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**Original Research Article**

# *Epimedium brevicornu Maxim* extract shows protective activity against Alzheimer's disease in mice

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## Abstract

***Purpose:*** *To investigate the protective effect of Epimedium brevicornu Maxim extract (EBME) against Alzheimer's disease in 3xTg-AD mice.*
***Methods:*** *The cognitive function of 3xTg-AD mice was assessed using Morris water maze test. The levels of amyloid beta deposits and NeuN in the mouse hippocampus were evaluated by immunohistochemistry. Brain neurotrophic-derived factor (BDNF) and tyrosine kinase B (TrkB) expressions were examined by western blot analysis.*
***Results:*** *EBME treatment significantly ameliorated learning and memory deficits in AD mice, as shown by the increased time spent in the target zone during probe tests. Compared with the 3xTg-AD mice (8.4 ± 1.1 s), the escape latency in animals treated with 600 mg/kg EBME (21.5 ± 1.1 s) was significantly increased ($p < 0.01$). In addition, EBME significantly decreased Aβ deposits, increased NeuN-positive cells, and upregulated the expressions of BDNF (1.5 ± 0.2, $p < 0.05$) and TrkB (1.6 ± 0.2, $p < 0.05$) in the 3xTg AD mice.*
***Conclusion:*** *EBME treatment may be a useful therapeutic strategy for managing memory impairment.*

***Keywords:*** *Epimedium brevicornu, Alzheimer's disease, Memory impairment, NeuN-positive cells, Amyloid beta deposits*



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## INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia in the elderly and accounts for between 50 and 75 % of all cases. By 2030, it is estimated that more than 65 million people will be living with dementia, with projections almost doubling every 20 years. Alzheimer's disease is a progressive neurodegenerative disorder characterized, at least in part, by abnormal accumulation of β-amyloid peptide (Aβ) in the brain [1]. The accumulated Aβ is believed to play an important role in the pathogenesis of AD [2]. Thus, Aβ continues to be an important target for prevention and treatment of AD [3].

Alzheimer's disease is the most common form of dementia relating to memory and cognitive decline. Alzheimer's disease is a progressive neurodegenerative disease in which dementia symptoms gradually worsen over a number of years [4]. Classical biochemical hallmarks of AD

include the accumulation of amyloid beta (Aβ) peptide oligomers and soluble hyperphosphorylated tau [5]. Brain-derived neurotrophic factor (BDNF) is a small dimeric protein, and BDNF acts through high affinity binding with its receptor, tyrosine kinase B (TrkB). BDNF modulates neuronal growth and survival, and BDNF is implicated in learning and memory processes; therefore, dysfunction in BDNF is accompanied by cognitive deficits. In particular, BDNF is involved in the AD-related decline of neurogenesis, and the nerve growth factor level also decreased in the prosess of AD [6].

Medical treatment for Alzheimer's disease patients is placing an increasing burden on physicians and families every year. Clinically, there are a variety of drugs available for AD, such as cholinesterase inhibitors, glutamate receptor antagonists, and free radical scavengers. However, these drugs do not closely target the pathogenesis of the disease and they have numerous side effects [7]. Therefore, it is important to elucidate the mechanism of AD pathophysiology and find a new treatment drug.

*Epimedium brevicornu Maxim,* has been used in China in the treatment of various disorders, including stress-induced physiological changes, inflammation, hypertension, and cancer. *Epimedium brevicornu Maxim* extract has been reported to have anti-oxidant [8], immunomodulatory [9], and anti-mutagenic activities [10]. In this study, investigation was carried out to evaluate the neuroprotective effects of EBME on learning and memory deficits in a triple-transgenic mouse model of Alzheimer's disease (3xTg-AD), which expresses $APP_{Swe}$, $PS1_{M146V}$, and $tau_{P301L}$ [11].

## EXPERIMENTAL

### Plant material

The plant material, *Epimedium brevicornu Maxim* were collected from Guilin City, Guangxi Province in China in May 2018. Taxonomic identification of the plant was performed by Professor Yonghe Li of Shandong University, China. A voucher specimen (no. EBME 201805008) was deposited in the herbarium of College of Pharmacy, Shandong University, China for future reference.

The herbal samples *Epimedium brevicornu Maxim* was dried in an oven. *Epimedium brevicornu Maxim* extract (EBME) was obtained by steeping the dried *Epimedium brevicornu Maxim* in water at 60 ℃ three times, each for one hour before first drying in an oven and then freeze-drying the EBME thus obtained. One gram powder was equivalent to about 1.6 g crude samples. The yield was 62.33 %.

### Animals

Alzheimer's disease (3xTg-AD) mice carrying a mutant APP (KM670/671NL), a human mutant PS1 (M146V) knock-in, and tau (P301L) transgenes (B6; 129-*Psen1*$^{tm1Mpm}$ Tg (APPSwe, tauP301L) 1Lfa/J) were purchased from the animal research institute, Nanjing University (Nanjing, China). The non-transgenic littermates were used as wild type (WT) controls. All animals were kept in a pathogen-free environment on a 12 h light/dark cycle and had free access to feed and water *ad libitum*.

### Animal groups

The mice were randomly divided into four groups as follows: (1) saline-treated WT group (WT, n = 8); (2) saline-treated 3xTg group (3xTg, n = 8); (3) 300 mg/kg EBME-treated 3xTg group (3xTgþ + EBME 300, n = 8) and (4) 600 mg/kg EBME-treated 3xTg group (3xTgþ + EBME 600, n = 8). Starting at 3 months of age, mice were given PBS and EBME once a week for 3 months until they were 6 months old. The drugs were dissolved in water, and administered using a 5-ml syringe with a 2-cm long gavage needle from the mouth to the mouth once daily for 3 weeks. The animal experiment was approved by the Animal Care and Use Committee of Yantai Yeda Hospital (approval ref no. 20130802) and was carried out in compliance with Directive 2010/63/EU on the handling of animals used for scientific purposes [12].

### Water maze test

A modified version of the water maze procedure described by Morris was used to test each mouse cognitive function [13]. The water maze was a circular pool 0.9 m in diameter and constructed of fiberglass. Water in the pool was maintained at 22 ± 2 ℃ and mixed with 1 kg of powdered skim milk to make it opaque. During testing in the water maze, a platform (6 cm in diameter) was fixed 1 cm below the surface of the water at identical location within the pool. The pool was surrounded by differing extra-maze cues. All mice were subjected to four trials per day at intervals of 15 min for four consecutive days. The proportion of time spent searching for the platform in the training quadrant, i.e., the previous location of the platform, was used as a measure of memory retention.

### Western blotting

At the end of the experiment, the mice were decapitated and the brains were rapidly removed and placed on ice. The hippocampus was quickly dissected by a scalpel and stored at -80 $^{o}$C fridge until use. The hippocampal tissue was homogenized in PRO-PREPTM Protein Extraction Solution (Shanghai Shengong, Shanghai, China). The homogenates were subsequently centrifuged at 12,000 g for 10 min at 4 $^{o}$C, and the supernatants were collected for protein concentration determination using a protein assay (Bio-Rad, Hercules, CA, USA).

Protein samples (30 μg) were separated on a sodium dodecyl sulfate-polyacryl-amide gel and transferred onto a nitrocellulose membrane. The membranes were incubated with 5 % skim milk in Tris-buffered saline containing 0.1 % Tween-20 and then incubated overnight at 4 $^{o}$C with the following primary antibodies: mouse β- actin antibody (1 : 1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit BDNF antibody (1 : 500; Santa Cruz Biotechnology), and rabbit TrkB antibody (1 : 1000; Santa Cruz Biotechnology). Subsequently, the membranes were incubated for 1 h with secondary antibodies (1 : 2000; Cell Signaling), and detection was performed using the enhanced chemiluminescence (ECL) detection kit.

### Densitometry analysis

Coronal sections of the hippocampus were examined from the rostral anteroposterior (- 2.1 mm) to the anteroposterior (- 4.5 mm) direction, as defined by the bregma of the brain atlas. Images were obtained at 10 × magnification using IMAGE PRO PLUS System (version 4.0; Media Cybernetics, Silver Spring, MD, USA) on a computer attached to a light microscope (Zeiss Axioskop, Oberkochen,
Germany), which interfaced with a charge coupled device video camera (Kodak Mega Plus model 1.4 I). To determine the density of the Aβ-immunoreactive staining in the hippocampus, a square frame of 500 × 500 μm$^2$ was placed in the dorsal part of the hippocampus. A second square frame of 200 × 200 μm$^2$ was placed in the corpus callosum to measure the background. As previously described, variations in background illumination were controlled by subtracting the average background density of the corpus callosum from the average density of the hippocampus in each section analyzed [14].

### Statistical analysis

Data are presented as mean ± standard deviation (SD) and were analyzed by one-way ANOVA followed by Tukey's multiple comparison using SPSS 16.0 software for Windows. Differences were considered statistically significant at $p < 0.05$.

## RESULTS

### EBME reversed spatial learning deficits at 6 months of age in 3xTgAD mice

To asess spatial memory, the performance of animals in the probe trial was compared to the time animals spent swimming to the platform. All animals were studied by a retention test that involved removing the platform from the pool on the fourth trial day. As seen in Table 1, the escape latency in animals treated with 600 mg/kg EBME was significantly increased as compared to the 3xTg-AD mice. 3xTg-AD mice displayed severely impaired spatial cognition in the water maze test compared with the WT controls, and the administration of EBME ameliorated these learning and memory deficits.

**Table 1:** Effect of EBME on behavior in a Morris water maze (n = 8)

| Group | Escape latency(s) |
|---|---|
| Control | 23.6 ± 1.2 |
| 3xTg | 8.4 ± 1.1$^{**}$ |
| EBME-L | 13.2 ± 1.0 |
| EBME-H | 21.5 ± 1.1$^{\#\#}$ |

$^{**}P < 0.01$, compared with control group; $^{\#\#}p < 0.01$, compared with 3xTg group

### Effect of EBME on Aβ pathology in 3xTg-AD mice

In 3xTg-AD mice, Aβ is present in the hippocampus by 6 months of age. Therefore, to investigate the link between neurogenesis and the development of AD pathology, the Aβ burden in brains from 3xTg-AD mice that began receiving EBME treatments 3 month prior to 6 months of age was studied. Aβ deposits in the CA1 region of the hippocampus were significantly increased in 6-month-old 3xTg animals when compared to the age-matched WT group ($p < 0.01$). In the CA3 region of the hippocampus, Aβ deposits were significantly increased in the 3xTg group ($p < 0.001$) when compared to the WT group ($p < 0.01$). Compared with the 3xTg group, Aβ deposits in the CA1 region were significantly decreased by EBME 600 mg treatment ($p < 0.05$). In addition, compared with the 3xTg group, Aβ deposition in the CA3 was significantly decreased by EBME 300 mg treatment ($p < 0.05$). However, there were no significant differences between the 3xTg

group and the EBME treatment rats(600 mg/kg) (Table 2).

**Table 2:** Effect of EBME on the deposition of Aβ in the hippocampus (n = 8)

| Group | Amyloid beta deposits in CA1 (% of 3xTg) | Amyloid beta deposits in CA3 (% of 3xTg) |
|---|---|---|
| Control | 58.6 ± 1.3 | 79.7 ± 1.3 |
| 3xTg | 121.4 ± 3.2** | 132.4 ± 2.4** |
| EBME-L | 86.2 ± 2.1 | 89.4 ± 2.2 |
| EBME-H | 75.6 ± 1.6# | 81.5 ± 1.7# |

Data are shown as mean ± SEM; ** $p < 0.01$, compared with control mice; # $p < 0.05$ compared with 3xTg group (one-way ANOVA and Tukey's post-hoc test)

### Effect of EBME on expressions of BDNF and TrkB in hippocampus

The effect of EBME treatment on the expressions of hippocampal BDNF and TrkB in mice was determined. The results are shown in Table 3. The expressions of hippocampal BDNF and TrkB of 3xTg mice treatment with saline were significantly lower compared to that of WT mice ($p < 0.05$). It was also demonstrated that EBME treatment significantly increased the expression of hippocampal BDNF and TrkB. These results show that induction of AD reduced BDNF and TrkB expressions in the hippocampus, whereas, EBME treatment enhanced BDNF and TrkB expressions in the hippocampus of the 3xTg mice.

**Table 3:** Effect of EBME on BDNF and TrkB expression in the hippocampus

| Group | TrkB/beta action ratio | BDNF/beta action ratio |
|---|---|---|
| Control | 1.9 ± 0.3 | 1.8 ± 0.3 |
| 3xTg | 0.5 ± 0.4* | 0.8 ± 0.4* |
| EBME-L | 0.9 ± 0.4 | 1.0 ± 0.5 |
| EBME-H | 1.6 ± 0.2# | 1.5 ± 0.2# |

* $P < 0.05$, compared to control mice; # $p < 0.05$, compared to 3xTg mice (one-way ANOVA and Tukey's post-hoc test)

## DISCUSSION

The present study demonstrated that EBME increased spatial learning, memory abilities, and the expression of hippocampal BDNF and TrkB in 3xTg-AD mice. In the present study, 3xTg-AD mice was used, a model derived from $APPS_{we}$, $PS1_{M146V}$, and $tau_{P301L}$ transgenes. The 3xTg-AD mice develop a progressive, age-related neuropathological phenotype that includes plaque and tangle pathologies. These hallmark lesions are mainly limited to the hippocampus, amygdala, and cerebral cortex – the brain structures most impacted by AD pathology [15]. Cognitive impairment has been found in 2-month-old 3xTg-AD mice, and Aβ deposits in the hippocampus and cortex have been found in 6-month-old 3xTg-AD mice [16]. These findings indicate that the pathological features that imitate AD in 3xTgAD mice remain stable. Classic symptoms of AD include problems with spatial learning and memory deficits. This study demonstrated that treatment with EBME resulted in a significant restoration of spatial learning and memory function in AD mice. These results suggest that EBME treatment may be effective in ameliorating cognitive impairment caused by AD.

Neuron-specific nuclear antigen (NeuN) is a neuronal-specific nuclear protein [17]. The expression of NeuN is observed in most neuronal cell types throughout the nervous system, with the exception of some neuronal populations that are NeuN-negative, but does not stain non-neuronal cells [18]. NeuN is a soluble nuclear protein that is localized to the cell nucleus and in the neuronal cytoplasm of postmitotic neurons. Within the hippocampus, NeuN can be used as a marker of postmitotic cells and labels both “normal” postmitotic neurons and newly generated postmitoticneurons. BDNF plays pivotal roles in learning, memory, and neuronal plasticity.

The levels of BDNF, and its main receptor TrkB, have been reported to decrease in AD. It was hypothesized that BDNF and its receptor may be involved in the protective role of EBME against memory impairment. This study has demonstrated that EBME intake significantly increases the expression of BDNF and its main receptor TrkB, in the brain, which is in agreement with the hypothesis. It has been demonstrated that BDNF and TrkB, are capable of protecting against memory impairment and regulate neurogenesis in the hippocampus of AD. A recent study also supports the role of BDNF signaling through TrkB in the pathophysiology and cognitive and its receptor involving EBME in AD [19].

## CONCLUSION

The findings of this study reveal that EBME attenuates learning and memory deficits in 3xTg-AD mice, and therefore, can potentially be developed as an alternative therapeutic agent for AD.

## DECLARATIONS

### *Conflict of interest*

No conflict of interest is associated with this work.

### *Contribution of authors*

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Zi-ran Zhao designed all the experiment and revised the paper. Jiang-hua Xu and Mei Li performed the experiment equally and they are co-first author, Guang-sheng Wang wrote the paper.

### *Open Access*



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