EFFECTS OF BUTYLPHTHALIDE ON THE EXPRESSION OF TNF-A AND IL-1B IN HIPPOCAMPUS OF RATS WITH VASCULAR DEMENTIA AND THE EXPRESSION OF 5-HT, DA AND NE IN CEREBRAL CORTEX

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ABSTRACT

Objective: *To investigate the expression of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in hippocampal tissues of rats with vascular dementia, and the monoamine transmitters 5-HT (5-HT), dopamine (DA) and norepinephrine (NE) in cerebral cortex.*

Methods: *120 clean healthy male SD rats were selected for adaptive culture for 2 weeks. Rats were randomly divided into model group, sham operation group, low-dose butylphthalide group and high-dose butylphthalide group, with 30 rats in each group. Sham operation group, only bilateral common carotid arteries were isolated without ligation. Model group: bilateral common carotid arteries were separated. Low-dose butylphthalide group: ligated bilateral common carotid artery and intraperitoneally injected 3 mg/kg butylphthalide sodium chloride injection; High-dose butylphthalide group: ligated bilateral common carotid artery and intraperitoneally injected 7 mg/kg butylphthalide sodium chloride injection. The model group and the sham operation group were given equal doses of saline intraperitoneally. Morris water maze experiment was used to detect the changes in the time of escape latency, the exploration time of the platform area, the time of effective zone residence and the number of errors. The expression levels of TNF- a and IL-1β in hippocampal tissues of rats were detected by elisa. The changes of 5-HT, DA and NE in cerebral cortex of rats were determined by immunofluorescence. The changes of hippocampal tissues in each group were observed by HE staining.*

Results: *Compared with the model group, the escape latency time and the number of errors in the high-dose and low-dose butylphthalide groups were significantly reduced, and the exploration time in the plateau area and the stay time in the effective area were significantly increased (P<0.05). Compared with the model group, the levels of TNF-α and IL-1β in the high-dose, low-dose and sham operation groups were significantly increased, and the levels of TNF-α and IL-1β in the model group were significantly higher than those at 1 and 6 weeks after the operation (P<0.05). Compared with the model group, the levels of 5-HT, DA and NE in the high-dose, low-dose and sham operation groups were significantly increased (P<0.05). The hippocampal nerve cells in the sham operation group were arranged normally, with dense and large round neurons, obvious nucleoli and distinct cell stratification. The shapes of hippocampal neurons in the high-dose and low-dose butylphthalide groups were regular, with obvious stratification, and there was no significant difference between the two groups. In the model group, the pyramidal cells in the hippocampal of rats were arranged in disorder, and the shape of the cells were irregular, connective tissue such as glial cells proliferate, nodular formation, and the level disappeared.*

Conclusion: *Butylphthalide can inhibit the expression of TNF-α and IL-1β in hippocampus of rats with vascular dementia, reduce its damage to the nervous system, and increase the levels of 5-HT, DA and NE in vascular dementia rats, and improve the learning and memory ability of rats.*

Keywords: *butyl phenyl peptide, vascular dementia, hippocampus, TNF-α, IL-1β, 5-HT, DA, NE.*

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Introduction

Vascular dementia refers to dementia syndrome caused by brain tissue damage, and which caused by a series of cerebrovascular factors, including cognitive ability, computational ability, social life ability and emotional personality changes[(1)]. With the development of society, the pace of people's life is accelerating, and the incidence of vascular dementia is increasing year by year.

According to statistics, the mortality rate of vascular dementia exceeds 65% within five years, and it has become the main factor affecting the health and quality of life of the elderly.

Kalaria et al[(2)] found that cerebral blood flow interruption and reperfusion can lead to

brain cell necrosis or apoptosis, and repeated ischemic cerebrovascular events can induce cognitive dysfunction and neurological dysfunction associated with cerebrovascular diseases. Adhesion molecules and cytokines produced early in ischemia-reperfusion are the basis of ischemic inflammatory damage[3]. The study found that serum inflammatory factor levels were significantly elevated in patients with vascular dementia, and their levels were significantly positively correlated with the degree of dementia. Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are two important pro-inflammatory cytokines, the occurrence and development of vascular dementia is closely related to the levels of TNF-α and IL-1β in hippocampus of cerebral ischemia.

Synapses are the key parts of the connection between neurons and receive information. The early stage of cerebral ischemic injury will affect the synaptic function and inhibit the release of neurotransmitters. When ischemia and hypoxia persist, the release of neurotransmitters is significantly reduced, and thus create obstacles to learning and memory functions[4].

Neurotransmitters act as messengers in the transmission of synapses, mainly monoamine neurotransmitters and peptides. 5-hydroxytryptamine (5-HE), dopamine (DA) and norepinephrine (NE) belong to monoamine transmitters, which mainly regulate human learning and memory ability and response level[5]. A large number of studies have found that in the pathogenesis of vascular dementia, the reduction of neurotransmitters in the brain, especially the reduction of monoamine neurotransmitters in the hippocampus and cerebral cortex, can cause insufficient blood supply to the brain, causing ischemia and deficiency of brain tissue.

The oxygen state is further aggravated, which accelerates the development of dementia[6]. At present, Western medicine mainly uses cholinergic agents, neuroprotective agents and brain metabolism improving agents to treat neuronal damage and reduce clinical symptoms in the treatment of vascular dementia.

Butylphthalide is a new type of anti-cerebral ischemic agent, which can reduce brain edema, improve microcirculation in cerebral ischemic area, and inhibit neuronal apoptosis[7]. Most studies on butylphthalide are focused on cerebral ischemia, while the relationship between butylphthalide and vascular dementia is less reported. In this study, rats were used as the research object to investigate the effects of butylphthalide on the expression of TNF-α, IL-1β and 5-HT, DA and NE in hippocampus of rats with vascular dementia.

Materials and methods

Experimental animals

120 healthy male SD rats (provided by JunKe Biological Co., Ltd., production license SCXK (Ning) 2017-0001), body weight (240 ± 30) g.

Main instruments and reagents

Centrifuge (Shanghai Precision Instrument Co., Ltd., model: GL-25MS); Microscope (Japan Keenshi Company, Cining Palace: XG-X); Incubator (Herry Tech Co., Ltd., model: DZ-75L); Oven (Tianjin Shunuo Instrument Technology Co., Ltd., Model: 202-00A); Low temperature refrigerator (Zhongke Meiling Cryogenics Co., Ltd., Model: YCD-EL289); Hematoxylin (Shanghai Meixuan Bioogical Science and technology Ltd.); Anhydrous ethanol (Suzhou Ott Chemical Co., Ltd.); Paraffin (Shanghai Thermo Fisher Scientific Co., Ltd.); Morris water maze (Jiangsu Cyrus Biotechnology Co., Ltd.); Phosphate buffer (prepared when use); 10% chloral hydrate (Qingdao Yulong Algae Co., Ltd., production batch number: 37172673); butyl hydrazine sodium chloride injection (CSPC NBP Pharmaceutical Co., Ltd., production batch number: 20170041, specification: 25 mg :100 mL).

Experimental grouping and establishment of animal models

All rats were acclimated for 2 weeks at a laboratory temperature of 23 ± 3 °C, a humidity of 56 ± 12%, and a 12-hour day and night, free diet and drinking water. Rats were randomly divided into model group, sham operation group, butylphthalide low dose group and butylphthalide high dose group, with 30 rats in each group. Sham operation group: Only bilateral common carotid arteries were isolated without ligation; Model group: Separation of bilateral common carotid arteries; Low-dose butylphthalide group: Ligated bilateral common carotid artery and intraperitoneally injected 3 mg/kg butylphthalide sodium chloride injection; High-dose butylphthalide group: Ligated bilateral common carotid artery and intraperitoneally injected 7 mg/kg butylphthalide sodium chloride injection. The model group and the sham operation group were intraperitoneally injected with an equal dose of physiological saline.

Establishment of a rat model of vascular dementia: Before the establishment of vascular dementia rat model, the rats fasted for 12 hours and forbade drinking for 8 hours. The rats were fixed on a laboratory bench and anesthetized by intraperitoneal injection of 0.5 mL of 10% chloral hydrate.

The common carotid arteries of both sides of the rat were ligated, the skin and various layers of muscles were sutured, and antibiotics were sprayed and injected at the surgical wound to prevent infection.

Experimental methods and indicators

Morris water maze test was used to test the learning and memory ability of the rats: The water maze was divided into four areas, and the water mark marking was set in each area to place a concealed circular platform in the third area. In the directional navigation test (3 times a day for 4 days), each time a region was randomly selected into the water, and the time to find the circular platform within 100s was the escape latency. Observe and record the time it takes for the rat to find and climb the platform. On the fifth day, the platform was removed for space exploration experiments, and water was taken from the original area to record the exploration time of the original platform area within 100 s. If the platform was not found within 100 s, the rats should be led to the platform. After 48 hours, the experiment was repeated, and the time of escape latency, the exploration time of the platform area, the residence time of the effective area and the number of errors were observed and recorded.

After the rat model was successfully prepared and grouped, 15 rats in each group were sacrificed at the first week, 3 weeks, and 6 weeks. The hippocampus tissues were isolated and stored in a freezer at a low temperature. The expression levels of TNF-α and IL-1β in rat hippocampus were detected by enzyme-linked immunosorbent assay. At the end of the behavioral experiment, 15 rats in each group were sacrificed and the brain tissue was taken out. The hippocampus tissue was isolated, the hippocampus tissue was homogenized, the supernatant was centrifuged, and the monoamines in the rat cerebral cortex were determined by immunofluorescence. The levels of 5-HT, DA and NE in the transmitter varied.

HE staining was used to observe the changes of hippocampus in each group.

Statistical methods

The SPSS23.0 software package was used for statistical data analysis. The measurement data were compared by single factor multi-sample comparison test; the count data were compared by $\chi 2$ test. The ranking data was compared using the Ridit test. The statistical results were statistically significant at $P<0.05$

Results

Comparison of escape latency time, platform area exploration time, effective area retention time and error times of each group of rats

Compared with the sham operation group, the evapotranspiration time and the number of errors in the high-dose, low-dose and model groups of butylphthalide group increased significantly, and the exploration time of the platform area and the residence time of the effective area were significantly reduced ($P<0.05$). Compared with the model group, the escape latency and the number of errors in the high-dose and low-dose groups of butylphthalide were significantly reduced, and the exploration time and effective time of the platform were significantly increased ($P<0.05$). See Table 1.

Group	*n*	escape latency time (s)			
		1st Day	2nd Day	3rd Day	4th Day
Sham operation	30	13.44±4.31[a]	10.27±4.87[a]	7.37±5.62[a]	6.27±2.48[a]
High-dose butylphthalide	30	17.42±6.33[ab]	15.02±4.18[ab]	13.15±5.19[ab]	10.48±0.98[ab]
Low-dose butylphthalide	30	20.97±7.06[ab]	18.91±6.94[ab]	17.19±6.81[ab]	13.54±3.08[ab]
Modle	30	31.38±8.05	29.61±12.11	28.45±14.35	27.86±7.69

Group	*n*	Platform area exploration time (seconds)	Effective area dwell time (seconds)	Number of errors
Sham operation	30	14.95±5.06[a]	34.27±4.98[a]	16.42±4.65[a]
High-dose butylphthalide	30	9.29±3.43[ab]	29.62±11.34[ab]	19.67±5.02[ab]
Low-dose butylphthalide	30	6.32±5.01[ab]	19.78±9.58[ab]	23.05±5.33[ab]
Modle	30	3.76±1.63	14.91±2.42	37.82±7.63

Table 1: Comparison of escape latency time, platform area exploration time, effective area residence time and error times of each group of rats ($\bar{x}\pm s$).

Note: A indicates [a]P<0.05 compared to the model group, and b indicates [b]P<0.05 compared with the sham operation group.

Comparison of TNF-α and IL-1β expression levels in hippocampus of rats in each group

Compared with the model group, the levels of TNF-α and IL-1β in the high-dose, low-dose of butylphthalide groups and sham operation groups were significantly increased, and the levels of TNF-α and IL-1β were significantly higher in the model group at three weeks after surgery. It was higher than 1 week and 6 weeks after operation (P<0.05). See Table 2, Table 3.

Group	*n*	1Weeks	3 Weeks	6 Weeks
Sham operation	15	163.65±53.86 [a]	164.57±43.88 [a]	168.72±53.05 [a]
High-dose butylphthalide	15	184.66±25.43[ab]	176.91±31.74[ab]	179.17±485.07[ab]
Low-dose butylphthalide	15	326.53±46.11[ab]	318.59±93.67[ab]	253.68±74.66[ab]
Modle	15	373.66±111.04	393.81±111.82	357.78±96.12

Table 2: Comparison of TNF-α expression levels in hippocampus of rats in each group ($\bar{x}$±s) (ng/L).
Note: a indicates [a]P<0.05 compared to the model group, and b indicates [b]P<0.05 compared with the sham operation group.

Group	*n*	1 Weeks	3 Weeks	6 Weeks
Sham operation	15	9.12±3.06 [a]	8.69±2.68 [a]	8.34±2.52 [a]
High-dose butylphthalide	15	13.43±2.14[ab]	12.98±1.78[ab]	11.68±1.78[ab]
Low-dose butylphthalide	15	21.31±4.48[ab]	23.79±3.91[ab]	15.18±3.48[ab]
Modle	15	26.33±3.46	30.73±5.91	21.59±3.11

Table 3: Comparison of IL-1β expression levels in hippocampus of each group of rats ($\bar{x}$±s) (ng/L).
Note: a indicates [a]P<0.05 compared to the model group, and b indicates [b]P<0.05 compared with the sham operation group.

Comparison of 5-HT, DA and NE levels in cerebral cortex monoamine transmitters in each group

Compared with the model group, the levels of 5-HT, DA and NE in the high-dose, low-dose of butylphthalide groups and sham operation groups were significantly higher.

Group	n	5-HT (ng/mL)	DA (ng/mL)	NE (ng/mL)
Sham operation	15	431.62±55.49 [a]	514.87±65.46 [a]	310.66±16.87 [a]
High-dose butylphthalide	15	402.55±40.08[ab]	477.69±40.59[ab]	290.24±29.54[ab]
Low-dose butylphthalide	15	270.36±20.09[ab]	341.57±20.99[ab]	206.16±11.86[ab]
Modle	15	238.29±20.13	301.66±15.66	174.19±21.57

Table 4: Comparison of 5-HT, DA and NE levels in the cerebral cortex monoamine transmitters of each group ($\bar{x}$±s).
Note: A indicates [a]P<0.05 compared to the model group, and b indicates [b]P<0.05 compared with the sham operation group.

Compared with the sham operation group, the levels of 5-HT, DA and NE in the high-dose and low-dose butylphthalide groups were significantly lower (P<0.05). See table 4.

Changes in hippocampus of rats in each group

Sham operation group

The hippocampal nerve cells in the sham operation group were arranged normally, with dense and large round neurons, obvious nucleoli and distinct cell stratification; Butylphthalide high dose group and butylphthalide low dose group: The shape of rat hippocampal neurons was regular and the layering was obvious. There was no significant difference compared with the sham operation group.

The neuronal cell death was significantly reduced compared with the model group. The model group: The hippocampal pyramidal cells were disorderly arranged, the cell shape was irregular, and the connective tissue such as glial cells proliferated, and there are nodules formed and the levels disappear. See Figure 1.

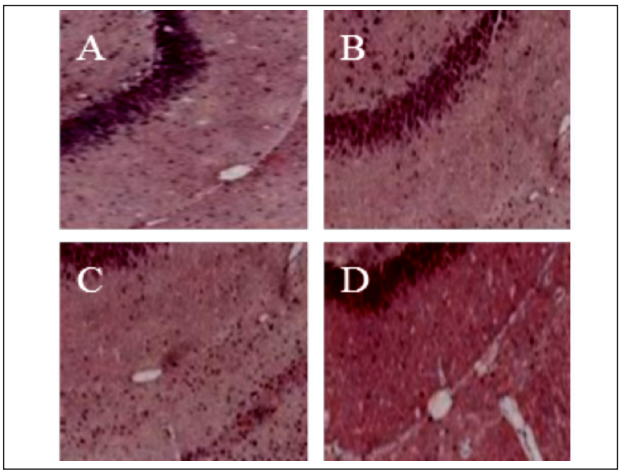

Fig. 1: Changes in hippocampus of rats in each group. ***Panel A:*** sham operation group; ***panel B:*** butylphthalide high dose group; ***panel C:*** butylphthalide low dose group; ***panel D:*** model group.

Discussion

Dementia is a acquired and persistent disorder syndrome caused by brain dysfunction, and is common in older people. Cerebrovascular disease is the main cause of vascular dementia. The pathogenesis of vascular dementia is not clear, and there is a lack of effective therapeutic drugs. Therefore, the establishment of vascular dementia rat model and the analysis and discussion of the occurrence mechanism of vascular dementia play an important role

in the early treatment, diagnosis and prevention of the disease.

Butylphthalide is a yellow oily liquid isolated and purified from celery seed. The mechanism of butylphthalide against cerebral ischemia is very complex, which is a multi-gene, multi-target and multi-link process. By reducing cerebral edema, improving brain energy metabolism and microcirculation in ischemic brain area, inhibiting neuron apoptosis, preventing cerebral thrombosis, platelet aggregation and inhibiting glutamate release, reducing intracellular calcium concentration, inhibiting free radicals and increasing antioxidant enzyme activity, thereby exerting the drug efficacy[8]. Kojima et al[9] found that butylphthalein can regulate the function of the cholinergic system, so as to minimize the accumulation of abnormal proteins in the blood vessels, reduce the damage of oxidative stress and increase the content of GSH-Px in the body.

Some scholars have confirmed that butylphthalide can improve the blood-brain barrier of local brain tissue and inhibit cerebral edema. Thomas et al[10] found that butylphthalide can improve the learning and memory ability of rats, inhibit protein non-enzymatic glycation, and reduce cell death. The Morris water maze is a classic method for evaluating spatial learning and memory functions in animals such as mice. In this study, compared with the model group, the escape latency and the number of errors in the high-dose and low-dose groups of butylphthalide were significantly reduced, and the exploration time of the platform area and the residence time of the effective area were significantly increased ($P<0.05$). This indicates that butylphthalide can improve the learning and memory ability of rats with vascular dementia.

Li et al[11] found that the occurrence of vascular dementia is closely related to cerebral cortex ischemia and hypoxia. After cerebral ischemia, neurons and endothelial cells are activated, and the levels of TNF-α and IL-1β are significantly increased, which promotes the release of other cytokines, forming a series of cascades, causing inflammatory responses to damage neurons and promoting the occurrence of vascularity dementia. TNF-α is a polypeptide cytokine produced by monocytes and macrophages. It has a variety of biological activities and is closely related to the body's immune response and inflammatory response. It can activate the release of other inflammatory factors (such as IL-6), trigger an inflammatory response, and cause apoptosis and necrosis. It is an important pro-inflammatory factor[12]. IL-1β is a member of the IL-1 chemokine family. IL-1β activates semi-cystic aspartitase (Caspase-3), induces neuronal apoptosis, and impairs cognitive function in mice.

Caspase-3 plays an irreplaceable role in apoptosis. Chie et al. believe that butylphthalide can inhibit the secretion of TNF-α and reduce further damage to ischemic and hypoxic tissue. In this study, compared with the model group, the levels of TNF-α and IL-1β in the high-dose, low-dose and sham operated groups of butylphthalide were significantly higher ($P<0.05$), which was consistent with the results of Memisoglu et al[13].

The hippocampal region of the brain is an important structure of learning and memory, and is also one of the brain regions that are very sensitive to ischemia and hypoxia. It has been found that the intelligent changes of vascular dementia are closely related to the release and metabolism of monoamine neurotransmitters. 5-HT, DA and NE are mainly expressed in synapses and play an important role in synaptic maturation and transmission. Many studies have shown that the changes of monoamine neurotransmitters are closely related to the occurrence of vascular dementia. 5-HT was first found in serum and is widely found in mammalian tissues, especially in the cerebral cortex and synapses, which can promote the differentiation of neurons[14]. DA is a key neurotransmitter in the hypothalamus and pituitary gland, which can be converted into norepinephrine under the action of dopamine p-hydroxylase, and is an important catecholamine neurotransmitter in the central nervous system. NE is mainly synthesized and secreted by sympathetic postganglion neurons and cerebral adrenalin, which can regulate the afferent activity of synapses, reduce the afferent of interfering stimuli, and better preserve information[15]. In this study, the levels of 5-HT, DA and NE in the high-dose, low-dose and sham operation groups were significantly higher than those in the model group. It is suggested that butylphthalide can improve the levels of 5-HT, DA and NE and improve the learning and memory ability of rats with vascular dementia.

In summary, butylphthalide can inhibit the expression of TNF-α and IL-1β in hippocampus of rats with vascular dementia, and alleviate the neurological damage caused by it. The levels of 5-HT, DA and NE in rats with vascular dementia were increased to improve the learning and memory ability of rats.

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