick paraffin sections were made, dewaxed with xylene, dehydrated with alcohol, stained with hematoxylin, washed with phosphate buffer, and then re-stained with eosin and dehydrated with 75%~100% gradient alcohol, transparent xylene and neutral gum mounting. The histopathological changes in the hippocampus tissues of the rats in each group were observed with a microscope.

Hippocampus tissues from five rats in each group were selected. The tumor necrosis factor-α (Tumor necrosis factor-α, TNF-α), interleukin-6 (interleukin-6, IL-6), interleukin-1 β (interleukin-1 β, IL-1 β), and other inflammatory factors were measured using enzyme-linked immunosorbent assay for expression levels in the hippocampus tissues of the rats in each group. Each hippocampus sample was taken, homogenized thoroughly and centrifuged. The supernatant was taken and fully diluted. Different concentrations of standard products were prepared and added to the standard and sample wells (except the blank wells); 100μL of the antibody was to be tested and added into each well, incubated for 1h under the condition of 37°C, and left standing for 2 minutes. The washing solution was absorbed and the test was repeated. Then 50uL of solution A and solution B were added into each well, incubated for 20min away from the light; a stop solution was added to terminate the reaction, and the absorbance was measured at 470nm using a microplate reader.

The expression levels of the phosphorylated protein kinase B (p-Akt), phosphorylated mammalian target of rapamycin (p-mTOR), apoptosis-related proteins Bax and Bcl-2, and autophagy-related protein Beclin 1, LC3II/I and p62 were measured using Western blotting. The hippocampus tissue samples of the remaining five rats were taken and the homogenate was lysed fully. The BCA method was used to obtain the target protein according to the operating method of the kit. After electrophoresis, the membrane was transferred and blocked, and the primary antibody was added, incubated overnight at 4°C and washed with TBST. The membrane was incubated at room temperature for 60min, and then developed by ECL. The expression levels of the target proteins of rats in each group was analyzed via the Quantity One software.

Statistical methods

In the present study, the measurement data were compared via an independent-sample t-test between two groups, and the single-factor multi-sample mean comparison among multiple groups was used, all expressed as ($\bar{x}\pm s$). SPSS20.0 software was used for statistical data analysis, and the result $P<0.05$ was regarded as statistically significant.

Results

Changes in the escape latency of rats in each group

Compared with the sham operation group, the escape latency of rats in the model group was significantly prolonged ($P<0.05$); compared with the model group on the first day, the escape latency of the rats in the oxiracetam group was shortened but there was no significant difference ($P>0.05$). Starting on the second day, the escape latency of rats in the oxiracetam group was significantly shortened ($P<0.05$). See Table 1.

Group	Escape latency (s)				
	1d	2d	3d	4d	5d
Sham group	61.13±6.13	49.29±6.18	30.36±3.43	15.49±2.50	7.38±1.29
Model group	77.38±7.31[a]	74.41±7.29[a]	71.89±4.05[a]	50.15±2.25[a]	44.12±3.59[a]
Oxiracetam group	72.49±6.14[a]	61.58±5.26[ab]	46.83±4.69[ab]	26.19±3.57[ab]	15.19±1.23[ab]
F	24.30	59.66	392.32	589.18	1049.37
P	<0.001	<0.001	<0.001	<0.001	<0.001

Table 1: Changes in the escape latency of rats in each group ($\bar{x}\pm s$).
Note: [a]P<0.05 means comparison with the sham operation group; [b]P<0.05 means comparison with the model group.

Comparison of the ratio of the residence time of rats of each group in the original platform quadrant to the total time

Compared with the sham operation group, the ratio of staying time in the original platform quadrant to the total time in the model group was significantly reduced ($P<0.05$); compared with the model group, the ratio of staying time in the original platform quadrant to the total time in the oxiracetam group was significantly increased ($P<0.05$). See Table 2.

Histopathological changes in the hippocampus of rats in each group

In the sham operation group, the hippocampal tissue cells were neatly arranged, the nuclear membrane was intact, the nucleolus was clear, and the staining was uniform; conversely, the cell arrangement in the model group was disordered, the number was significantly reduced, and the nucleolus disappeared. In the oxiracetam group, the number of hippocampal tissue cells was significantly

increased and the pathological tissue changes were significantly improved compared with the model group. See Figure 1.

Group	The ratio of the residence time of the original platform quadrant to the total time (%)
Sham group	43.43±4.68
Model group	24.86±2.72[a]
Oxiracetam group	33.31±3.63[ab]
F	91.58
P	<0.001

Table 2: Comparison of the ratio of the residence time of rats in each group in the original platform quadrant to the total time ($\bar{x}\pm s$).
Note: [a]P<0.05 means comparison with the sham operation group; [b]P<0.05 means comparison with the model group.

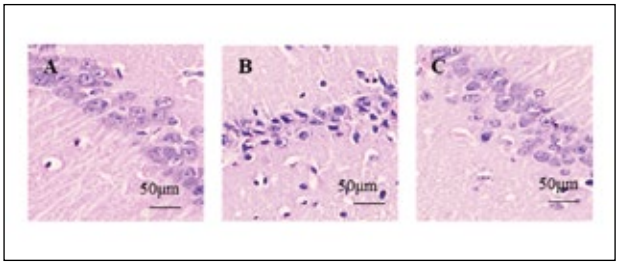

Figure 1: Histopathological changes in the hippocampus of rats in each group.
Note: Figure 1A: Sham operation group; Figure 1B: Model group; Figure 1C: Oxiracetam group.

Comparison of the expression levels of inflammatory factors in the hippocampus of rats in each group

Compared with the sham operation group, the expression of inflammatory factors in the hippocampus in the model group was significantly increased (P<0.05); compared with the model group, the expression of inflammatory factors in the oxiracetam group was significantly reduced (P<0.05). See Table 3.

Group	Case	TNF-α (pg/mg)	IL-6 (pg/mg)	IL-1β (pg/mg)
Sham group	5	9.13±3.42	10.07±3.34	11.54±2.16
Model group	5	21.51±4.95[a]	22.82±4.79[a]	24.96±3.48[a]
Oxiracetam group	5	15.49±3.56[ab]	16.93±2.58[ab]	16.55±2.87[ab]
F		11.76	14.99	27.58
P		0.002	0.001	<0.001

Table 3: Comparison of expression levels of inflammatory factors in the hippocampus of rats in each group ($\bar{x}\pm s$).
Note: [a]P<0.05 means comparison with the sham operation group; [b]P<0.05 means comparison with the model group.

Comparison of the expression levels of p-Akt, p-mTOR, Bcl-2/Bax, Beclin 1, LC3II/I, and p62 in the hippocampus of rats in each group

Compared with the sham operation group, the expression levels of p-Akt, p-mTOR, Beclin 1, and LC3II/I in the hippocampus tissues in the model group were significantly increased, while the expression levels of Bcl-2/Bax and p62 were significantly decreased (P<0.05). Compared with the model group, the expression levels of p-Akt, p-mTOR, Bcl-2/Bax, and p62 in the oxiracetam group were significantly increased, and the expression levels of Beclin 1 and LC3II/I were significantly decreased (P<0.05). See Table 4.

Group	Case	p-Akt	p-mTOR	Bcl-2/Bax	Beclin 1	LC3II/I	p62
Sham group	5	0.43± 0.08	0.40± 0.05	1.69± 0.17	1.06± 0.18	0.11± 0.02	1.10± 0.22
Model group	5	0.58± 0.08[a]	0.56± 0.12[a]	1.06± 0.16[a]	1.68± 0.23[a]	0.25± 0.04[a]	0.47± 0.12[a]
Oxiracetam group	5	0.78± 0.11[ab]	0.78± 0.13[ab]	1.46± 0.14[ab]	1.39± 0.17[ab]	0.17± 0.05[ab]	0.80± 0.24[ab]
F		18.57	16.15	20.57	12.64	16.44	12.37
P		<0.001	<0.001	<0.001	0.001	<0.001	0.001

Table 4: Comparison of the expression levels of p-Akt, p-mTOR, Bcl-2/Bax, Beclin 1, LC3II/I, and p62 in the hippocampus tissues of rats in each group ($\bar{x}\pm s$).
Note: [a]P<0.05 means comparison with the sham operation group; [b]P<0.05 means comparison with the model group.

Discussion

Vascular dementia is a type of cognitive dysfunction syndrome, mainly caused by cerebral hemorrhage, cerebral ischemia, acute and chronic cerebral hypoxia, and other cerebrovascular diseases. It primarily manifests as dysfunctions in memory, language, calculations, vision, space perception, etc. According to relevant statistics, the incidence of vascular dementia accounts for about 25% of all types of dementia. The rapid growth of the "social economy" and of pressures in people's lives have led to a significant increase in the incidence of this ailment and adverse impact on the quality of life[5].

Although the pathogenesis of vascular dementia remains unclear, it is believed that it may have an important relationship with nerve cell apoptosis, inflammatory response, free radical damage, and the abnormal activation of signal pathways[6]. Therefore, early intervention and the procurement of relevant drugs for treatment are crucial. Oxiracetam is a novel cyclic aminobutyric acid derivative that can promote learning, enhance memory and protect

the damaged central nervous system. Studies have found that oxiracetam can selectively act on the cerebral cortex and hippocampus to activate, protect or extend the functional recovery of nerve cells[7]. Parnetti et al[8] found that oxiracetam had an important effect on improving brain injury, hypoxia and chronic cerebral insufficiency.

Cognitive dysfunction is the most basic and typical symptom of vascular dementia. Animal behavior testing is often used in many fields of neuroscience, especially in evaluating animal models of cognitive dysfunction-related diseases[9]. The present study mainly explored and analyzed the neuroprotective effect of oxiracetam on vascular dementia in rats, along with its mechanisms. It used the Morris water maze experiment to measure the learning and memory functions of rats in each group.

The study found that the spatial learning and memory functions of rats with vascular dementia were significantly decreased. This may be because the hippocampus is a key brain area for learning and memory, and hippocampal neuron damage is an important cause of memory dysfunction. When vascular dementia occurs, the hippocampal neuron membrane flap develops phospholipid metabolism disorder; a large number of free radicals are produced and excitatory amino acids and intracellular calcium experience an overload, which causes the loss of hippocampal neurons, further affecting the spatial learning and memory ability of rats[10]. The results indicate that oxiracetam can significantly improve spatial learning, memory function and hippocampal neuron injury in rats.

Inflammation is the body's defense response to irritation and damage sustained from various internal and external substances. In recent years, a large number of studies have shown that inflammation response is involved in the development of most diseases[11]. TNF-α, IL-6 and IL-1β are all inflammatory cytokines; microglia are the main effector cells of neuronal inflammation in the brain and are closely related to the occurrence and development of various nervous systems. When pathological stimulation occurs, microglia are activated and a large number of inflammatory factors are released, which aggravate the inflammatory response, thus causing injury to neurons[12]. This study found that the levels of inflammatory factors in the hippocampus of rats with vascular dementia were significantly increased, and that oxiracetam could significantly inhibit the development of hippocampal tissue inflammation.

Apoptosis is a normal process, and its development is regulated by a variety of genes. Both Bcl-2 and Bax belong to the Bcl-2 family and play an important role in the process of cell apoptosis. Among them, Bcl-2 can reduce the concentration of Bax, affect the permeability of cell membranes, and inhibit the occurrence of cell apoptosis; Bax is a pro-apoptotic gene, which can promote cell apoptosis.

Meanwhile, autophagy is a basic life process that degrades and recycles macromolecules and organelles in cells[13]. Studies have indicated that the injury and protection of autophagy in cerebral ischemia-reperfusion injury itself may be closely related to brain maturity and damaged areas[14]. According to reports, the PI3k/Akt/mTOR signaling pathway plays an important role in regulating neuronal autophagy[15]; mTOR is an important regulator of autophagy and a downstream factor of the PI3k/Akt signaling pathway. Similarly to this study's results, Li et al[16] observed that when the PI3k/Akt/mTOR signaling pathway was activated, it could promote cell survival and inhibit autophagy. The present study found that oxiracetam could activate the PI3k/Akt/mTOR signaling pathway, promote the expression levels of Bcl-2/Bax and p62, inhibit the expression levels of Beclin 1 and LC3II/I, promote cell apoptosis, and inhibit autophagy.

In conclusion, oxiracetam can significantly inhibit the inflammatory response in the hippocampus tissues of rats with vascular dementia and improve pathological changes in the hippocampus. The drug has obvious neuroprotective effects, which may be activated by the PI3k/Akt/mTOR signaling pathway, so as to promote neuronal apoptosis and autophagy in the hippocampus.

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