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Original Article

Dl-3n-butylphthalide protects against the permeability changes of blood brain barrier by influencing tight junctions in Alzheimer's disease

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Abstract: Alzheimer's disease (AD) is a leading cause of morbidity among old people. Dl-3n-butylphthalide (NBP) is a novel agent and has a potential role on angiogenic activity and a good curative effect against cerebral ischemia. However, the molecular mechanism remains unclear. In order to explore the effect of NBP on the incidence and development of AD, we selected Male Wistar rats for study and separated them into 3 groups: NBP group (rats were intraperitoneal injected with NBP after permanent bilateral occlusion of the common carotid arteries), control group (rats underwent the same aforementioned protocol except for permanent bilateral occlusion of the common carotid arteries and NBP injection) and 2-VO group (rats underwent permanent bilateral occlusion of the common carotid arteries). The MWM task was performed to explore the potentiation of NBP on the cognitive and memory function of rats with AD. Rat cortex and hippocampus tissues were isolated. Immunohistochemistry and western blotting assays were used to determine the protein expression of APP, Aβ, VEGF and TJ claudin-5. Result showed the protein expression of VEGF and TJ claudin-5 decreased significantly in 2-VO group, while the expression of Aβ increased. However, Aβ expression decreased, and VEGF and TJ claudin-5 expression increased in NBP group compared with those in 2-VO group. Our results indicated NBP could affect the process of AD by influencing the tight junctions (TJs) of blood brain barrier (BBB).

Keywords: Alzheimer's disease, blood brain barrier, tight junctions, dl-3n-butylphthalide

Introduction

Alzheimer's disease (AD) represents one of the great unsolved medical needs confronting society during this millennium [1]. It is a neurodegenerative disease characterized by the presence of cerebral amyloid plaque, a highly ordered protein aggregate defined by its insolubility and fibrillar structure [2, 3]. It is a leading cause of morbidity and mortality among the elderly [4]. Slowly but surely, AD patients lose their memory and their cognitive abilities, and even their personalities may change dramatically [5]. Despite considerable work during the past quarter century, no medicines exist that attack the underlying pathophysiology of the disease [1]. Therefore, to explore the pathogenesis of VD and make effective treatment has become a hotspot in research field of medicine.

The development of AD is probably influenced by both genetic and environmental risk factors [6]. Its pathogenesis is complicated. The β-amyloid deposition in senile (diffuse and neuritic) plaques and the decrease of cholinergic neuron are the main pathological change of AD [7, 8]. Amyloid β (Aβ) is a small self-aggregating peptide produced from its precursor protein, amyloid precursor protein (APP), by proteolytic processing at its N- and C-termini by β- and γ-secretase enzymes, respectively [9, 10]. Overproduction of Aβ is appears to be directly neurotoxic [11, 12]. It can be detected at the earliest stages of AD and, in fact, before cognitive dysfunction is detectable [13]. Long time cerebral hypoperfusion can induce a series of pathophysiological changes in brain, and clinical manifestations were mainly progressive learning, memory and other cognitive dysfunction [14, 15]. Chronic cerebral hypoperfusion

(CCH) is commonly happened in elderly and can increase the risk of AD through several biologically plausible pathways, but the relationship between CCH and the development of AD remains uncertain [15, 16]. Rat models with CCH were constructed by permanent bilateral occlusion of the common carotid arteries. It well simulates the pathological process of vascular dementia in clinical, and is a good model for the study of AD [17]. Previous studies show that chronic hypoperfusion can lead to the permeability change of blood brain barrier (BBB), which may be one of the causes that result in the occurrence of vascular dementia [18]. BBB is formed by the endothelial cells that line cerebral microvessels, and has an important role in maintaining a precisely regulated microenvironment for reliable neuronal signaling [19, 20]. Tight junctions (TJ) between endothelial cells of brain capillaries are the most important structural elements of the BBB [21]. Claudin-5 is a major cell adhesion molecule of TJ in BBB [22, 23]. Vascular endothelial growth factor (VEGF), a potent mediator of endothelial proliferation and migration, has an important role also in brain edema formation during hypoxia and ischemia [24].

Dl-3n-butylphthalide (NBP) was extracted from the seeds of Apium graveolens Linn. (Chinese celery) and has a potential angiogenic activity [25, 26]. It is a novel agent for the treatment of stroke, acting to reduce the ischemic injury in brain via multiple mechanisms [27]. It has effect on the expression of VEGF and basic fibroblast growth factor (BFGF) in protein [28]. Mechanism of NBP against cerebral ischemia is extremely complex and the process is constituted by multiple genes, multiple targets and multiple links. It can alleviate encephaledema, improve physiological metabolism of the brain tissue, improve microcirculation in ischemia brain region, inhibit neural apoptosis, resist thrombus formation, inhibit glutamate release and reduce the intracellular calcium concentration [27, 29]. Therefore, we speculate NBP can influence the permeability of BBB by affecting the expression of TJ proteins in BBB, which can reverse the production of amyloids. However, there have no reports about the effect of NBP on TJ proteins of BBB in chronic ischemic pathology.

In this study, we aimed to study whether NBP affects the development and progression of AD by influencing the TJ proteins of BBB. We explored the change of TJ proteins in BBB of rat models with CCH. It may provide new approaches for the diagnosis of AD, which has a very highly clinical significance.

Methods

Experimental groups

Male Wistar rats (approximately 10 weeks old) weighing 250 to 270 g were used for the study and maintained on a 12-hour light/dark cycle with continuous access to food and water. All animals were treated according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal procedures were conducted after gaining the approval of the animal care committee of Shanghai resident standardization training base.

Rats were divided at random into the following 3 groups: (1) the NBP group: rats of this group (n=60) were intraperitoneal injected with NBP (Shijiazhuang Pharmaceutical Group Ouyi Pharma Co., Ltd) at 6 mg/kg per day for 2 weeks after permanent bilateral occlusion of the common carotid arteries; (2) the control group: rats of this group (n=60) underwent the same aforementioned protocol except for permanent bilateral occlusion of the common carotid arteries and NBP injection; (3) the 2-VO group: rats of this group (n=60) underwent permanent bilateral occlusion of the common carotid arteries. The 3 groups were compared after permanent bilateral occlusion of the common carotid arteries for 2 and 4 weeks, respectively.

Morris water maze

The Morris water maze (MWM) is a test of spatial learning for rodents that relies on distal cues to navigate from start locations around the perimeter of an open swimming arena to locate a submerged escape platform [30]. It was described 20 years ago as a device to investigate spatial learning and memory in laboratory rats [31].

One month after operation, the MWM task was performed to evaluate the cognitive function in rats of the 3 groups as described previously with minor modification [32]. Briefly, the MWM consisted of a circular pool, 1.8 m in diameter and 0.6 m in height, filled to a level of 35 cm

with water maintained at a temperature of 25±1°C. The hidden escape platform (diameter: 9 cm) was submerged 2 cm below the surface of the water and was invisible from the water level. Swimming paths were registered by a computerized video imaging analysis system. All rats received four trials per day for four consecutive days with a constant interval of 1 h. The animals were gently placed in water in one of four quadrants, facing the wall of the pool, and the starting quadrant was varied randomly over the trials. Rats were allowed a maximum of 90 s to find the escape platform, where it remained for 30 s. Rats that failed to locate the platform at the end of 90 s were manually guided to the platform. For all training trials, swim speed and escape latency before reaching the platform were measured. All space probe testing consisted of a 60 s trial with the platform removed and conducted immediately after the four-day period. Time spent in the target platform location and the number of target crossings over the previous location of the target platform was recorded.

Immunohistochemistry

The rats were anesthetized with 10% chloral hydrate (350 mg/kg, i.p.), the brain was quickly removed, and the whole hippocampus was carefully dissected. Six sham-operated animals were used as control at 20 weeks after operation. Tissues were snap-frozen in liquid nitrogen and stored at -80°C. The left hippocampus was used for immunohistochemistry and the right hippocampus was used for western blotting. Rat cortex of the 3 groups was also used for immunohistochemistry and western blotting assay.

The tissues were put into a 24-well microtiter plate which consisted of citric acid-sodium citrate buffer (0.01 M, pH 6.0). Then it was heated in microwave with power of 50% for 10 min. Triton-X100-PBS (0.2%) was used to wash the tissues for 5 min. Nonspecific binding sites were blocked with 3% bovine serum albumin in PBS-0.2 Triton-X100 for 30 min and incubated with the polyclonal anti-MAP-2 (Sigma) at a dilution of 1:200 in PBS overnight at 4°C. The tissues were washed three times for 10 min each in PBS at room temperature. The sections were immunostained overnight at 4°C using a rabbit polyclonal antibody against APP at a dilution of 1:500 and 4 μg/ml propidium iodide (PI, Sigma), a rabbit polyclonal antibody against Aβ, a rabbit polyclonal antibody against VEGF and a rabbit polyclonal antibody against TJ claudin-5. The sections were then treated with secondary antibodies. The sections were washed three times in PBS, mounted on glass slides, and coverslipped using Gelvatol. The slides were analyzed on a LSM 510 META laser-scanning confocal microscope (Zeiss).

Wsetern boltting

To further determine the expression changes of APP, Aβ, VEGF and TJ claudin-5, western boltting was used. Frozen tissues were washed thrice with PBS and transferred to buffer containing 25 mM HEPES; 2.5 mM EDTA; 0.1% Triton X-100, 1 mM PMSF, 5 μg/ml leupeptin. The mixture was centrifuged at 3000 r/min for 10 min and the supernatant was stored at 4°C. Total protein concentrations were determined with a UV spectrophotometer using a modified Bradford assay (Beckman Coulter, Fullerton, CA, USA). Equal amounts of protein from each sample (40 Ag) were mixed with 15-20 Al sample buffer and boiled for 5 min Samples were separated by electrophoresis on 7.5-12% polyacrylamide gels. Bands of proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The PVDF membrane was blocked TBS buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl) containing 0.1% Tween-20 (TBST) for 1 h at room temperature and then incubated with antibodies against TJ claudin-5, VEGF, APP and Aβ for 3 h at room temperature. After an additional incubation for 1 h with horseradish peroxidase-conjugated secondary antibodies, the binding of antibodies to the PVDF membrane was and measured by KS 400 image analysis system (Carl Zeiss, Vision, Oberkochen, Germany).

Statistical analysis

Values presented in this study are expressed as mean ± SEM. One-way ANOVA followed by post hoc Fisher protected least significant difference test was used to determine the significance of differences in various indexes among the different groups. P value < 0.05 denoted the presence of a statistically significant difference.

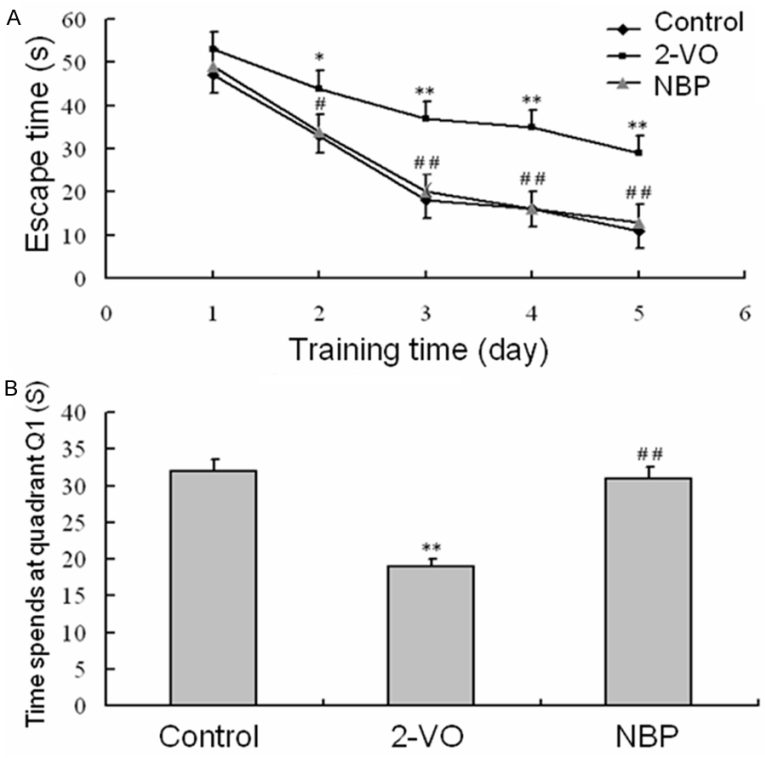

Figure 1. Effects of 2-VO and NBP on Morris water maze performance of Wistar rats. A. Changes in escape latency during the training day. B. Time spent in quadrant Q1 during probe trial (swimming 60 s without platform). All values are mean ± S.E.M. (n=3). *P < 0.05 and **P < 0.01, compared with the control group, escape time or time spends at quadrant Q1 in 2-VO group had significant difference; #P < 0.05 and ##P < 0.01, compared with 2-VO group, escape time or time spends at quadrant Q1 in NBP group had significant difference. 2-VO: bilateral carotid artery occlusion; NBP: Dl-3n-butylphthalide. Q1: quadrant 1, where the platform was placed.

Results

Morris water maze

CCH was induced in Wistar rats by permanent bilateral occlusion of the common carotid arteries (2-VO) as described in the method part. Rats were divided into 3 groups. Rats in the 3 groups were subjected to the MWM. As shown in **Figure 1A**, escape latency decreased significantly during training and there was a significant difference among the 3 groups. On day 5, rats in control group immediately swam toward the platform when placed in the maze, whereas the rats in 2-VO group swam a significantly longer distance before finding the platform. Rats in NBP group swam toward the platform for almost the same time with that in control group. To assess spatial memory more directly, the rats were subject on the day after the end of training to a space probe trial in which the platform was removed, and the rats were allowed 60 s to search. As shown in **Figure 1B**, there was a significant difference in time spent in the target quadrant between rats in control and 2-VO group, 2-VO and NBP group (P < 0.05). The rats in control group spent less swim time in the target platform position. The rats in NBP group also crossed the previous platform position significantly less times as compared to that in 2-VO group (P < 0.05). These results indicated that the rats of CCH have an impairing effect in learning and memory capacity, and NBP can improve the learning and memory capacity.

Protein expression changes by immunohistochemistry

In order to measure the immunoreactivity changes of Aβ, VEGF and TJ claudin-5, immunohistochemistry assay was conducted. The immunostaining of Aβ, VEGF and TJ claudin-5 were assessed using antibodies against Aβ, VEGF and TJ claudin-5, respectively. The results were shown in **Figure 2**. VEGF and TJ claudin-5 expression level was lower in 2-VO group compared to the control group. Aβ expression was significantly higher in 2-VO group compared to the control group, and it decreased in NBP group.

Protein expression of TJ claudin-5, VEGF, and Aβ

To determine the effect of NBP on protein expression of Aβ, VEGF and TJ claudin-5, western blotting was explored. As shown in **Figure 3**, CCH led to significantly decreased protein levels of VEGF and TJ claudin-5 in the hippocampal regions of the model rats compared with the control rats. However, the protein level of them in rats in NBP group was almost the same with the control rats. At the same time, CCH led

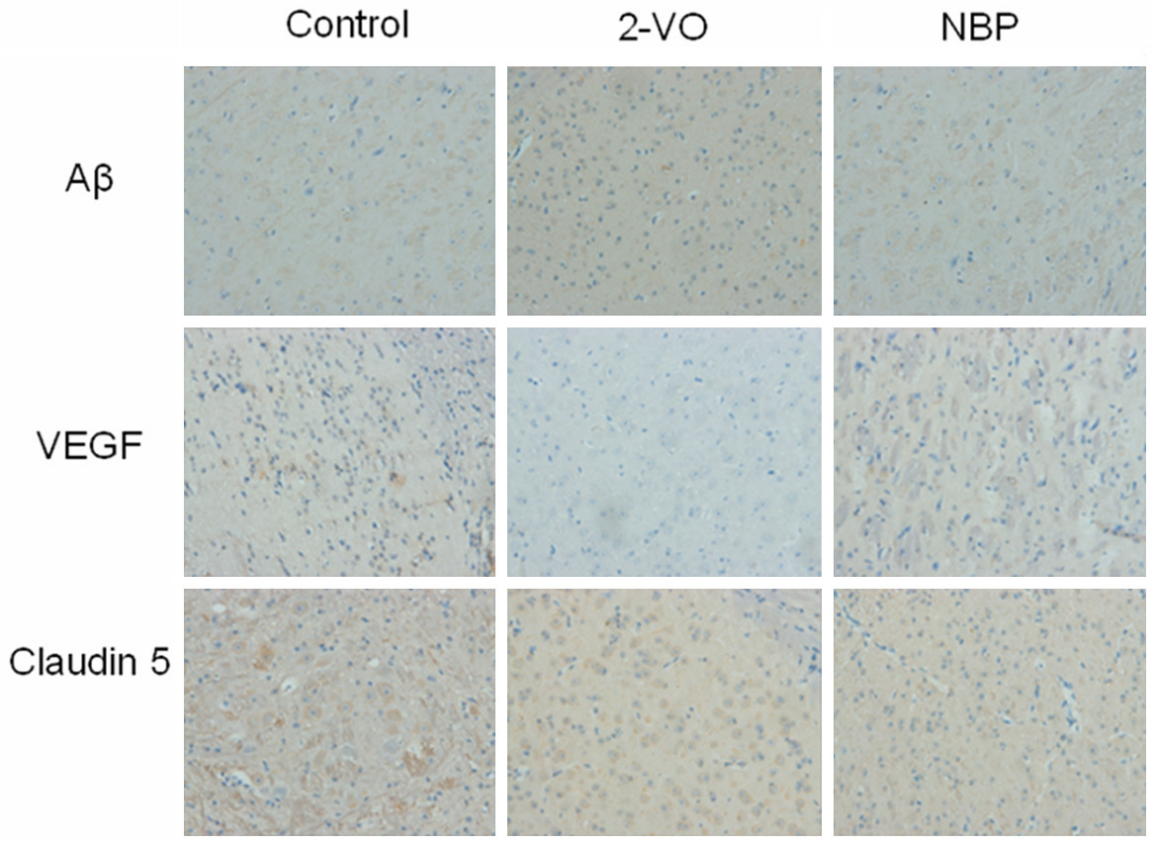

Figure 2. Immunostaining of Aβ, VEGF and TJ claudin-5 in NBP, 2-VO and control group by immunohistochemistry.

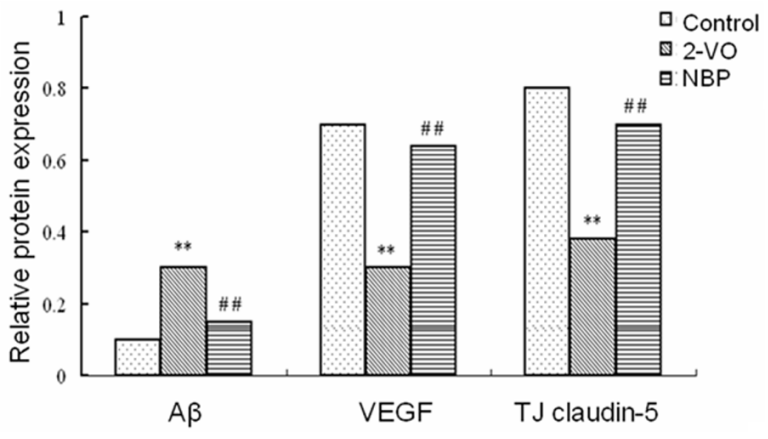

Figure 3. Analysis on the effect of NBP on the protein levels of Aβ, VEGF and TJ claudin-5 by Western blotting. *P < 0.05 and **P < 0.01, compared with the control group, protein expression values of Aβ, VEGF and TJ claudin-5 in 2-VO group had significant difference; #P < 0.05 and ##P < 0.01, compared with 2-VO group, protein expression values of Aβ, VEGF and TJ claudin-5 in NBP group had significant difference.

to significant increase in protein level of Aβ in the hippocampal region of rats in 2-VO group compared with the control group. After rats were treated with NBP, the protein expression level of Aβ decreased to the similar level in control rats.

Discussion

AD is the sixth leading cause of all deaths in the United States and is the fifth leading cause of death in Americans aged 65 years. It characteristically produces a remarkably pure impairment of memory [3, 33]. Although other major causes of death have been on the decrease, deaths because of AD have been rising dramatically [34]. The pathophysiological process of Alzheimer's disease (AD) is thought to begin many years before the diagnosis of AD dementia [35]. Rapid progress in deciphering the biological mechanism of AD has

arisen from the application of molecular and cell biology to this complex disorder of the limbic and association cortices [36]. The recent advances in use of reliable biomarkers of AD that provide in-vivo evidence of the disease has stimulated the development of new research criteria [37]. AD is characterized by neurofibrillary tangles, oxidative stress, progressive neuronal deficits, increased levels of Aβ peptides, and their deposition in neuritic plaques and cerebral blood vessels [38]. Much evidence indicates that abnormal processing and extracellular deposition of Aβ peptides is central to the pathogenesis of Alzheimer's disease [39, 40]. Aβ peptides are derived from the APP by sequential proteolysis, catalysed by the aspartyl protease BACE, followed by presenilin-dependent g-secretase cleavage [41]. Inhibition of neocortical β-amyloid (Aβ) accumulation may be essential in an effective therapeutic intervention for Alzheimer's disease (AD) [7]. Tarkowski et al reported patterns of local release of vascular endothelial growth factor

(VEGF) have a pivotal role in the process of AD. The expression of VEGF represents one potential mechanism whereby vascular and AD pathologies are related [42]. Recent evidence indicates that VEGF facilitates memory and learning through stimulating angiogenesis and neurogenesis in the rat hippocampal dendate gyrus [43]. Cerebrovascular dysfunction contributes to cognitive decline and neurodegeneration in AD [44]. As VEGF determines important neurotrophic and neuroprotective actions, we postulated serum VEGF levels could be abnormally low in patients with AD [45]. The study by Solerte et al showed the decreased VEGF secretion by peripheral immune cells of AD subjects could have a negative role for brain angiogenesis, neuroprotection and for brain microvascular permeability to nutrients, increasing brain frailty towards hypoxic injuries [43]. Amyloid-β could increase VEGF mRNA and protein levels in ARPE-19 cells [46]. They have been described to inhibit angiogenesis both in vitro and in vivo, and deregulation of angiogenic factors. The blood-brain barrier (BBB) is formed by the endothelial cells of cerebral microvessels, providing a dynamic interface between the peripheral circulation and the central nervous system [47]. It is the regulated interface between the peripheral circulation and the central nervous system [20]. The tight junctions (TJs) between the endothelial cells serve to restrict blood-borne substances from entering the brain [48]. They regulate the passage of ions and molecules through the paracellular pathway in epithelial and endothelial cells [49]. The rapid changes in the TJ protein phosphorylation state may serve as a mechanism by which VEGF regulates endothelial barrier permeability [50]. VEGF-induced changes in tight junction protein phosphorylation state may be a fundamental mechanism by which vascular permeability is regulated [51]. In brain, claudins-1 and -5, together with occludin, have been described to be present in endothelial TJs forming the BBB [52]. Claudin-5 is a major cell adhesion molecule of tight junctions in brain endothelial cells [22]. In BBB breakdown, the expression of claudin-5 decreased [53].

Dl-3n-Butylphthalide (NBP), extracted from Apium graveolens Linn, has displayed a broad spectrum of neuroprotective properties [54]. Appropriate restoration of blood flow via angiogenesis is critical for the recovery from ischemic stroke and the treatment with NBP increases the number of local potent cerebral microvessels [29, 55]. NBP could reduce ischemic brain injury and inhibit further formation of cerebral infarction by improving blood flow in the occlusive lesions [25, 56]. However, the mechanism about how NBP play a role in ischemic brain injury remains unclear. Therefore, in this study, Male Wistar rats were selected for study and separated into 3 groups: NBP group (rats were intraperitoneal injected with NBP after permanent bilateral occlusion of the common carotid arteries), control group (rats underwent the same aforementioned protocol except for permanent bilateral occlusion of the common carotid arteries) and 2-VO group (rats underwent permanent bilateral occlusion of the common carotid arteries). The MWM task was performed to evaluate the cognitive and memory function. Rat cortex and hippocampus tissues were selected for immunohistochemistry and western blotting assay to test the immunoreactivity and protein expression of TJ claudin-5, VEGF and Aβ. Resluts showed immunoreactivity and protein expression of VEGF and TJ claudin-5 significantly decreased in rats underwent permanent bilateral occlusion of the common carotid arteries compared the control rats. However, they are extremely higher in rats injected with NBP after permanent bilateral

occlusion of the common carotid arteries. The immunoreactivity and protein expression of Aβ increased markedly in rats underwent permanent bilateral occlusion of the common carotid arteries compared the control rats and decreased almost to the same levels of the control rats after injected with NBP. We concluded in rats underwent permanent bilateral occlusion of the common carotid arteries, the abnormal metabolism of APP resulted in the anomalous deposition of Aβ, and the decrease of Aβ activity inhibit the VEGF mRNA and protein levels. VEGF was reported has a role in regulating TJ protein expression and so the expression of TJ claudin-5 decreased. TJs between endothelial cells of brain capillaries are the most important structural elements of the BBB [21]. BBB is essential for maintaining brain homeostasis and low permeability [57]. It plays a critical role in regulating cell trafficking through the central nervous system (CNS) due to several unique anatomical features, including the presence of interendothelial tight junctions that form impermeable seals between the cells [58]. Abnormalities in the human BBB contribute to the onset and progression of dementia AD type [59, 60]. Therefore, we concluded NBP could be used to treat AD patients by influencing the TJs of BBB.

In conclusion, we explored the molecular mechanism how NBP affect the incidence and development of AD, which would help us find new ways for the diagnosis and treatment of AD. It will have a very high clinical significance.

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Disclosure of conflict of interest

None.

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