

FULL LENGTH ARTICLE

Effect of the electro-acupuncture on senile plaques and its formation in APP⁺/PS1⁺ double transgenic mice

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Abstract Alzheimer's disease (AD) is a progressive neurodegenerative disease and its incidence will increase with age and is aggravating. The senile plaques (SPs) are one of three main pathological features in AD patients, which are formed by amyloid β -protein ($A\beta$) over-accumulation. β -amyloid precursor protein (APP), β -site APP cleavage (BACE1), and insulin degrading enzyme (IDE) proteins participate in the process of $A\beta$ production and degradation. At present, the pathogenesis of AD is not yet clear and the current treatment methods can only relieve the related symptoms of AD. The electro-acupuncture (EA) is a traditional Chinese medicine treatment combined the acupuncture and electrical stimulation and the treatment effect can also be controlled by transform the electrical frequency. Thus, in this experiment, we carried out behavioral test, immunohistochemistry (IHC), and Western Blot (WB) after different period treatments to the model mice by electro-acupuncturing "Baihui" and "Shenshu" acupoints in APP⁺/PS1⁺ double transgenic mice. It was found that the EA therapy can improve the ability of learning, memory and spatial exploration, and reduce the deposition of SPs in brain of AD model mice, and reduce the expressions of APP and BACE1, increase the expression of IDE protein. These results prompt that EA can effectively alleviate the pathological process of AD. We speculate that EA may play a comprehensive role in preventing the development of AD, considering the previous data.

Abbreviations: AD, Alzheimer's disease; $A\beta$, amyloid β -protein; SPs, senile plaques; APP, β -amyloid precursor protein; BACE1, β -site APP cleavage; IDE, insulin degrading enzyme; EA, electro-acupuncture; IHC, immunohistochemistry; SOD, superoxide dismutase; Ach, acetylcholine; ChAT, acetylcholine transferase; AchE, cholinesterase.

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease with progressive memory deficits and cognitive impairments. It is estimated to have more than 30 million AD patients worldwide in 2010 and expected 106 million by 2050.¹ In China, the incidence of AD is 2.57% for people over 55 years old and 4.6% for over 65 years old.² The incidence of AD will increase with age and is aggravating, brings heavy burden to the family and society.³ The current physical and medical treatment methods which can only relieve the related symptoms to a certain degree have the corresponding side effects at the same time.⁴ Although the study of AD has made great progress, however, there is not an absolutely effective treatment of AD so far. Thus, the identification of effective and safe treatments with clear functional mechanisms is urgently needed.

The electro-acupuncture (EA) application is a traditional Chinese medicine treatment way combined the acupuncture and electrical stimulation and the treatment effect can also be controlled by transform the electrical frequency.^{5,6} Some previous literature reported that manual acupuncture or electro-acupuncture some acupoints could improve the cognitive symptoms of AD model animals which were mainly made by injected intraperitoneally D-galactose or AlCl_3 .^{7,8} Additionally, several studies have found that the effect of EA for improving cognitive symptoms of AD animals is associated with a lot of pathways. For example, EA at the "Baihui" and "Shenshu" acupoints improves learning and memory function through down-regulating Notch signaling pathway or increasing PPAR- γ expression and reducing p-p38MAPK expression and attenuating A β in the AD rat model.^{9,10}

As is known, the main pathological features of AD patients are the senile plaques (SP), neurofibrillary tangles and loss of neurons. Potential mechanisms causing the pathology has not been elucidated, and many hypotheses about, including gene mutations, tau hyperphosphorylation, cholinergic neuronal damage.⁷ The A β cascade hypothesis is recognized as the cause of the generation of SP in AD patients.¹¹ Therefore, many studies focused on it. A previous study has demonstrated that EA at "Baihui" acupoint ameliorated learning and memory deficits and reduced A β 42 deposit in APP⁺/PS1⁺ mice and propose that the promotion of neurogenesis in the hippocampus and cortex would be the underlying mechanism of EA.¹² Anyway, the therapeutic effect of EA treatment for AD has shown in many works. However, it is seldom reported that EA stimulation regulates the number of SPs and the expression of its related proteins in the APP⁺/PS1⁺ model mice.

In view of the above, according to the theory of Traditional Chinese Medicine, acupuncture points of "Baihui" and "Shenshu" were applied in this experiment.¹³ We evaluated the effect of EA on the formation mechanism of SPs in the APP⁺/PS1⁺ double transgenic mice. Therefore, Morris water

maze test, IHC and WB technique were used to observe spatial learning and memory ability, the expression of SP and level of the related proteins APP, BACE and IDE of mice.

Materials and methods

Animals and ethics

Male APP⁺/PS1⁺ double transgenic mice (APPswe and PS1 Δ E) and age-matched wild-type (C57BL/6J) mice were purchased from Beijing Huafukang Biotechnology Co., Ltd. (Beijing, China). Mice were housed in a controlled environment (21–25 °C; 50 $\pm$ 10% relative humidity and automatic 12 h light/dark cycle) with access to food and water ad libitum in the Animal Experiment Center of Chongqing Medical University.

Furthermore, all operations were carried out strictly in accordance with international ethical guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Gene identification

The procedure of DNA identification was carried out to determine the reliability of APP⁺/PS1⁺ double transgenic mice. The tails of mice were cut with a length of about 3–5 mm after the mice were fed for about a week. Total DNA was isolated from the mouse by using the DNA extraction kit (Beijing Tiangen Biochemical Technology Co., Ltd.) and was amplified by PCR.

The PCR reaction system was under the following conditions: initial denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 40 s at 58 °C, and extension for 40 s at 72 °C followed by a final extension for 10 min at 72 °C. The PCR products were resolved on agarose gels and quantitated using the Bio-Rad gel imager (90 V constant voltage, continued for 30min). Primers used for PCR in this study are set as following. APP: forward 5'-GAC TGA CCA CTC GAC CAG GTT CTG - 3' and reverse 5'-CTT GTA AGT TGG ATT CTC ATA TCC G - 3'; PS1: forward 5'-AAT AGA GAA CGG CAG GAG CA - 3' and reverse 5'-GCC ATG AGG GCA CTA ATC AT - 3'.

Grouping and method of EA treatment

Mice were randomly divided into four groups: the control group (wild type mouse), the model group (APP⁺/PS1⁺ double transgenic mouse), the 2 courses of EA treatment group (APP⁺/PS1⁺ double transgenic mouse which were dealt with EA for 2 weeks) and the 3 courses of EA treatment group (APP⁺/PS1⁺ double transgenic mouse which were dealt with EA for 3 weeks), six mice in each group. The control group was consisted of the wild type mice, the

other three groups were consisted of APP⁺/PS1⁺ double transgenic mice.

According to *Experimental Acupuncture*, the point of "Baihui" (DU 20) and "Shenshu" (BL 23) of the mouse could be located precisely. The EA needle (diameter, 0.18 mm, Beijing Zhongyantai Medical Devices Co., Ltd., Beijing China) was inserted at a depth of 2 mm into the DU20 acupoint, which is located at the intersection of the sagittal midline and the line linking the two ears. The BL23 acupoint was inserted at a depth of 4 mm, which is located 1 mm next to the spinous process of the second vertebral body. Stimulation was generated using EA apparatus (model 6805-All; Shantou Medical Equipment Factory, Guangdong, China) and the stimulation parameters were set as disperse waves of 2 Hz and electric current of 2 mA for 15 min once a day, and 7 days/one course, 1 day rest between two courses.

Morris water maze assay

After the EA treatment, cognitive function was tested by the Morris water maze apparatus, which was a stainless steel circular tank with a diameter of 120 cm and a height of 50 cm. The tank was filled with water (21–23 °C) to the depth of 40 cm and divided into four equal quadrants.

Visual platform experiment: On the first day, a platform (diameter, 6 cm) was placed in the third quadrant and above the surface of the water about 1 cm. Each group of mice was placed in water from four quadrants in turn according to the group number. We can stop the experiment if the mouse find the platform in 60 s. If a mouse did not find the platform within 60 s, it would be removed from the water and placed on the platform for 10 s by researchers. Record the escape latency and the search path of the mouse to find the platform.

Covert platform experiment: From the 2nd day to the 6th day, a platform was submerged 0.5 cm below the surface of the water. Mice were allowed to swim in pool during a period of 60 s to find the hidden platform. Recorded the escape latency and the search path of the mouse to find the platform.

Space exploration experiment: On the 7th day, the platform was removed and each mouse was allowed to swim freely for 60 s. Recorded the times of passing the hidden platform within 60 s to assess spatial reference learning and memory.

Tissue preparation

After the completion of the water maze experiment, animals were terminally anesthetized with 3.5% chloral hydrate (10 mL/kg) by intraperitoneal injection and transcardially perfused with 0.9% sodium chloride solution. Brains were immediately dissected. For Immunohistochemistry (IHC) assay, the brain tissues for each animal were postfixed for 24–48 h in the 4% paraformaldehyde solution, and then dehydrated with gradient alcohol and wrapped in paraffin. Finally, serial coronal cryosections (5 µm thick) were cut on a paraffin slicer (Leica RM2235). For Western blot analysis, the fresh tissues of cortex and hippocampus of each animal were separated and stored at –80 °C independently.

Immunohistochemistry assay

IHC was performed on the 5 µm-thick coronal paraffin sections. The number of SPs was examined with DAB kits according to the manufacturer's protocols. Dissolved the insoluble Aβ in senile plaques for 10 min with 88% formic acid solution. The sections were incubated in 3% hydrogen peroxide at 37 °C for 5 min to block the non-specific protein binding. Blocked with 5% BSA blocking solution at 37 °C for 30 min. Sections were incubated with primary anti-4G8 antibody (1: 250; mouse, monoclonal; Convance Inc, USA) at 4 °C overnight and subsequently incubated with secondary antibody at 37 °C for 30 min. SPs were stained brown by the DAB staining liquid and hematoxylin was used to visualize the nuclei of all cells. Images of SP deposition in the hippocampus were captured using an optic microscope (Leica DM2000, Germany). All experiments were repeated three times independently.

Western Blot assay

The cortex and hippocampal samples were homogenized and centrifuged, then the supernatant was separated. BCA method was used to detect the concentration of protein. The extracted proteins were inactivated by protein loading buffer.

A total of 50 µg protein was separated by electrophoresis (80 V for 120 min) on 10% SDS-PAGE gels (Nanjing Kingsley Biological Technology Co., Ltd. China.). Proteins were transferred onto PVDF membranes (Millipore, USA). The membranes were blocked for 2 h with blocking solution at 37 °C and incubated with antibodies against APP (1: 1000; rabbit, monoclonal; Abcam, USA), BACE1 (1: 500; rabbit, monoclonal; Cell Signaling Technology, USA), IDE (1: 1000; rabbit, monoclonal; Abcam, USA), β-actin (1: 1000; Nanjing Zhongding Biological Technology Co., Ltd.) at 4 °C overnight and subsequently incubated with secondary antibody (1:10000; goat anti-rabbit) for 30 min. The protein bands were visualized with enhanced chemiluminescence luminescence and imaged with the Bio-Image Analysis system. All experiments were repeated three times independently. The ratio of the protein band intensities to the β-actin was determined as the statistical data for analysis.

Statistical analysis

All data were presented as means ± SD. All data were analyzed by the SPSS statistical and Graphpad software. Independent sample *t* test was used for the comparison between the control group and the model group, one-way ANOVA was used for the comparison between the other three groups. The difference was statistically significant with *p* < 0.05.

Results

Gene identification

The samples from the APP⁺/PS1⁺ double transgenic mice had APP and PS1 expression bands at the same time, while

the samples from the WT mice were negative in APP and PS1 bands. The results showed that all the mice that we used in this study were credible (Fig. 1A).

The effect of the learning, memory and spatial exploration ability by EA treatment

In the visual platform experiment, the escape latency and the search path between each group mice had no statistical significance. This result showed that the vision between the four groups mice has no significant difference.

In covert platform experiment, the escape latency and the search path of the model group mice were increased compared with the control group ($p < 0.05$). The two treatment groups mice were decreased compare with the model group ($p < 0.01$). It changed obviously with the 3 periods of EA treatment group mice than that of the 2 periods of EA treatment group ($p < 0.05$).

In space exploration experiment, the model group mice had less the times of passing the hidden platform than the control group ($p < 0.001$). The treatment group mice had more passing times than the model group ($p < 0.01$, $p < 0.001$). The passing times of the 3 periods of EA treatment group mice were increased than that of the 2 periods of EA treatment group ($p < 0.05$) (Fig. 1 B–D).

These results suggested that EA treatment markedly improved the earning, memory and spatial exploration ability.

The effect of the SP deposition by EA treatment

The model group had more SPs both in the hippocampus ($p < 0.0001$) and the cortex ($p < 0.0001$) than the control group. The amount of SPs of the two treatment groups mice were distinctly reduced in the hippocampus ($p < 0.01$, $p < 0.0001$) and the cortex ($p < 0.001$) than that of the model group. The 3 periods of EA treatment group mice were obviously reduced in the hippocampus ($p < 0.01$) compared with the 2 periods of EA treatment group. There were no statistically significant differences between the 2 periods of EA treatment group and not only the model group but also the 3 periods of EA treatment group in the cortex (Fig. 2).

This result suggested that EA treatment markedly decreased the SP deposition of the hippocampus and cortex in APP⁺/PS1⁺ double transgenic mice.

The effect of the expression of the SP-related proteins by EA treatment

The expression of APP in the hippocampus ($p < 0.0001$) and cortex ($p < 0.001$) had increased compared with the control group. The expression of APP in the two treatment group mice were significantly decreased both in the hippocampus ($p < 0.0001$) and the cortex ($p < 0.05$, $p < 0.01$) than that of the model group. The 3 periods of EA treatment group mice had less expression of APP in the hippocampus

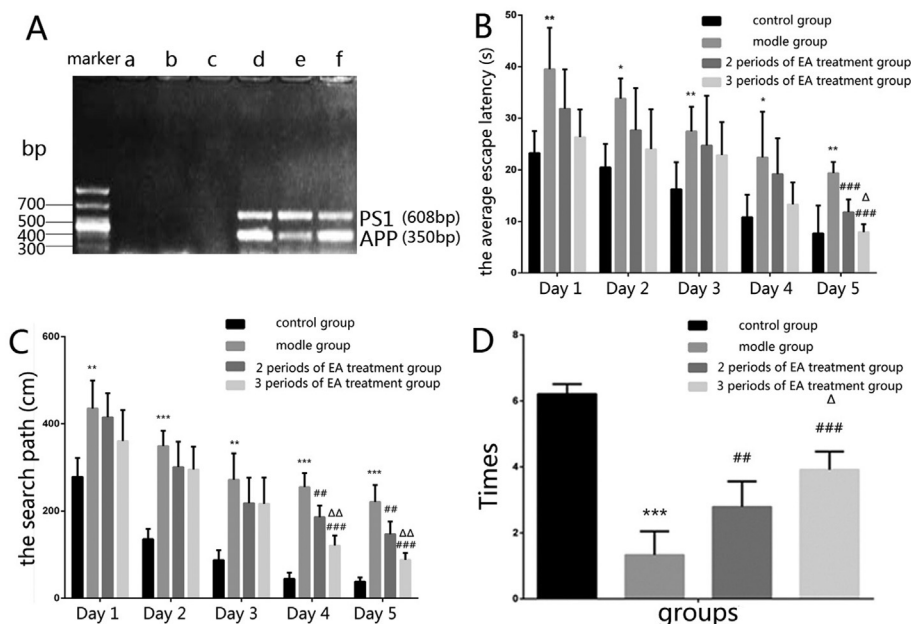


Figure 1 A Gene identification of experimental model mice. a,b,c were wild-type C57BL/6J mice, d,e,f were APP⁺/PS1⁺ double transgenic mice. B The comparison of the average escape latency time (s) by each group of mice in the covert platform experiment. * $p < 0.05$ and ** $p < 0.01$ versus the control group, ### $p < 0.001$ versus the model group, Δ $p < 0.05$ versus the 2 periods of EA treatment group. C The comparison of the search path (cm) by each group of mice in the covert platform experiment. ** $p < 0.01$ and *** $p < 0.001$ versus the control group, ## $p < 0.01$ and ### $p < 0.001$ versus the model group, Δ $p < 0.05$ versus the 2 periods of EA treatment group. D The comparison of the times of passing the hidden platform in the space exploration experiment. *** $p < 0.001$ versus the control group, ## $p < 0.01$ and ### $p < 0.001$ versus the model group, Δ $p < 0.05$ versus the 2 periods of EA treatment group ($n = 6$ in each group).

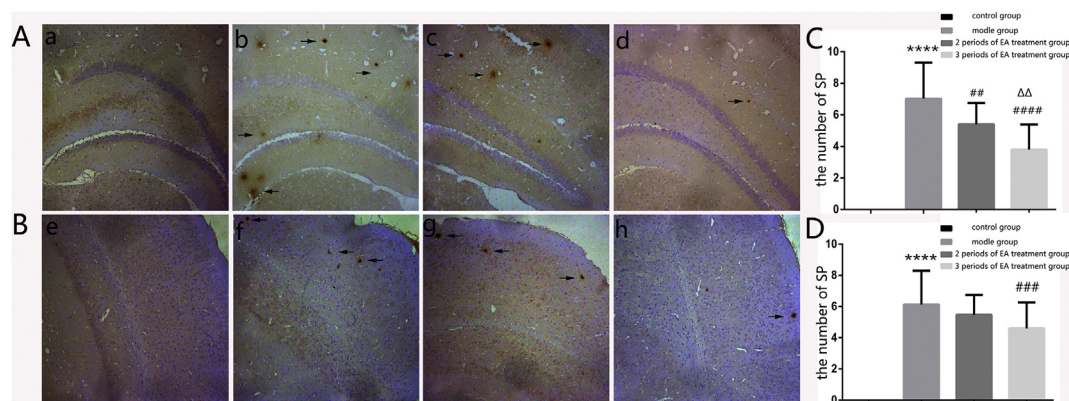


Figure 2 A The changes of senile plaques (SPs) in the hippocampus. B The changes of SPs in the mice cortex. The stained SPs were indicated by the black arrow, a,e: the control group, b,f: the model group, c,g: the 2 periods of EA treatment group, d,h: the 3 periods of EA treatment group. C The number of staining for SPs in the hippocampus of each group. **** $p < 0.0001$ versus the control group, ** $p < 0.01$ and **** $p < 0.0001$ versus the model group, $\Delta \Delta p < 0.01$ versus the 2 periods of EA treatment group. D The number of staining for SPs in the cortex of each group. **** $p < 0.0001$ versus the control group, **** $p < 0.0001$ versus the model group ($n = 6$ in each group).

($p < 0.001$) and cortex ($p < 0.05$) than the 2 periods of EA treatment group mice (Fig. 3 A and D).

The expression of BACE1 in the model group mice were significantly increased in the hippocampus ($p < 0.0001$) and the cortex ($p < 0.001$) compared with the control group. The expression of BACE1 in the treatment groups mice were decreased both in the hippocampus ($p < 0.01$, $p < 0.001$) and cortex ($p < 0.05$) compared with the model group. The 3 periods of EA treatment group mice had less expression of BACE1 in the hippocampus ($p < 0.05$) than the 2 periods of EA treatment group (Fig. 3 B and E).

The expression of IDE in model group mice were significantly decreased compared with the control group in the hippocampus ($p < 0.0001$) and cortex ($p < 0.001$). The expression of IDE in the two treatment groups mice were distinctly increased both in the hippocampus ($p < 0.001$, $p < 0.0001$) and cortex ($p < 0.05$) compared with the model group. It was obviously increased in the hippocampus ($p < 0.05$) of the 3 periods of EA treatment group mice compared with that of the 2 periods of EA treatment group (Fig. 3 C and F).

There were no statistically significant differences between the 2 periods of EA treatment group and not only the model group but also the 3 periods of EA treatment group about the expression of BACE1 and IDE in the cortex (Fig. 3 E and F).

These results suggested that EA treatment markedly decreased the expression of APP and BACE1 and increased the IDE of the hippocampus and cortex in APP⁺/PS1⁺ double transgenic mice (Fig. 3 G and H).

Discussion

AD is a degenerative disease of the central nervous system. The A β cascade hypothesis is the most accepted one in many hypotheses of AD. This hypothesis agrees that the SPs in AD patients' brain is formed by the accumulation of A β fragments produced by the abnormal processing of APP.¹⁴

The APP⁺/PS1⁺ double transgenic mouse is characterized by the progressive increase in SPs deposition as the month grows. It expresses a mutated allele of the human APP and PS1 genes.¹⁵ The deposition of SPs is the main pathological features of AD patients, and also most likely the direct cause of AD. So, it is the ideal animal model of AD to simulate the pathological features of AD patients in all aspects.¹¹ Therefore, in this study, the wild-type mice and APP⁺/PS1⁺ double transgenic AD model mice were selected as the experimental animals.

The SP which is formed by A β over-accumulation is one of the typical pathological features of the AD. A β is produced by the cutting of APP through the β - and γ -secretase. There are two forms of A β : A β 40 and A β 42. A β 40/A β 42 is equal to 10:1 under normal circumstance, too much A β 42 tends to form SPs in the brain.¹⁵ The concentration of A β in the body is mainly regulated by the process of its formation and degradation. BACE1 is a kind of β -secretase and is the key rate-limiting enzyme in the A β production process, which increases its concentration, and IDE is an important hydrolase that catalyzes the degradation of A β , which reduces its concentration.^{16–18} In conclusion, the level of APP, BACE1 and IDE were considered to be important factors affecting the deposition of SPs.

In previous numerous animal studies, EA and manual acupuncture have demonstrated therapeutic utility for the treatment of AD.^{19–29} In this work, the results of Morris water maze experiment suggest that the EA treatment can significantly improve the learning, memory and spatial exploration ability of APP⁺/PS1⁺ double transgenic mice. This result is consistent with the existing research conclusion. Previous studies reported that acupuncture has positive effects on cognition in AD and dementia by modulating neuronal signaling pathways. These pathways, which are suggested to mediate the beneficial effects of acupuncture on cognitive and physiological functions in animal models, involve those related to cell survival, apoptosis, and glucose metabolism.^{12,19,23,25,27,29,30} However, little studies show the effect of EA on the mechanism about deposition

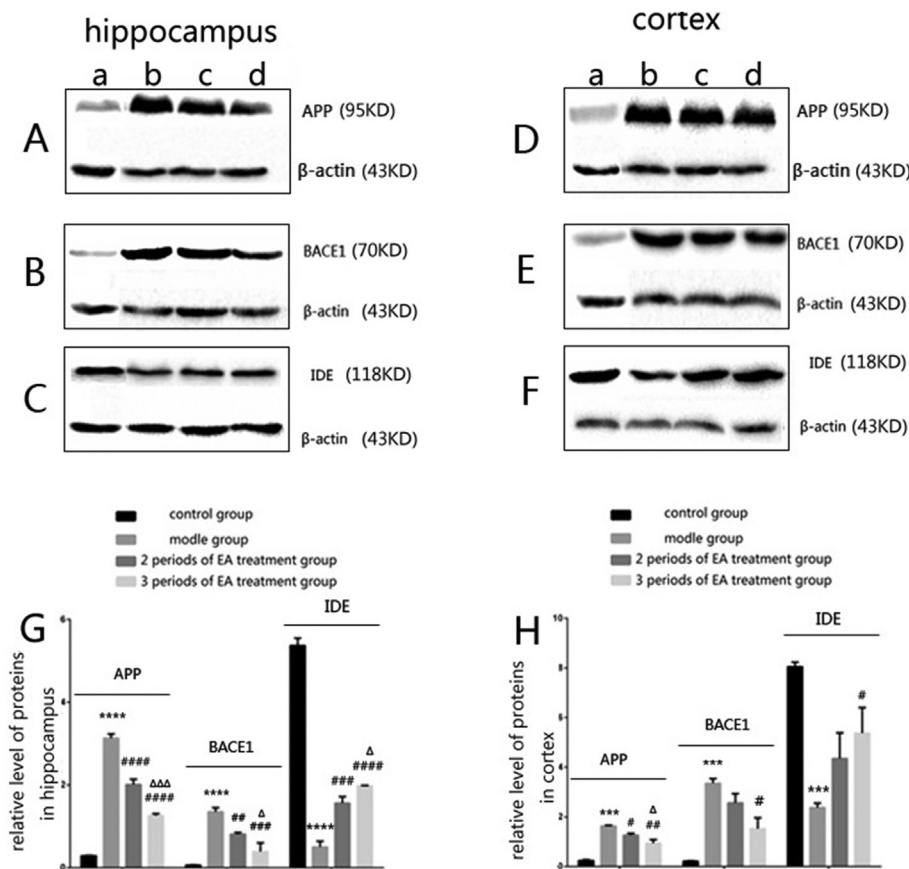


Figure 3 A, B, C The expression of APP, BACE1, IDE in the hippocampus of each group of mice. D, E, F The expression of APP, BACE1, IDE in the cortex of each group of mice. a: the control group. b: the model group. c: the 2 periods of EA treatment group. d: the 3 periods of EA treatment group. G The level of APP, BACE1, IDE in the hippocampus was indicated by column chart of each group of mice. APP: **** $p < 0.0001$ versus the control group, ##### $p < 0.0001$ versus the model group, $\Delta \Delta \Delta p < 0.001$ versus the 2 periods of EA treatment group. BACE1: **** $p < 0.0001$ versus the control group, ## $p < 0.01$ and ### $p < 0.001$ versus the model group, $\Delta p < 0.05$ versus the 2 periods of EA treatment group. IDE: **** $p < 0.0001$ versus the control group, ### $p < 0.001$ and #### $p < 0.0001$ versus the model group, $\Delta p < 0.05$ versus the 2 periods of EA treatment group. H The level of APP, BACE1, IDE in the cortex was indicated by column chart of each group of mice. APP: *** $p < 0.001$ versus the control group, # $p < 0.05$ and ## $p < 0.01$ versus the model group, $\Delta p < 0.05$ versus the 2 periods of EA treatment group. BACE1: *** $p < 0.001$ versus the control group, # $p < 0.05$ versus the model group. IDE: *** $p < 0.001$ versus the control group, # $p < 0.05$ versus the model group, ($n = 6$ in each group).

of SPs and its related proteins in the APP⁺/PS1⁺ model mice. We observed that EA treatment cut down the level of SP, APP and BACE1, and also enhance the level of IDE in the hippocampus and cortex of the APP⁺/PS1⁺ model mice, which suggest that the EA treatment can effectively lessen the deposition of SPs by reducing the expression of APP and BACE1 and increasing the expression of the IDE in the APP⁺/PS1⁺ model mice.

The earliest expression of AD is the loss of memory caused by the hippocampal neuron lesions, then there is cerebral cortex lesions, eventually lead to memory loss and cognitive functions like language and space decreased.³¹ As was reported, there is fiber projection between the hippocampus and the cortex to connect each other.³² Thus, the neurons of both districts play a critical role in the development of AD. The results in this study revealed that, in the cortex, there are no significantly effects after 2 periods of EA treatment about the amount of SPs and the expression of BACE1 and IDE, but the EA

treatment of 3 periods has a significant effect. Nevertheless, SP and these related proteins in hippocampus have already changed in 2 periods of EA treatment. The insufficient treatment time may be the cause of the phenomenon, this prompts us. At the same time, given the different manifestations of the cortex and hippocampus, we speculate that it may rescue the hippocampus neurons at first by EA treatment, and the effects of EA on the cortex may be produced by the recovering of the fiber projection from hippocampus to cortex, and this recovering duration takes time. Therefore, we can extend the treatment time to assess the relationship between the time and efficacy of the EA treatment.

As is reported, from the neurotransmitter point of view, it has been found that the EA was given "Baihui", "Shen-shu", "Zusanli" can improve the activity of the acetylcholine transferase (ChAT) and inhibit the cholinesterase (AChE) in cortex of rat. Thus, it increases the content of acetylcholine (ACh) in brain tissue and reverses the decline of its

memory.³³ In terms of Anti-oxidize effect, some researchers believe that the EA was given "Baihui", "Dazhui", "Shenshu", "Taixi", "Zusanli" can improve the activity of free radical scavenging enzymes-superoxide dismutase (SOD) in the brain and thus improve the learning and memory abilities of AD model rats.³⁴ In the aspect of brain morphology and histopathology, researchers found that cells in the hippocampus of AD model rats are arranged more neatly after EA treatment of "Shenshu", "Neiguan", "Dazhui".³⁵ These above studies of AD model animals for EA treatment and the results in this work together suggest that EA treatment may exert a comprehensive effect in preventing the development of AD. However, some problems are present in these studies, such as, there is no uniform standard in aspect of acupoint option. Moreover, the therapeutic mechanism of EA simulation was explored in different studies through using various acupoints. But, we have not yet known whether there has the definite link between the different acupoints and its corresponding treatment mechanisms. In addition, the pathogenesis of AD is also associated with tau hyperphosphorylation, estrogen and insulin, etc. How EA affects those factors and their relationship, we don't know yet. So, we will explore them in the further studies.

In summary, the EA treatment can reduce the formation of SP on the AD patients. The results of these studies provide evidences for the widespread application of the EA in the AD clinical treatment.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

Acknowledgments

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