

## Original Article

# The therapeutic effect and mechanism of dl-3-n-butylphthalide in Alzheimer's disease

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Received May 24, 2018; Accepted September 12, 2019; Epub October 15, 2020; Published October 30, 2020

**Abstract:** Objective: To investigate the efficacy and mechanism of dl-3-n-butylphthalide in the treatment of Alzheimer's disease. Methods: Ninety-six patients with Alzheimer's disease were randomly divided into a conventional treatment group and a dl-3-n-butylphthalide group. Cognitive function evaluations were performed before and after the treatment in the two groups using the Alzheimer's Disease Assessment Scale-Cognitive section, and the patients' serum  $\beta$ -amyloid protein, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  levels were quantified using Elisa. Thirty APP/PS1 transgenic mice were randomized into the control group and the dl-3-n-butylphthalide group, and expressions of BDNF and TrkB in the mice's brain tissues were determined using Western blot. Results: At the end of the treatment period, the cognitive function scores and the serum  $\beta$ -amyloid protein levels of the patients in the dl-3-n-butylphthalide group were significantly lower compared with the conventional treatment group ( $t=5.210$ ,  $P=0.000$ ;  $t=4.816$ ,  $P=0.000$ ). The serum IL-1 $\beta$  and TNF- $\alpha$  levels were significantly reduced, but there was no significant difference in IL-6 ( $t=2.441$ ,  $P=0.017$ ;  $t=2.271$ ,  $P=0.026$ ;  $t=0.569$ ,  $P=0.571$ ). Compared with the control group, the BDNF and TrkB expression levels in the mice's brain tissues in the dl-3-n-butylphthalide group were significantly increased ( $t=18.070$ ,  $P=0.000$ ;  $t=12.690$ ,  $P=0.000$ ). Conclusion: dl-3-n-butylphthalide activates the BDNF-TrkB signaling pathway and therefore plays a therapeutic role in Alzheimer's disease.

**Keywords:** Alzheimer's disease, dl-3-n-butylphthalide, brain-derived neurotrophic factor, tyrosine receptor kinase B

## Introduction

Alzheimer's disease (AD), a major cause of dementia, is a common degenerative disease of the nervous system with progressive cognitive impairment as the main clinical manifestation and causing great economic and psychological burdens to the patients and their families [1-3]. It was reported that in 2016, there were 46.8 million AD patients in the world, making it the third leading cause of death in the elderly after cardiovascular and cerebrovascular diseases and malignant tumors. The global aging trend further aggravates the severity of AD [4]. Hence, it is of great clinical significance to actively conduct research on the treatment and mechanism of Alzheimer's disease. The pathological mechanisms of Alzheimer's disease have not been clearly elucidated, such as the abnormal

deposition of  $\beta$ -amyloid proteins (A $\beta$ ), the formation of neurofibrillary tangles by the hyperphosphorylation of tau proteins, cholinergic neuron injury, neuroinflammation, oxidative stress injury and so on. Thus, according to its pathological mechanisms, a series of medications are applied to treat Alzheimer's disease, such as cholinesterase inhibitors, N-methyl-D-aspartate receptor antagonists, and antioxidants [5-9]. Dl-3-n-butylphthalide has been widely used to treat nervous system diseases, such as ischemic brain injury, radiation brain injury, traumatic brain injury, epilepsy, acute cerebral infarction, and cerebral hemorrhage, due to its biological effects of anti-inflammation, anti-oxidation, and anti-apoptosis, and its vascular endothelial cell protection [10-12]. Zhao et al. found that dl-3-n-butylphthalide can improve motor function and reduce the degree

of post-traumatic depression in mice with traumatic brain injuries, and these therapeutic effects might occur because dl-3-n-butylphthalide inhibits the trauma-induced activation of the NF- $\kappa$ B signaling pathway, resulting in the reduction of the expressions of the inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$  and the increase of the expressions of the protective factors such as brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor, endothelium-derived nitric oxide synthase, and matrix metalloproteinase 9 [13]. Therefore, 96 patients with Alzheimer's disease were recruited for this study to observe whether dl-3-n-butylphthalide can improve the behavioral manifestations of patients with Alzheimer's disease, and to explore the effect of dl-3-n-butylphthalide on the  $\beta$ -amyloid protein and the inflammatory factors. To further explore the mechanism of dl-3-n-butylphthalide, we investigated BDNF and tyrosine receptor kinase B (TrkB) using a mouse model of Alzheimer's disease.

### Materials and methods

#### General data

Ninety-six patients with Alzheimer's disease admitted to our hospital from January 2014 to December 2017 were randomly assigned to two groups: the conventional treatment group and the dl-3-n-butylphthalide group. The patients in the conventional treatment group received oral donepezil hydrochloride (qd, 10 mg/time) and oral dl-3-n-butylphthalide (tid, 0.2 g/time) was added to the conventional treatment in the dl-3-n-butylphthalide group. The patients in both groups were treated for 6 months [14]. Informed consents were obtained, and the relevant ethical regulations were followed in all aspects of this experiment.

There were 48 patients ranging in age from 60 to 81 (68.17 $\pm$ 6.42) years old in the conventional treatment group, including 29 males and 19 females; the duration of the disease was from 2 to 12 (6.15 $\pm$ 1.18) years. There were 48 patients ranging in age from 61 to 80 (68.02 $\pm$ 6.80) years old in the dl-3-n-butylphthalide group, including 31 males and 17 females; the duration of the disease was from 3 to 12 (6.22 $\pm$ 1.41) years. There were no significant differences in the general clinical data between the two groups ( $P > 0.05$ ).

#### Inclusion and exclusion criteria

Inclusion criteria: 1) Patients who were diagnosed with dementia through clinical examinations and rating scales. 2) Patients with progressive severity. Exclusion criteria: 1) Patients with cognitive impairments caused by trauma or other causes. 2) Patients who had severe hepatic or renal dysfunction. 3) Patients suffering from malignancies, rheumatic immunologic diseases, infectious diseases, etc.

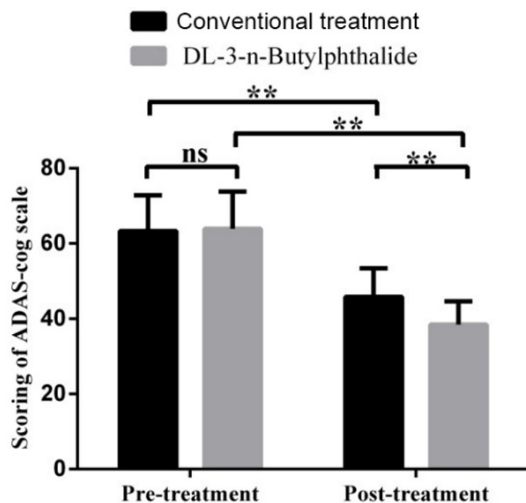
#### Observation indexes

Cognitive function evaluations were performed before and after the treatment in both groups using the Alzheimer's Disease Assessment Scale-Cognitive section (ADAS-cog). The ADAS-cog scale included 12 items: word recall, naming objects and fingers, commands, constructional praxis, ideational praxis, orientation, word recognition, remembering test instructions, spoken language ability, comprehension, word-finding difficulty, and attention. The total scores ranged from 0-75, and the higher the score, the more serious the cognitive impairment.

Blood samples were collected from all patients before the treatment and at the end of the treatment. The serum  $\beta$ -amyloid protein, IL-1 $\beta$ , IL-6, and TNF- $\alpha$  levels were quantified using Elisa kits (Neobioscience). The kits were calibrated for 30 minutes at room temperature, and standard substances and samples were diluted in proportion. 50  $\mu$ L of standard substances and samples were added to each well and incubated at 37 $^{\circ}$ C for 30 minutes. Then the wells were washed and patted dry with absorbent paper 5 times. Next, 50  $\mu$ L enzyme-labeled reagent was added to each well, and then the wells were incubated again at 37 $^{\circ}$ C for 30 minutes. After the incubation, the wells were washed 5 times. Subsequently, 100  $\mu$ L color-substrate solutions was added to each well, and they were incubated at 37 $^{\circ}$ C in the dark for 15 minutes. The optical density was measured at 450 nm. The concentration of the target protein in the sample was calculated according to the standard curve.

#### A mouse model of Alzheimer's disease and treatments

Thirty APP/PS1 transgenic mice, purchased from Beijing Vital River Laboratory Animal



**Figure 1.** Comparison of the patients' ADAS-cog scores before and after the treatment. The conventional treatment group was orally administered donepezil hydrochloride, and the dl-3-n-butylphthalide group was orally administered donepezil hydrochloride and dl-3-n-butylphthalide for 6 months. <sup>ns</sup>P>0.05; <sup>\*\*</sup>P<0.01.

Technology Co. Ltd., China, were randomly divided into two groups, with 15 mice in each group: the control group and the dl-3-n-butylphthalide group. The dl-3-n-butylphthalide was dissolved in ddH<sub>2</sub>O at a concentration of 25 µg/mL, which was the mice's drinking water in the dl-3-n-butylphthalide group for 6 months. The mice in the control group received ddH<sub>2</sub>O as their drinking water, and the remaining treatments were the same as those in the dl-3-n-butylphthalide group.

#### *Measuring the BDNF and TrkB expressions in the mice's brain tissues using Western blot*

The mice's brains were ground on the ice at a low temperature, and a proteinase inhibitor (1:10, Sigma) was added to inhibit protein degradation. The total protein concentration was measured using BCA Protein Quantitative Kits (TransGen Biotech). The same amount of total protein was added for the SDS-PAGE, transmembrane, and blocking. After incubation overnight at 4°C with 1:500 anti-BDNF antibodies, 1:500 anti-TrkB antibodies, and 1:1000 anti-GAPDH antibodies (Abcam), the membrane was washed and incubated for 1 hour at room temperature with HRP-labeled secondary antibodies (Boster). Images were obtained after the color development.

#### *Statistical analysis*

All the statistical analyses were performed using SPSS 13.0 software, and the measurement data were expressed as the mean ± standard deviation. Student's t-tests were used for the comparisons between two groups, and chi-square tests were used for the comparisons between enumeration data. There was no statistical difference when P>0.05, and it was expressed as ns. There was a statistical difference when P<0.05, and it was presented as \*. There was a significant statistical difference when P<0.01, and it was represented as \*\*.

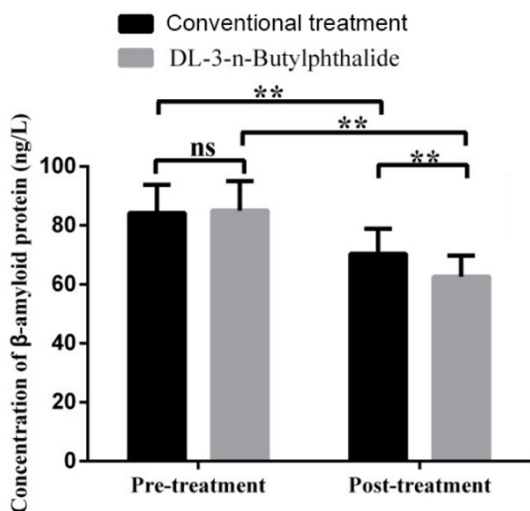
#### **Results**

##### *Comparison of the patients' ADAS-cog scores before and after the treatment*

To compare the patients' cognitive functions in the two groups before and after the treatment, an ADAS-cog evaluation was performed on each patient. The results in **Figure 1** show that there was no statistical difference in the scores between the two groups before the treatment (t=0.307, P=0.760). The patients' scores in the conventional treatment group and in the dl-3-n-butylphthalide group were significantly decreased after the treatment compared with the scores before the treatment (t=9.923, P=0.000; t=15.050, P=0.000). The patients' scores in the dl-3-n-butylphthalide group at the end of treatment were significantly lower than the scores in the conventional treatment group, suggesting that the addition of dl-3-n-butylphthalide can improve cognitive functions in patients with Alzheimer's disease (t=5.210, P=0.000).

##### *Comparison of the patients' serum β-amyloid protein levels before and after the treatment*

The results shown in **Figure 2** suggest that the serum β-amyloid protein levels were not significantly different in the two groups before the treatment (t=0.421, P=0.675). Compared with before the treatment, the serum β-amyloid protein levels in the conventional treatment group and the dl-3-n-butylphthalide group decreased significantly after the treatment (t=7.512, P=0.000; t=12.660, P=0.000). Compared with the conventional treatment group, the serum β-amyloid protein levels in the dl-3-n-butylphthalide group were significantly lower at the end



**Figure 2.** Comparison of the patients' serum  $\beta$ -amyloid protein levels before and after the treatment. The conventional treatment group was orally administered donepezil hydrochloride, and the dl-3-n-butylphthalide group was orally administered donepezil hydrochloride and DL-3-n-Butylphthalide for 6 months. <sup>ns</sup>P>0.05; <sup>\*\*</sup>P<0.01.

of the treatment, illustrating that the addition of dl-3-n-butylphthalide reduced the serum  $\beta$ -amyloid protein levels in the patients with Alzheimer's disease ( $t=4.816$ ,  $P=0.000$ ).

#### Comparison of the patients' serum inflammatory factor levels before and after the treatment

A quantitative determination of the serum IL-1 $\beta$ , IL-6, and TNF- $\alpha$  levels in the two groups before and after the treatment was conducted using Elisa kits. The results in **Figure 3** show that there was no significant difference in the serum IL-1, IL-6, and TNF- $\alpha$  levels in the two groups before the treatment ( $t=1.148$ ,  $P=0.254$ ;  $t=0.605$ ,  $P=0.547$ ;  $t=0.569$ ,  $P=0.571$ ). Compared with the conventional treatment group, the serum IL-1 $\beta$ , and TNF- $\alpha$  levels were significantly lower in the dl-3-n-butylphthalide group at the end of the treatment ( $t=2.441$ ,  $P=0.017$ ;  $t=2.271$ ,  $P=0.026$ ), and there was no statistical difference in the IL-6 levels ( $t=0.569$ ,  $P=0.571$ ), demonstrating that the addition of dl-3-n-butylphthalide was able to alleviate the Alzheimer's disease patients' inflammatory levels.

#### Comparison of the BDNF expressions in the brain tissues in the two groups of mice

To further explore the therapeutic mechanism of dl-3-n-butylphthalide, a mouse model of

Alzheimer's disease was formed, and the BDNF expressions in the mice's brain tissues were determined using Western blot. The results in **Figure 4** demonstrate that the expressions of BDNF in the brain tissues of the mice treated with dl-3-n-butylphthalide increased significantly compared with the control group ( $t=18.070$ ,  $P=0.000$ ), illustrating that dl-3-n-butylphthalide promotes the expression of BDNF in the brain tissues of mice with Alzheimer's disease.

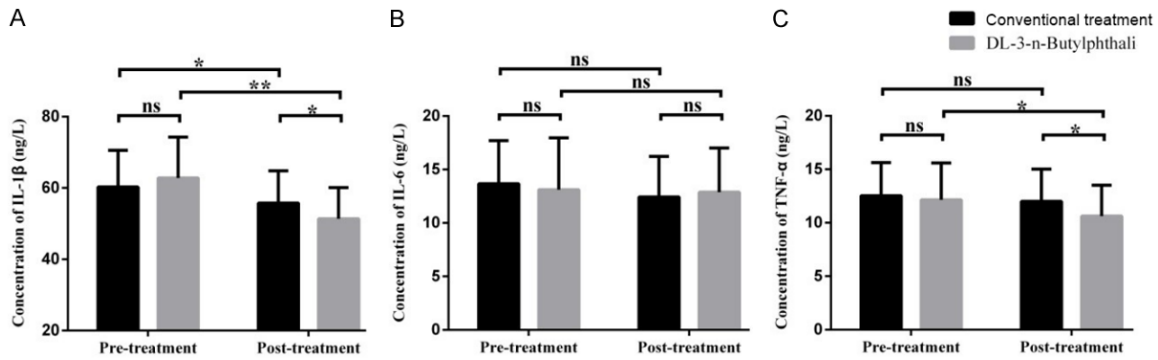
#### Comparison of the TrkB expressions in the mice's brain tissues

To further study the precise mechanism of dl-3-n-butylphthalide in promoting BDNF expression, the expression of TrkB, an important regulator of the BDNF signaling pathway, was determined using Western blot. The results in **Figure 5** suggest that the expressions of TrkB in the brain tissues of the dl-3-n-butylphthalide group was significantly increased compared with the control group ( $t=12.690$ ,  $P=0.000$ ), indicating that dl-3-n-butylphthalide can promote the expression of TrkB in the brain tissues of Alzheimer's mice and plays an indispensable role in the activation of the BDNF-TrkB signaling pathway.

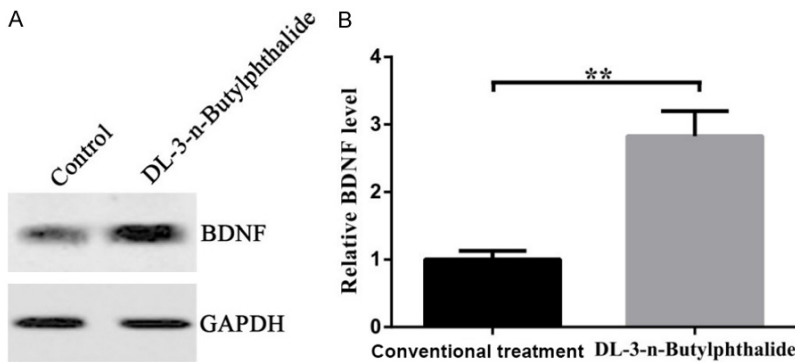
## Discussion

In the study of cerebral ischemia, it has been found that dl-3-n-butylphthalide can limit the area of cerebral infarction by reducing oxidative damage and neuronal apoptosis, inhibiting inflammation and other biological effects. Therefore, dl-3-n-butylphthalide was approved by the China Food and Drug Administration in 2002 for use in stroke patients [15]. In addition, dl-3-n-butylphthalide plays a protective role in central nervous system diseases, such as parkinsonism, cerebral ischemia-reperfusion injury, epilepsy, and traumatic brain injury [10, 12, 13, 16]. However, whether dl-3-n-butylphthalide plays a therapeutic role in Alzheimer's disease has not been reported. Hence, the role of dl-3-n-butylphthalide in Alzheimer's disease was examined in this study.

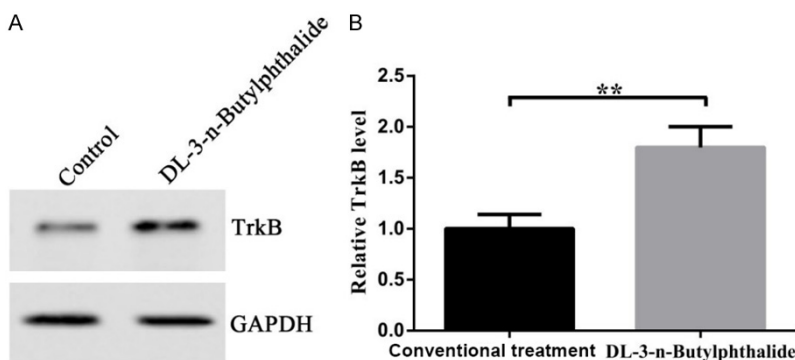
In this study, the results showed that the addition of dl-3-n-butylphthalide can improve patients' cognitive functions and reduce their serum  $\beta$ -amyloid protein levels, illustrating that dl-3-n-butylphthalide can alleviate the condition of patients with Alzheimer's disease.



**Figure 3.** Comparison of the patients' serum inflammatory factor levels before and after the treatment. A: IL-1 $\beta$ ; B: IL-6; C: TNF- $\alpha$ . The conventional treatment group was orally administered donepezil hydrochloride, and the dl-3-n-butylphthalide group was orally administered donepezil hydrochloride and dl-3-n-butylphthalide for 6 months. <sup>ns</sup>P>0.05; \*P<0.05; \*\*P<0.01.



**Figure 4.** Comparison of the BDNF expressions in the mice's brain tissues. A: Protein bands using Western blot; B: Western blot statistical chart. The control group was given normal drinking water, and the dl-3-n-butylphthalide group was given drinking water containing 25  $\mu$ g/mL dl-3-n-butylphthalide. \*\*P<0.01.



**Figure 5.** Comparison of TrkB expressions in the mice's brain tissues. A: Protein bands using Western blot; B: Statistical chart. The control group was given normal drinking water, and the dl-3-n-butylphthalide group was given drinking water containing 25  $\mu$ g/mL dl-3-n-butylphthalide. After treatment for 6 months, the brain tissues were removed for the extraction of proteins, and GAPDH was used as an internal control. \*\*P<0.01.

Alzheimer's disease, which might be related to the inhibition of the activation of neuroglial cells such as microglial cells and astrocytes by dl-3-n-butylphthalide [17, 18]. Kamphuis et al. found that a great number of activated microglia were distributed around the  $\beta$ -amyloid protein, which secretes IL-1 $\beta$ , IL-6, TNF- $\alpha$  and other inflammatory factors, thereby aggravating the degeneration of the central nervous system [19].

BDNF plays a significantly protective role in Alzheimer's disease. The expressions of BDNF in transgenic mice with Alzheimer's disease are significantly decreased, and the treatment of exogenous BDNF can alleviate the pathological progress of Alzheimer's disease [20]. Therefore, in order to further study the therapeutic effect of dl-3-n-butylphthalide, the BDNF-TrkB signaling pathway of Alzheimer's disease transgenic mouse model was explored. We found that dl-

Moreover, dl-3-n-butylphthalide reduces the serum IL-1 $\beta$  and TNF- $\alpha$  levels in patients with

3-n-butylphthalide can promote the expressions of BDNF and its key downstream mole-

cule, TrkB, suggesting that the therapeutic mechanism of dl-3-n-butylphthalide might be closely related to the activation of the BDNF-TrkB signaling pathway.

In conclusion, we studied the therapeutic effects and specific mechanisms of dl-3-n-butylphthalide through clinical experiments and animal models and found that dl-3-n-butylphthalide can activate the BDNF-TrkB signaling pathway, thus playing a therapeutic role in Alzheimer's disease. However, there are still many shortcomings in our study. We will explore the treatment mechanism of dl-3-n-butylphthalide in depth by examining the following three questions: 1) Does dl-3-n-butylphthalide play an anti-inflammatory role in Alzheimer's disease by inhibiting the activation of neuroglial cells such as microglia cells and astrocytes; 2) Does dl-3-n-butylphthalide promote the expressions of other neurotrophic factors, such as vascular endothelial growth factor, endothelium-derived nitric oxide synthase, and matrix metalloproteinase-9?; 3) Is the therapeutic effect of dl-3-n-butylphthalide on Alzheimer's disease related to the induction of stem cell migration and repair? Some research has found that dl-3-n-butylphthalide can induce the migration of endothelial progenitor cells and repair damaged brain tissues in ischemic brain injury [11].

#### Acknowledgements

This work was supported by the Public Projects of Zhejiang Provincial Science and Technology Department (2017C33027) and Medical Health Science and Technology Project of Zhejiang Provincial Health Commission (Grant No. 2013RCA036).

#### Disclosure of conflict of interest

None.

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