THE THERAPEUTICAL POTENTIAL OF GABAPENTIN COMBINED WITH DIOSCOREA OPPOSITA THUNB EXTRACTS ON A MURINE MODEL OF VASCULAR DEMENTIA BY MODULATING P2RX7 RECEPTORS

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Abstract
This paper aimed to investigate the therapeutically effect of gabapentin (GBP) combined with Dioscorea opposita Thunb extracts (DOTE) on vascular dementia (VD) and the underlying mechanism related to P2X7 receptor (P2RX7) modulation using a murine model. Sixty Wistar rats were randomly divided into normal group, model group, GBP group, DOTE group, and GBP + DOTE group, 12 animals per group. The VD rat model was obtained by repeatedly clipping the bilateral common carotid artery-reperfusion method. The Morris water maze test was used to evaluate animal behaviour, and qPCR was used to detect the change of P2RX7 protein expression in hippocampal tissues. The results showed that compared with the normal group, the elusive incubation period of the rats in the model group was significantly prolonged, and the number crossing the original platform quadrant was significantly reduced. Compared with the model group, the GBP group, the DOTE group, and the GBP + DOTE group, all had significantly shortened escape latency and significantly increased the number of passes through the original platform quadrant, with the GBP + DOTE group having the shortest escape latency and the most access through the original platform. The treatment significantly decreased the morphological changes observed in the model group and downregulated the P2X7R expression, the effect being superior in the GBP + DOTE group. In conclusion, GBP combined with DOTE extract can improve VD rats’ learning and memory ability and protect neuronal cells.

Keywords: gabapentin, Dioscorea opposita Thunb extracts, vascular dementia, P2RX7 receptor

Introduction
Vascular dementia (VD) refers to a syndrome of severe cognitive impairment triggered by diffuse atrophy and degenerative changes in brain tissue due to ischemic stroke, haemorrhagic stroke and cerebrovascular disease. Vascular dementia determines hypoperfusion in brain regions, affecting memory, cognition, and behaviour mainly related to cerebral atherosclerosis, manifesting as an organic lesion with slowly progressive mental retardation [1]. According to epidemiological statistics, VD patients account for 10% ~ 20% of the total patients with dementia in countries such as Europe and the United States. In China, the prevalence of VD is 1.1% ~ 3.0% of the population [2]. VD has become the second leading cause of senile dementia after Alzheimer’s disease (AD) in Asia and most developing countries globally. The incidence, prevalence, disability and mortality of vascular dementia due to cerebrovascular disease and its risk factors are increasing as the ageing process of the population accelerates [3]. VD seriously impairs the daily living ability and social function of patients reduces patients’ quality of life, imposes heavy care and economic burden on families and society, and has become one of the major public health problems. Because there are many pathogenic factors and complex pathogenesis of VD, which has not been fully elucidated until now [4].
treatments targeting cognitive symptoms are relatively limited, mainly in the two main categories of drugs acetylcholinesterase inhibitors and Glutamate N-methyl-D-aspartate (NMDA) receptor antagonists [5]. Other drugs such as the calcium antagonist nimodipine have been studied less clinically to treat VD, and there is insufficient evidence for efficacy in the treatment of these patients. The effects of potentially effective antioxidants, nonsteroidal anti-inflammatory drugs and hormone replacement therapy that improve brain circulation and promote brain cell metabolism remain controversial [6].

The human P2X7 receptor gene is located on chromosome 12 at 12q24.31. It encodes a 595 amino acid protein, and the P2X7 subunit mainly exists as a homotrimer complex and can also associate with the P2X4 subunit to form a heteromer [7]. The P2X7 subunit has an N-terminal residue and C-terminal tail, one extracellular domain containing the ATP binding site, and two transmembrane helices. Unlike other subtypes of the P2X family, the P2X7 receptor has an extended carboxy-terminus. This region has essential physiological functions, such as posttranslational modifications, formation of large pores and activation of signalling pathways [8]. The P2X7 acceptor has ten spliceosomes (P2X7A-J), is highly polymorphic, and more than 150 nonsynonymous single nucleotide polymorphism sites have been reported [9]. P2X7 receptors are widely distributed in the body, such as the heart, pancreas, kidney, muscle, etc. They are expressed in almost all immune cells, such as T lymphocytes, macrophages, dendritic cells, etc. As one of type 2 purinergic receptor family (P2 receptor) members, the purinergic ligand channel 7 (P2X7) receptors have a wide range of roles and are involved in embryonic development, immune system maturation, inflammation and cancer, among others [10]. Recently, much attention has been paid to the mechanism of the P2X7 receptor in the related aspects of cardiovascular disease. However, its specific mechanism has not been demonstrated. Gabapentin is a GABA analogue initially recorded for treating epilepsy and lately permitted for specific neuropathic pain. Gabapentin alternative suppression of the α2-δ subunits of voltage-sensitive calcium channels (VSCC) is involved in reducing allodynia for it triggers lessening of neural excitement and regulation of neurotransmitter release [11]. Dioscorea opposita Thunb is a widely cultivated species in China for food and medicinal purposes. The extracts have been used in traditional Chinese medicine for tonic properties and to improve the stomach, spleen, lung and kidney function. Modern studies showed its antioxidant activity and beneficial anti-diabetic, anti-hypertensive and anti-hypercholesterolemia properties [13-15]. This current study aimed to elucidate if the combination of gabapentin and Dioscorea opposita Thunb extracts can prevent the formation and development of vascular dementia and if the mechanism is related to P2X7R modulation.

Materials and Methods

Animals
Sixty healthy SPF grade Wistar rats (male, aged 8-10 weeks and with a body mass (200 ± 20 g)) were purchased from Nanjing Junke Bioengineering Co. Ltd., China acclimatized to the new laboratory conditions for one week before starting the experiment. The rats were housed at controlled room temperature (22 ± 2°C) and humidity (50 ± 10%) with free access to water and standard animal feed. The administration and treatment of rats during this study were under regulations for the protection of experimental animals, and the protocol was approved by the Ethical Committee of The First Affiliated Hospital of Jiamusi University, Jiamusi, China.

Animal grouping and VD model preparation
Wistar rats were randomly divided into sham group, model group, GBP group, DOTE group and GBP + DOTE group, with 12 rats in each group. A modified method was prepared for a rat model of VD by permanent ligation (2-VO) of bilateral common carotid arteries. The animals have fasted 12 h before operation, and they did not receive water for 6 h. Then the animals were anaesthetized with intraperitoneal injection with 10% chloral hydrate (70 mg/kg body weight (bw), Perfermiker, USA), then fixed in a supine position on the operating table, and the limbs were fixed to the periphery of the operating table with a suture. The surgical neck field was shaved, the local skin was exposed, disinfected with iodophor disinfectant solution (Iosan, Novartis Animal Health, Ltd., Whittlesford, UK), and the skin was incised with a razor blade along the median cervical incision. The surrounding tissues were carefully separated with microsurgical scissors and ophthalmic scissors to avoid pulling and damaging the vagus nerve, the right common carotid artery was exposed, and the proximal and distal ends of the right common carotid artery were ligated with a No. 0 surgical thread (West Chester, USA). Then the incisions were sutured layer by layer sequentially and were swabbed around the incision again with iodophor antiseptic solution. The wound was closed with an external application of erythromycin ointment (0.5%, Hengyuan, China). After surgery, the animals were put in a cage to be fed normally after waking up. One week later, the left common carotid artery was ligated and snipped under the same conditions.

After being anaesthetized with the animals from the sham group by intraperitoneal injection of 10% chloral hydrate (70 mg/kg body weight), the rats were fixed in a supine position on the surgical table, routinely sterilized, and tissues such as muscle and fascia were bluntly separated. The bilateral carotid arteries were exposed and immediately sutured. The incision was...
sutured postoperatively, disinfected again, and then placed in a cage to be awake and fed normally.

**Treatment**
The animals in the sham group and model group received by gavage a volume of 2 mL/kg bw normal saline solution once per day from day 1 after modelling continuously for 4 weeks. The animals from the GBP group received 30 mg/kg bw per day gabapentin (Santa Cruz Biotechnology, Dallas, TX, USA) dissolved in saline solution in the same volume as the sham and the model group. The animals from the DOTE group were treated with 30 mg/kg bw per day gabapentin + 201 mg/kg bw per day DOTE extract. The treatment was administered every day for 4 weeks.

**Histological analysis**
After the treatment period, the rats were anaesthetized with 10% chloral hydrate (70 mg/kg body weight) and intracardiac perfused with a 5% paraformaldehyde in PBS (phosphate-buffered saline). Brains were gathered and post-fixed for 2 h, transported in 20% PBS and segmented with a sliding freezing microtome (Leica, Wetzlar, Germany) in the coronal direction with a thickness of 5 µm. H&E staining was used to observe the pathological changes of the hippocampus and cortex. P2X7 receptor expression in the hippocampus was determined by Western blot analysis.

The protein concentration in hippocampal tissue was measured by the bicinchoninic acid (BCA) assay, subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, Cold Spring Harbour Protocol, Cold Spring Harbor Press, NY, USA). The membrane was blocked for 2 h at room temperature on a decolourized shaker with 5% non-fat milk (Sanyuan, China). Rabbit anti-rat P2X7R antibody (Bio-Rad Laboratories, Hercules, CA, USA) was diluted in blocking solution (1:1000) and incubated overnight at 4°C with the samples. The following day, the membrane was washed three times for 5 min each with TBST (0.1% Tween 20) on a destained shaker at room temperature. After that, 1:3000 dilution of secondary antibody Goat anti-rabbit IgG-HRP conjugate (Bio-Rad Laboratories, Hercules, CA, USA) was added. After incubation for 30 min at room temperature, the membrane was washed with TBST three times for 5 min each. After electrophoretic transfer, the target bands were analysed in rows by alpha software processing system (Novagen, Madison, WI, USA).

**Statistical analysis**
All the measurement data were expressed as means ± standard error of the mean (x±s), and statistical analysis was performed using SPSS 20.0 statistical software (SPSS Inc., Chicago, USA). The comparisons between groups were analysed by analysis of variance, with p < 0.05 considered statistically significant.

**Results and Discussion**

**Morris water maze test results**
Compared with the sham group, the escape latency of rats in the model group was significantly increased, and the number of times crossing the original platform quadrant and duration of sitting on the original platform was significantly reduced (p < 0.05).

**Figure 1.**
Water maze test results. A. Escape latency in seconds. B. Number of crossing the original platform quadrant. C. Duration of sitting on the original platform in seconds.

##p < 0.05 compared with the sham group; ##p < 0.05 compared with the model group
Histomorphometric changes in the hippocampus

Observation and comparison of hippocampal morphology of rats in each group under light microscopy showed that the hippocampal CA1 pyramidal cells of the rats in the sham group were regular and complete in morphology, with a high number of cells, clear borders, well-defined, arranged orderly and tight, and evenly stained, with larger and rounder nuclei, uniform colouration, clear nucleoli, and abundant cytoplasm (Figure 2).

In the model group, the pyramidal cells in the CA1 region of the hippocampus showed irregular morphology, blurred cell borders, disordered and lost arrangement, the number of normal cells was reduced, necrotic neurons were seen, the cell membrane and nuclear membrane were not clear, the nucleus was irregular, the colouration was pale, the nucleolus was pyknotic, deeply stained, and the cytoplasm was turbid (Figure 2). Pyramidal cells in the hippocampal CA1 region of the GBP + DOTE group, compared with the model group, showed a regular cell morphology with a clear boundary, well-defined and orderly arrangement, a significantly higher number of normal cells, fewer necrotic neurons, a regular nucleus with a clear nuclear membrane and nucleolus, and abundant cytoplasm. Pyramidal cells in the CA1 region of the hippocampus of rats in the GBP group, compared with the model group, showed a regular cell morphology, a larger number of normal cells, a more regular nucleus, a clear nuclear membrane, and nucleolus, visible some nucleoli pyknosis, deep staining, rich cytoplasm. Pyramidal cells in the CA1 region of the hippocampus of the DOTE group, compared with the model group, the cells and nuclei were more regular in morphology, more orderly arranged, fewer necrotic neurons, clear nuclear-cytoplasmic boundary, pyknotic nucleoli, and less deeply stained (Figure 2). Among the treatment groups with VD, the GBP + DOTE group showed the lowest pathological changes, close to the normal hippocampal structure observed in the sham group.

Figure 2.
Morphological structure of hippocampal neurons in each group (HE, ×400)

Figure 3.
Comparison of the relative expression of P2X7R in the hippocampus of rats in each group

**p < 0.05 compared with the sham group; *p<0.05 compared with the model group; **p < 0.001 compared with the model group. ②p < 0.05 compared with the GBP group; ③p < 0.05 compared with the DOTE group
P2X7R expression in the hippocampus

P2X7R expression was significantly upregulated in the VD model group compared with the sham group (p < 0.01) and significantly decreased after treatment in the GBP group, DOTE group as well as in GBP + DOTE group compared with the model group (p < 0.05, p < 0.05, p < 0.01) (Figure 3). The comparison of P2X7R expression between the treatment groups revealed that P2X7R expression significantly decreased in the GBP + DOTE group compared with the GBP group or DOTE group (p < 0.05), the difference in P2X7R expression between the GBP and DOTE groups was not statistically significant (p > 0.05) (Figure 3).

The pathogenesis of VD is closely related to several factors, such as hypertension, diabetes mellitus, hyperlipidaemia, hyperhomocysteinemia, cerebral infarction, intracerebral haemorrhage, leukoaraiosis, and chronic cerebral ischemia. However, the underlying molecular mechanisms involve impairment of the cholinergic system, excitotoxicity of excitatory amino acids, inflammatory responses, synaptic plasticity and genetics [12, 15]. Chronic ischemia hypoxia due to cerebral hyperperfusion activates pathological processes such as oxidative stress and inflammatory responses, which appear as blood-brain barrier breakdown, endothelial cell damage, and initial immune activation, leading to impaired brain cell function and causing cognitive dysfunction. Because there are many pathogenic factors and complex pathogenesis of VD, which has not been fully defined until now, treatments targeting cognitive symptoms are relatively limited, mainly in the two main categories of drugs, acetylcholinesterase inhibitors and NMDA receptor antagonists [17]. In terms of current research progress, these drugs can improve patients' cognitive and behavioural abilities and relieve symptoms to some extent. Still, they have limited long-term outcomes and multiple toxic side effects.

Vascular dementia, a severe manifestation of vascular cognitive impairment, results from the development of vascular cognitive impairment. As one of the core research themes in cognitive vascular disorders, cognition is the higher-order function of the brain, is the intellectual processing process of acquiring knowledge and organisal cognition, involves memory, learning, language, spirit, emotion, thinking, figure orientation ability and other temporal and spatial series of psychological and social behaviours [18, 19]. Cognitive impairment is defined as the occurrence of abnormalities in the high-level mental processing of the brain related to the above learning and memory as well as thinking judgment, which can cause severe learning and memory impairment, can be accompanied by aphasia, apraxia or agnosia and other pathological changes, and in severe cases can lead to dementia. Learning and memory impairment is the most important manifestation of cognitive impairment. Although research on cognition has progressed considerably in recent years, the mechanisms involved in learning and memory remain incompletely understood. The Morris water maze belongs to the classical experiments in studying spatial cognitive abilities in rodents. It is a form of associative learning that uses the platform's location as a reference point, and the memory formed is a type of spatial reference memory by having rats repeatedly learn, find the line that best approximates the platform, or use the shortest time [20].

It is also the most commonly used and highly sensitive assessment of cognitive function in animal experiments of VD. It can specifically reflect hippocampal function and assess the degree of impaired spatial learning and memory in experimental animals. In this study, compared with the sham group, the escape latency, the number of crossing the original platform, and the length of stay in the open field were significantly longer in the VD model group, indicating that the learning and memory abilities of the VD rat model made by the modified 2VO method were decreased, which is consistent with the related literature reports [21]. Gabapentin is aminobutyric acid (GABA) receptor agonist involved in the treatment of vascular dementia [11]. DOTE extracts are extensively used in a variety of food products in China and countries in the Far East [13]. Due to the increasing concern about the influence of foods on health conditions, we have investigated the effect of the combination of aqueous extract of DONE plus gabapentin on VD. The results showed that combined therapy could effectively improve cognitive dysfunction in VD rats compared with a single therapy.

The P2X7 receptor is a nonsellective cation channel receptor, and ATP is its natural ligand. Under physiological conditions, P2X7 receptors are permeable to K+, Na+, Ca2+; upon repeated or sustained stimulation by high concentrations of ATP, their cation channels transform into nonselective membrane pores that are permeable to macromolecules up to 900 DA and cause cell swelling, vacuolization and even cell death. Studies have shown that the P2X7 receptor is associated with the activation of several inflammatory pathways, including the nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome, the release of the inflammatory cytokines interleukin-1 β (IL-1 β) and IL18 [22]. The P2X7 receptor regulates extracellular ATP levels, immune cell infiltration, and expression of extracellular nucleotides and also enhances oxidative phosphorylation and glycolysis, regulating phosphatidylinositol-3 kinase/protein kinase B (PI3K/PKB, also known as Akt) and hypoxia-inducible factor/vascular endothelial growth factor, HIF1α/VEGF) axis [22].

Conclusions

In summary, GBP combined with DOTE extract can improve VD rats' learning and memory ability and has a protective effect on nerve cells. The mechanism may
be related to the downregulation of P2RX7R that is upregulated in theVD murine model. Further studies should confirm these findings in humans.

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**Conflict of interest**

The authors declare no conflict of interest.

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