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Pre-moxibustion and moxibustion prevent Alzheimer's disease

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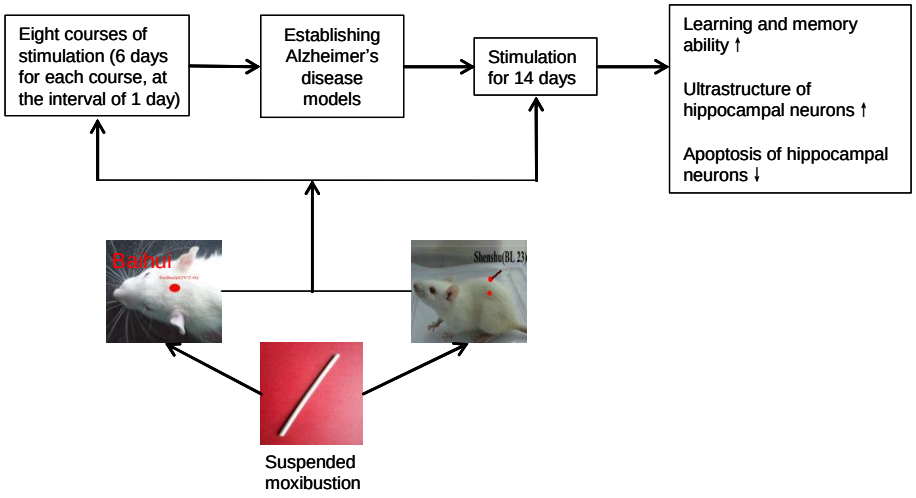
Graphical Abstract



Abstract

The Alzheimer's disease model in Wistar rats was established by injection of amyloid β-peptide ($A\beta_{1–42}$) into the hippocampal CA1 region. Rats were treated with suspended moxibustion on *Baihui* (GV20) and *Shenshu* (BL23) acupoints. Prior to and post $A\beta_{1–42}$ exposure. Results showed no evidence of apoptosis in hippocampal neurons, a significantly reduced apoptosis rate of neurons and improved learning and memory abilities were observed in the Alzheimer's disease model. In particular, moxibustion prior to $A\beta_{1–42}$ exposure was more effective than moxibustion after $A\beta_{1–42}$ exposure in protecting the neuronal structure and lowering the apoptosis rate. Our findings indicate that a combination of preventive and therapeutic moxibustion has a beneficial effect for the prevention of Alzheimer's disease development.

Key Words

neural regeneration; acupuncture; moxibustion; Alzheimer's disease; dementia; apoptosis; hippocampus; grants-supported paper; neuroregeneration

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Conflicts of interest: None declared.

Ethical approval: All experiments conducted on these samples were approved by the Animal Experimental Committee of Hubei Chinese Medical University in China.

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INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia in the elderly, affecting several millions of people worldwide[1]. Pathological changes in the AD brain include the presence of amyloid plaques, neurofibrillary tangles, loss of neurons and synapses, and oxidative damage. Substantial neuronal loss is one of the characteristic pathological features of AD[2]. Apoptosis, a major cause for neuronal loss, is a possible explanation for the progression of AD[3-7], resulting in learning and memory deficits. AD-related pathological factors, including amyloid β-protein (Aβ) and its accumulation[8], deregulation of calcium homeostasis[9], and high concentrations of free radicals[10], can induce neuronal apoptosis, leading to the onset of AD with the manifestation of associated clinical symptoms. Aβ is suggested to play a critical role in the pathogenesis of AD[11]. Aβ accumulation induces neuronal apoptosis by triggering caspase cascades and regulating the expression of genes involved in apoptosis[12]. Oligomeric Aβ is a small aggregate of the main Aβ species, and has been shown to induce neurodegeneration in AD[13]. Oligomeric Aβ induces apoptotic neuronal death in rat and mouse neurons *in vitro* and *in vivo* [14-15]. Furthermore, the degree of neuronal apoptosis was shown to closely correlate with the severity of AD[9-10]. However, the molecular mechanism(s) underlying its neurotoxic effect remains elusive[16].

Aβ is formed by the sequential cleavage of the amyloid precursor protein, yielding both $Aβ_{1–40}$ and $Aβ_{1–42}$. Based on recent *in vitro* and *in vivo* studies in rat and mouse neurons, $Aβ_{1–42}$ was shown to generate critical neurotoxic effects, aggregation of amyloid fibrils, and apoptotic neuronal death in a more similar manner to AD[17-19] than $Aβ_{25–35}$ (synthesized by artificial shearing) or $Aβ_{1–40}$. Furthermore, $Aβ_{1–42}$ is more easily accumulated than $Aβ_{1–40}$, and is thus the main component of senile plaques[20]. $Aβ_{1–42}$ is more neurotoxic than $Aβ_{1–40}$ or $Aβ_{25–35}$[21]. Both $Aβ_{1–40}$ and $Aβ_{1–42}$ downregulate Bcl-2 levels, resulting in the inhibition of apoptosis. $Aβ_{1–42}$ was shown to be more efficient in this response. In addition, the levels of proapoptotic Bcl-2-associated X protein (Bax) are upregulated by $Aβ_{1–42}$[22], but not by $Aβ_{1–40}$[22]. Therefore, to better mimic the pathological features of AD, the AD animal model of the current study was injected with peptide $Aβ_{1–42}$.

Acupuncture and moxibustion is well known to possibly stimulate body organs or tissue, showing benign and bidirectional adjustment action to its physiology and pathology process. By stimulating human body-related acupuncture points (*via* mechanical, thermal, drug, electrical, or light stimulations, or by other physical chemical means), acupuncture and moxibustion can thus affect meridian *Qi* coordinates, *Yin* and *Yang*, and regulate human body functions. Thus, different patterns of acupuncture and moxibustion can significantly inhibit apoptosis in various diseases of the central nervous system[23-26], such as stroke, Parkinson's disease, vascular dementia and AD. Growing evidence[27-28] on the molecular biology of acupuncture therapy in the treatment of AD has revealed that it plays a particular role in inhibiting cell apoptosis[29-30] and reducing neuronal loss[31-32], as well as a .positive role in controlling neurotransmitters[33-34], regulating protein content in the brain[35-37], resisting free radical oxidation damage[38], promoting brain energy metabolism, modulating synaptic changes[39-40]. An increasing number of clinical and animal studies have confirmed that acupuncture is effective for the treatment of AD[27-40]. Acupuncture and moxibustion may be more suitable long-term therapies because drugs exhibit side-effects including mental confusion, dizziness, headache, agitation and constipation. Wang *et al* [41] reported that moxibustion is more effective than electro- acupuncture for improving space-recognizing memory ability in aged mice. These results suggested that moxibustion is another alternative or complementary therapy used to treat AD.

The "preventive treatment of diseases" (published in the book, "Canon of Medicine") is a

theory pertaining to disease prevention and health promotion encompassing preventive treatment of diseases, control of the progression of existing diseases, and prevention of the deterioration of existing diseases. Thus far, no effective treatment has been found for the prevention of AD or the inhibition of its progression[42]. Given the insidious pathology of AD, diagnosing this disease at its early onset has proven to be unsuccessful. In addition, patients exhibiting signs of cognitive decline will often consider them as a normal part of aging rather than early clinical symptoms of AD[43]. Once amyloid plaques are formed they cannot be removed. Therefore, preventing Aβ accumulation or retarding the progression of AD is the possible solution, by means of measures that can be easily adopted in daily life, which take on the long-term social importance. According to the traditional Chinese medicine theory, the emptiness of the sea of marrow and apraxia are the main causes of AD. The *Baihui* (GV20) acupoint is located on the top of the head and belongs to the governor vessel, which originates from the cinnabar field and is closely related to the brain and spinal cord. This acupoint functions to enrich the brain and open the puzzle, and belongs to the vicinity-selecting acupoints. The *Shenshu* (BL23) acupoint belongs to the *Taiyang* bladder meridian, located at the back of the waist collecting the kidney essence and marrow. It is the origin of congenital constitution. The *Shenshu* acupoint functions to reinforce the kidney, dredging *Du* meridian, regulating the mind, and resuscitating consciousness. The consistency of *Shenshu* and *Baihui* acupoints can reinforce kidney essence and marrow, and restores intelligence. Based on the theory of "preventive treatment of diseases" in Chinese medicine, we hypothesized that stimulation of *Shenshu* and *Baihui* acupoints enhances the rat's ability to cope with neurodegenerative damage. In the present study, we only used suspended moxibustion (also named warming moxibustion, scarring moxibustion, or herb-partition moxibustion) on *Baihui* and *Shenshu* acupoints to observe the action of pre- moxibustion on preventing apoptosis in a rat AD model.

RESULTS

Quantitative analysis of experimental animals

Forty healthy rats were randomly divided into four groups: control group, model group, moxibustion group and pre-moxibustion group. The latter three groups were treated with intracerebral injection of $A\beta_{1-42}$ to establish an AD-like pathology. The moxibustion group received suspended moxibustion on *Baihui* and *Shenshu* acupoints for 14 days after $A\beta_{1-42}$ injection. The pre- moxibustion group was treated with moxibustion for eight courses (each course lasting for 6 days) prior to the exposure and 14 days after $A\beta_{1-42}$ exposure. The final analysis incorporated all rats.

Pre-moxibustion improved spatial learning and memory ability in AD rats

The Morris water maze navigation test showed that latency of rats exposed to $A\beta_{1-42}$ alone was significantly ($P < 0.01$) prolonged compared with the control group (Figure 1). $A\beta_{1-42}$-exposed rats treated with moxibustion markedly ($P < 0.01$) shortened the latency (Figure 1). The therapeutic effect of the pre-moxibustion group was significantly ($P < 0.05$) better than that of the moxibustion group (Figure 1).

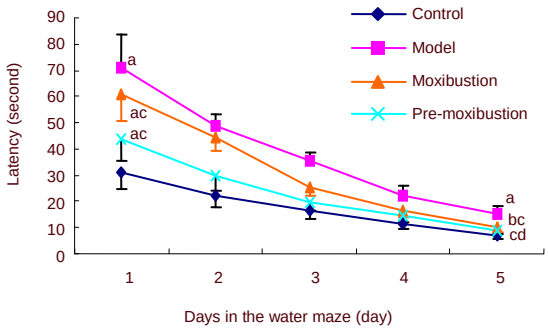

Figure 1 Effects of pre-moxibustion in Alzheimer's disease rats on spatial learning and memory ability using the Morris water maze navigation test.

The longer latency in the water maze represents learning and memory deficits. Data are expressed as mean ± SEM of ten rats in each group. Multiple comparisons between groups were conducted *via* the multivariate analysis of variance (ANOVA), followed by pairwise comparisons *via* paired *t*-tests. $^{a}P < 0.01$, $^{b}P < 0.05$, *vs*. control group; $^{c}P < 0.01$, *vs*. model group; $^{d}P < 0.05$, *vs*. moxibustion group.

The Morris water maze spatial probe test showed that the number of platform crossings and time spent in the quadrants were significantly ($P < 0.01$) decreased in rats exposed to $A\beta_{1-42}$ alone compared with the control group (Figure 2). Moxibustion treatment to $A\beta_{1-42}$-exposed rats markedly ($P < 0.01$) increased the number of platform crossings and time spent in the quadrants (Figure 2). The curative effect of the pre-moxibustion group was similar to that of the moxibustion group (Figure 2).

Pre-moxibustion alleviated the ultrastructural change of the hippocampus in AD rats

Transmission electron microscopy showed that hippocampal neurons of control rats contained distinct and

complete nuclear membranes with compact mitochondrial matrices and clear ridges (Figure 3). Apoptotic characteristics, such as pyknosis, nuclear invagination, karyorrhexis, and increased numbers of lysosomes were clearly observed in rats exposed to $A\beta_{1-42}$ alone (Figure 3). However, these apoptotic features were absent in both the moxibustion and pre-moxibustion groups (Figure 3). Some cytoplasmic lipofuscin formation occurred in the moxibustion and pre-moxibustion groups, indicating the effects of aging (Figure 3). However, lipofuscin granules were still visible in both groups, with a smaller number in the pre-moxibustion group than in the moxibustion group (Figure 3).

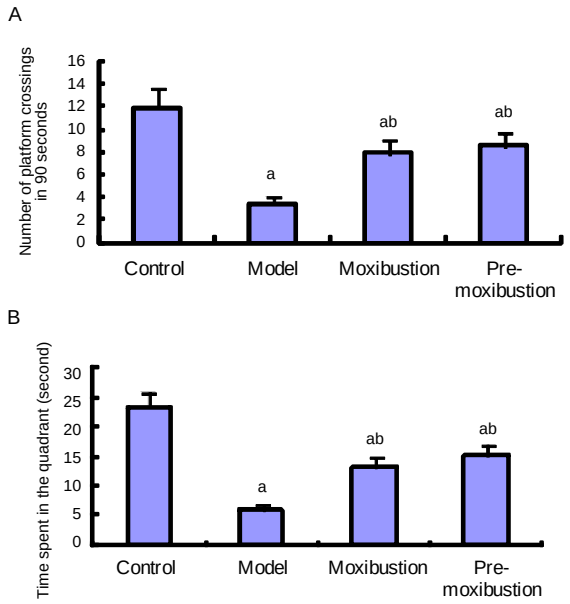

Figure 2 Effects of pre-moxibustion in Alzheimer's disease rats on the spatial probe test of the Morris water maze.

In the spatial probe test, both the number of platform crossings and time spent in the quadrant were significantly higher and longer in the moxibustion or pre-moxibustion group than in the model group, indicating that memory ability was better on the original platform. Data are expressed as mean ± SEM of ten rats in each group. Multiple comparisons between groups were conducted *via* the multivariate analysis of variance, followed by pairwise comparisons *via* paired *t*-tests. [a]$P < 0.01$, *vs*. control group; [b]$P < 0.01$, *vs*. model group.

Pre-moxibustion significantly decreased the apoptosis rate of hippocampal neurons in AD rats

Flow cytometry results showed that the apoptosis rate of hippocampal neurons in the control group was 1.75 ± 2.02% (Figure 4). The apoptosis rate in rats exposed to $A\beta_{1-42}$ alone was significantly increased (15.45 ± 1.49%) ($P < 0.01$; Figure 4). The apoptosis rate was significantly decreased in $A\beta_{1-42}$-treated rats exposed to moxibustion (8.78 ± 1.03%) or in the pre-moxibustion group (6.69 ± 0.58%) compared with rats exposed to $A\beta_{1-42}$ alone ($P < 0.01$; Figure 4). Notably, the apoptosis rate in the pre-moxibustion group was significantly ($P < 0.05$) lower than in the moxibustion group (Figure 4).

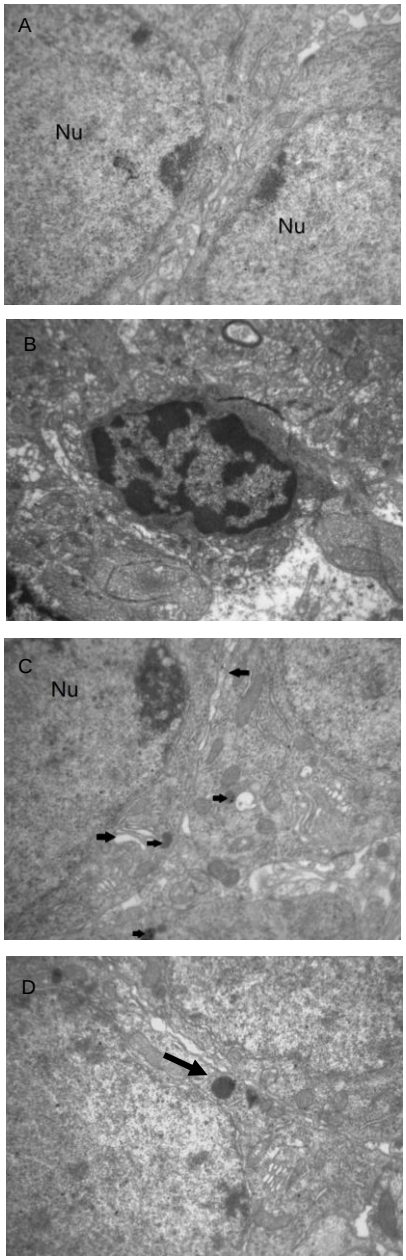

Figure 3 Effect of pre-moxibustion in Alzheimer's disease rats on hippocampal ultrastructure related to apoptosis (× 15 000).

Transmission electron microscopy of hippocampal neurons showing that neurons in the control group (A) are normal. Apoptotic features, such as pyknosis, nuclear invagination, karyorrhexis, and increased numbers of lysosomes are evident in rats treated with $A\beta_{1-42}$ alone (B). Rats treated with moxibustion (C) or pre-moxibustion (D) show reduced severity of neuronal damage compared with (B). Arrows indicate lipofuscin granules. Nu: Nucleus.

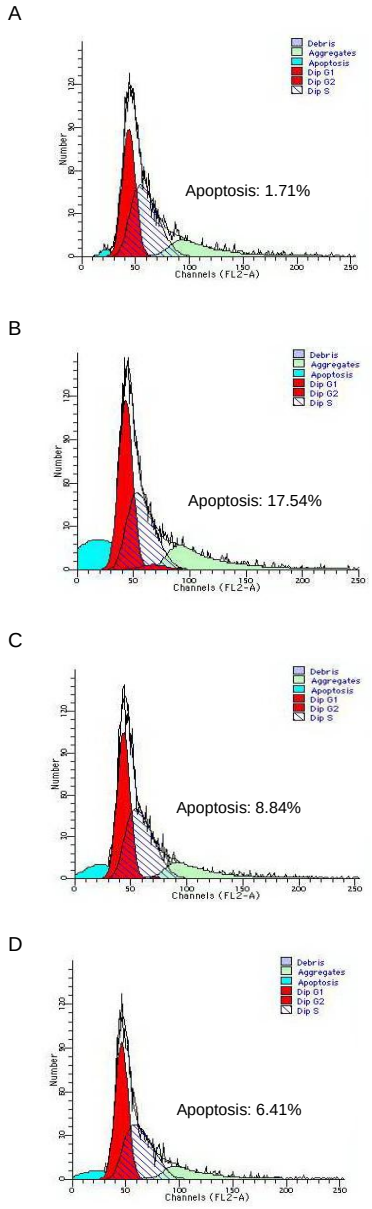

Figure 4 Effects of pre-moxibustion on apoptosis rate in hippocampal neurons of Alzheimer's disease rats.

Flow cytometry analysis to investigate hippocampal apoptosis rate. (A) Control group. The apoptosis rates of hippocampal neurons in the moxibustion group (C) and pre-moxibustion group (D) were significantly lower than those of rats treated with $A\beta_{1-42}$ alone (B). The apoptosis rate in the pre-moxibustion group was significantly lower than that of the moxibustion group.

DISCUSSION

The theory of "preventive treatment of diseases" in traditional Chinese medicine meets the requirements of AD treatment, and has become a breakthrough point for the search of effective AD treatments. Acupuncture and moxibustion may stimulate the body or organ through disease-related acupoints at a satisfactory tolerance level for patients. Therefore, timely intervention with acupuncture and moxibustion may trigger the endogenous protective mechanism of the body, thereby reducing or resisting disease harm, delaying degenerative changes in tissues and organs, and finally improving human body resistance and tolerance.

In this study, pre-moxibustion treatment in the rat AD model embodies the theory of "preventive treatment of diseases". Furthermore, moxibustion is simple to use and cost-effective. Therefore, moxibustion may be suitable for the prevention and treatment of AD. According to the traditional Chinese medicine theory, the emptiness of the sea of marrow and apraxia are the main causes of AD. Therefore, treatment of AD may be based on reinforcing kidney, dredging *Du* meridian, regulating mind, and resuscitating consciousness. The *Baihui* acupoint is located on the median parietal bones and belongs to the governor vessel, which originates from the cinnabar field, and is closely related to the brain and spinal cord. The governor vessel is an important pathway through which kidney essence, heart spirit and nutrients are transported, from other organs, to the brain to enrich its nourishment. Therefore, the *Baihui* acupoint can enrich the brain and open the puzzle. It belongs to the vicinity-selecting acupoints. The *Shenshu* acupoint belongs to the *Taiyang* bladder meridian and is located at the back of the waist. It collects the kidney essence and marrow, and is the origin of congenital constitution. The *Shenshu* acupoint functions to reinforce the kidney, dredge *Du* meridian, regulate the mind, and resuscitate consciousness. These activities counteract the pathological characteristics of AD. The consistency of *Shenshu* and *Baihui* acupoints can reinforce the kidney essence and marrow, and restore intelligence. The present study shows that moxibustion can reinforce the kidney essence and restore intelligence, thereby ameliorating AD-like pathology induced by the emptiness of the sea of marrow.

The severity of neuronal apoptosis has been shown to be positively correlated with learning and memory deficits[43]. In the present study, both moxibustion and pre-moxibustion improved the number of platform crossings and the time spent in the quadrant of the Morris maze test, thereby confirming the success of moxibustion treatment on improving the cognitive status of AD rats. Our results also revealed that moxibustion effectively reduced apoptosis of hippocampal neurons in rats with AD. A previous study has shown a preventive effect of

pre-moxibustion for AD, by comparing pre-stimulation therapy with pre-moxibustion and moxibustion in a rat model of AD. At present, the mechanism responsible for the inhibition of AD-affected hippocampal apoptosis by pre-moxibustion is not completely clear. However, pre-moxibustion in aged rats or in a rat model of AD was shown to inhibit neuronal apoptosis by regulating the levels of Bcl-2, nuclear factor kappa B, and Bax-all of which are related to mitochondrial apoptosis[44-45].

Reentry of neurons into the cell cycle is one of the pathological mechanisms of AD[46]. Pre-moxibustion inhibits apoptosis of AD-affected neurons, by downregulating the expression of the cell-cycle positive regulatory factor, CyclinA-cyclin-dependent kinases, upregulating the expression of the cell-cycle negative regulatory factor, p27Kip1, repairing DNA during the G1 phase, and blocking the progression of the cell cycle[42]. Overall, pre-moxibustion may regulate various targets through multiple pathways before and during AD pathogenesis. Pre-moxibustion may eliminate direct or indirect factors that induce apoptosis, and promote the expression of factors to protect neurons. Further research should focus on determining the mechanism of action of moxibustion in the prevention and treatment of AD for its effective application in clinical practice.

MATERIALS AND METHODS

Design

A randomized, controlled animal study.

Time and setting

The experiment was performed in the Acupuncture & Moxibustion Laboratory at Hubei University of Chinese Medicine, China in March 2012.

Materials

Male Wistar rats (12 months old; 500 ± 20 g), of specific pathogen free grade, were obtained from the Experimental Animal Center of Huazhong University of Science and Technology (Wuhan, China), with license No. SCXK (E) 2008-0005. Rats were housed at 22°C in a controlled environment and received 12-hour artificial light per day. They were allowed access to normal laboratory chow and water *ad libitum*. Experiments were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals* formulated by the Ministry of Science and Technology of China[48].

Methods

Establishing the AD models

An aliquot of 1 mg/μL solution (dimethyl sulfoxide in PBS) containing $A\beta_{1-42}$ peptides (Sigma, St. Louis, MO, USA) was prepared, and then incubated at 37°C for 7 days to produce neurotoxic oligomers. Rats were placed in the stereotaxic instrument (without anesthetic) (Chengdu TME Technology Co., Ltd., Chengdu, Sichuan Provicne, China), and bregma was exposed *via* an incision of the skin. After craniotomy was performed with a dental drill (Chengdu Kangfa Medical Equipment Co., Ltd., Chengdu, Sichuan Provicne, China) at the hippocampal CA1[49] (coordinates: 3.3 mm posterior to the bregma, 1.5 mm lateral to the midline, and 3 mm under the cortex), 5 μL of $A\beta_{1-42}$ solution was injected at a flow rate of 1 μL/min. After injection, the drilling bore was sealed with a denture base powder, and the wound was sutured. To avoid infection, all surgically treated rats were given 1 mL of penicillin (100 000 U/mL) every day for the first 3 days after the surgery. The sham group received a 5-μL saline injection.

Moxibustion treatment

After the hair around the acupoints was shaved, an ignited moxa-stick (diameter 6 mm; Nanyang Shennong Aaicao Appliance Company, Nanyang, Henan Province, China; a round long stick made of moxa floss, also called moxa roll), was suspended perpendicularly 2 cm above the acupoints. *Baihui* (located in the middle of the parietal bone[50]) and *Shenshu* (located under the second lumbar on both sides[50]) acupoints were simultaneously given suspended moxibustion. Each treatment consisted of a 15-minute moxibustion, keeping the spot warm and red but not burnt. Generally, the skin temperature was kept at 43 ± 1°C during the moxibustion procedure. Rats (not anesthetized before suspended moxibustion) were fixed in a prostrate position on the one-gloved hand. The gentle moxibustion (*i.e.*, a type of moxa-stick moxibustion) was performed once a day for 6 consecutive days as one treatment (course). The pre-moxibustion group was treated with eight courses of moxibustion before modeling (*i.e.*, 48 days) with a 1-day interval between two treatments. After the induction of AD (*i.e.*, $A\beta_{1-42}$) to rats, both the pre-moxibustion group and the moxibustion group were treated with moxibustion for 14 consecutive days. The rats of the normal and $A\beta_{1-42}$ alone groups were not treated with moxibustion but handled in the same way.

The Morris Water Maze navigation task

The water maze (Chengdu TME Technology Co., Ltd.), consisted of a circular pool (120 cm in diameter and 60 cm in depth) and a circular platform (10 cm in di-

ameter), placed at a fixed location in the center of the Northwest quadrant, submerged 2 cm below the water surface. The maze was bisected by two crossing principal axes, and the ends of axes demarcated four cardinal points: north (N), south (S), east (E) and west (W). The water temperature was maintained at 22 ± 1°C throughout the experiment. A video camera connected to an image analysis system was placed above the center of water maze.

According to Hu *et al* [51], data of the maze navigation were tracked, digitized, and stored for subsequent behavioral analysis. Each trial started with the release of an animal into the water maze, to immediately face the wall of the maze, at one of the four starting points along the wall of the pool. The rat was given 90 seconds to locate the hidden platform. If the rat failed to find the platform within this time, it was gently guided onto the platform and stayed there for 15 seconds. After an interval of 5 minutes, the rat was immediately returned to the pool. The starting location varied in each trial, and the order of the entry points was: (1) E–S–W–N for the first day, (2) S–W–N–E for the second day, (3) W–N–E–S for the third day, (4) N–E–S–W for the fourth day, and (5) E–W–N–S for the fifth day. The rats were trained for 5 consecutive days with four trials per day (total of 20 trials).

During the platform training, the latency (the time taken from entry into the water to reaching the platform) was recorded and indicated as the escape latency.

Spatial probe test

A spatial probe test was conducted after the completion of 20 trials of the navigation task, to evaluate the retrieval of spatial memory. In the probe test, the platform was removed from the pool, and the rats were released from the quadrant opposite to the platform (the target quadrant) and allowed to swim freely for 90 seconds. The swimming patterns of mice were recorded by a video camera mounted on the ceiling, and the time spent in the target quadrant of the water maze was measured in the spatial memory retrieval trials.

Flow cytometry analysis

After behavioral testing was complete, the bilateral hippocampus was removed, after rapid decapitation (without anesthesia), and kept on an ice pad as soon as moxibustion treatment ended. The hippocampus was repetitively minced by pipetting in PBS. The mixture was then filtered through a 300-nm mesh nylon net to obtain a single-cell suspension. After centrifugation (1 500 r/minute for 10 minutes), the supernatant was discarded, and 1.5 mL of 75% ethanol aqueous solution was added. The mixture was shaken and sealed. After incubation (at 4°C, overnight), the mixture was centrifuged (1 500 r/minute for 10 minutes). The supernatant was discarded and 1–2 mL of PBS was added, followed by centrifugation at 1 500 r/min and the removal of the supernatant. The above steps were repeated twice. The remaining cells were digested with RNAase (Sigma) (0.5 mg/mL) (at 37°C for 30 minutes), then the mixture was stained with propidium iodide (Sigma) solution (2.5 μg/mL) for 15–20 minutes (in the dark). Flow cytometry analysis was then performed (Becton Dickinson, San Jose, CA, USA).

Transmission electron microscopy

Normal saline (0.9%) was infused into the rat heart. Then 4% paraformaldehyde was rapidly infused for 5 minutes. The bilateral hippocampus was removed after rapid decapitation, and the tissue was fixed with 2.5% glutaraldehyde solution overnight, then with 1% osmic acid for 1 hour, followed by full rinsing with PBS. After dehydration with gradient ethanol, immersion with acetone, embedding, and 50-nm ultra-thin section preparation, the tissue was examined and photographed with a Hitachi 1200ES transmission electron microscope (Hitachi, Japan).

Statistical analysis

All data are expressed as mean ± SEM, and were analyzed with multivariate analysis of variance, followed by a pairwise comparison with the paired *t*-test, using SPSS 16.0 software (SPSS, Chicago, IL, USA). A $P < 0.05$ level was considered statistically significant.

Research background: The pathological development of Alzheimer's disease (AD) and the resultant cell apoptosis are caused by neurons failing to enter the cell division cycle because of the abnormal regulation of this cycle.

Research frontiers: Studies suggest that moxibustion is better than electro-acupuncture to improve the learning and memory abilities in aging mice, suggesting that moxibustion is a potential complementary therapy for the treatment of AD.

Clinical significance: Early moxibustion on *Baihui* (GV20) and *Shenshu* (BL23) acupoints can reduce cell apoptosis in the hippocampus and improve the learning and memory abilities in AD, thus providing a new method for clinical prevention and treatment of AD.

Academic terminology: Preventive treatment of disease means to take preventive measures to block the occurrence and development of the disease, and is the basic rule of traditional Chinese medicine theory and one of the core concepts of

Chinese medicine.

Peer review: Preventive moxibustion has been proven to reduce apoptosis of hippocampal neurons in AD rats, with an effect on the prevention and early treatment of AD. Therefore, the experimental results may be of great value under clinical settings for the treatment of AD.

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