Electroacupuncture with Bushen Jiannao improves cognitive deficits in senescence-accelerated mouse prone 8 mice by inhibiting neuroinflammation

Wang Xu, Li Zhaolong, Li Chunri, Wang Yue, Yu Song, Ren Lu

OBJECTIVE: To investigate effect of electroacupuncture (EA) with Bushen Jiannao on learning and memory ability in senescence-accelerated mouse prone 8 (SAMP8) mice and the related mechanisms.

METHODS: 8-month-old senescence-accelerated-resistant (SAMR1) and SAMP8 mice were treated with EA at Baihui (GV 20), Shenshu (BL 23), and Taixi (KI 3) acupoint once a week for 8 weeks. The Morris water maze, enzyme linked immunosorbent assay, immunohistochemistry, and Western blot were used to assess Alzheimer’s disease (AD)-associated cognitive and neuroinflammatory phenotypes.

RESULTS: Our data showed that EA treatment decreased activation of microglia and astrocytes, decreased levels of inflammatory factors including tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-17, and improved spatial memory deficits in SAMP8 mice. EA therapy with Bushen Jiannao exhibited anti-inflammatory properties and improved cognitive function.

CONCLUSION: The present study indicates that EA treatment based on the interaction between kidney and brain can improve learning and memory ability by inhibiting activation of astrocytes and microglia and decreasing expression of pro-inflammatory cytokines, TNF-α and IL-17. EA treatment based on the interaction between kidney and brain may be an effective treatment for AD.

Abstract

INTRODUCTION

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by progressive learning and memory deficits. There are two neuropathological hallmarks in brain tissues of AD patients: extracellular senile plaques composed of amyloid β-peptide (Aβ) and intracellular neurofibrillary tangles. Increasing evidence shows that AD is also characterized by prominent neuroinflammation, as manifested by reactive astrogliosis, microgliosis, and elevated levels of inflammatory factors. Reactive glial cells, including microglia and astrocytes, produce multiple inflammatory factors, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6, to cause brain injury and cognitive impairment. Previous study showed that inhibiting production of inflammatory cells and inflammatory factors could slow AD progression and therefore offer new hope to those who receive treatment soon after diagnosis.
Acupuncture is known to be effective in treating various diseases including neurodegenerative diseases, pain, and mood disorders. In recent years, an increasing number of Traditional Chinese Medicine (TCM) scientists have incorporated acupuncture into treatment protocols for AD patients and achieved notable curative effects. Electroacupuncture (EA) or acupuncture, which is a TCM treatment that stimulates certain acupoints, has been shown to induce significant neuroprotective effects and improve cognitive conditions among AD animal models and AD patients. Although the pathological changes of AD occur in the brain, the viscera dialects of Chinese medicine considered that the root of the disease is related to renal dysfunction. According to the theory of TCM, brain is closely associated with the kidneys and kidneys contain the essence and medulla. Furthermore, Medulla can produce bone marrow and promote the functions of brain. Thus, normal brain function depends on renal essence. Chinese medicine of tonifying kidney, removing blood stasis and phlegm shows certain curative effects for AD. Our previous studies demonstrated that EA at Baihui (GV 20), Shenshu (BL 23), and Taixi (KI 3) points according to the interaction between kidney and brain in senescence-accelerated mouse prone 8 (SAMP8) mice, which is a mouse model of AD, inhibited formation of Aβ. However, it is unclear whether EA stimulation can improve learning and memory by inhibiting neuroinflammation in AD. To evaluate this hypothesis, the SAMP8 mice were chosen to evaluate the effects of EA treatment at Baihui (GV 20), Shenshu (BL 23), and Taixi (KI 3) points based on the interaction between kidney and brain on spatial learning and memory impairment and discussed the related mechanisms in this study. Our results demonstrate that EA with Bushen Jiannao based on the interaction between kidney and brain may be a promising strategy for AD treatment.

MATERIALS AND METHODS

Animals and experimental protocols
Eight-month-old male SAMP8 and age-matched senescence-accelerated-resistant (SARM1) mice were purchased from Beijing Huafukang Biotechnology Company. They were housed on a 12 h/12 h day/night cycle and housed with free access to food and water. All procedures using animals were conducted in accordance with the care and use of medical laboratory animals (Ministry of Health PR China, 1998) and the guidelines of the laboratory animal ethical standards of Liaoning University of Traditional Chinese Medicine. A total of 18 male SAMP8 mice and 6 male homologous SAMR1 mice were randomly assigned to the following 4 groups (n = 6 per group): SAMR1 normal control group, SAMP8 model group, SAMP8 EA group, and SAMP8 memantine medicine group. In the EA group, EA treatment was performed daily for 15 min over a period of 2 months. The needles were inserted at a depth of 0.5 cm into the Baihui (GV 20), Shenshu (BL 23), and Taixi (KI 3) acupoints. The locations of these points were determined according to the "Laboratory Animal Acupuncture Atlas" developed by the National Acupuncture Society for Experimental Research. Electroacupuncture device, and EA stimulation parameters were as follows: sparse wave, 2 Hz, 2 V, and 0.6 mA current intensity. In the memantine group, the drug (20 mg/kg) was administered intragastrically (i.g.) every 24 h for 2 months.

After testing in the Morris water maze (MWM), mice were killed by decapitation, and the brains were immediately removed and divided into halves. The right hemisphere was fixed in 4% paraformaldehyde, and routine paraffin sections (6 μm) were prepared for morphological analysis. The cerebral cortex was dissected from the left hemisphere. The cortex was stored at −80 °C for biochemical, Western blotting, and enzyme-linked immunosorbent assay (ELISA) analysis.

Morris water maze test
The spatial learning and memory of the mice were evaluated through the MWM test, as previously described with few modifications. The test included 1 d of visible platform tests, 4 successive days of hidden platform tests and 1 d of probe trial. In brief, mice were trained individually for 1 d (3 trials with an interval of 30 min) to find the visible escape platform. Next, the mice were given a place navigation test for 4 consecutive days. The platform was placed just below the water surface, and each mouse was subjected to two trials per day at an interval of 60 s. If the mouse failed to find the platform within 60 s, it was guided to the platform, and the escape latency was recorded as 60 s. On the last day of the test (day 6), a probe test was performed. The platform was removed and each mouse was given 60 s to locate where the platform was originally placed. Finally, the recorded indicators including swimming speed, escape latency, and the number of passing times were analyzed.

Immunohistochemistry
Immunohistochemistry was conducted as previously described. The right half of each brain was fixed in 4% paraformaldehyde and routine paraffin sections (6 μm) were prepared for morphological analysis. The paraffin sections were dewaxed, rinsed, and blocked with 5% normal goat serum for 25 min. The sections were incubated overnight at 4 °C with the primary antibodies. The primary antibodies used in immunohistochemical staining included rabbit polyclonal antibodies against Iba-1 (1:300 dilution Abcam, Copenhagen, Denmark) and glial fibrillary acidic protein (GFAP) (1:300 dilution Abcam, Copenhagen, Denmark). On the second day, sections were performed using biotinylated secondary anti-rabbit IgG (1:500 dilution Beijing HANS-LH202 electroacupuncture device, and EA stimulation parameters were as follows: sparse wave, 2 Hz, 2 V, and 0.6 mA current intensity. In the memantine group, the drug (20 mg/kg) was administered intragastrically (i.g.) every 24 h for 2 months.

After testing in the Morris water maze (MWM), mice were killed by decapitation, and the brains were immediately removed and divided into halves. The right hemisphere was fixed in 4% paraformaldehyde, and routine paraffin sections (6 μm) were prepared for morphological analysis. The cerebral cortex was dissected from the left hemisphere. The cortex was stored at −80 °C for biochemical, Western blotting, and enzyme-linked immunosorbent assay (ELISA) analysis.

Morris water maze test
The spatial learning and memory of the mice were evaluated through the MWM test, as previously described with few modifications. The test included 1 d of visible platform tests, 4 successive days of hidden platform tests and 1 d of probe trial. In brief, mice were trained individually for 1 d (3 trials with an interval of 30 min) to find the visible escape platform. Next, the mice were given a place navigation test for 4 consecutive days. The platform was placed just below the water surface, and each mouse was subjected to two trials per day at an interval of 60 s. If the mouse failed to find the platform within 60 s, it was guided to the platform, and the escape latency was recorded as 60 s. On the last day of the test (day 6), a probe test was performed. The platform was removed and each mouse was given 60 s to locate where the platform was originally placed. Finally, the recorded indicators including swimming speed, escape latency, and the number of passing times were analyzed.

Immunohistochemistry
Immunohistochemistry was conducted as previously described. The right half of each brain was fixed in 4% paraformaldehyde and routine paraffin sections (6 μm) were prepared for morphological analysis. The paraffin sections were dewaxed, rinsed, and blocked with 5% normal goat serum for 25 min. The sections were incubated overnight at 4 °C with the primary antibodies. The primary antibodies used in immunohistochemical staining included rabbit polyclonal antibodies against Iba-1 (1:300 dilution Abcam, Copenhagen, Denmark) and glial fibrillary acidic protein (GFAP) (1:300 dilution Abcam, Copenhagen, Denmark). On the second day, sections were performed using biotinylated secondary anti-rabbit IgG (1:500 dilution Beijing HANS-LH202 electroacupuncture device, and EA stimulation parameters were as follows: sparse wave, 2 Hz, 2 V, and 0.6 mA current intensity. In the memantine group, the drug (20 mg/kg) was administered intragastrically (i.g.) every 24 h for 2 months.
Zhongshan Gold-en Bridge Biological Technology Co. Ltd., (China) for 1 h and streptavidin peroxidase for 1 h at room temperature. The staining procedures followed the instructions of the 3, 3’-diaminobenzi-dine tetrahydrochloride (DAB). To assess the Iba1-positive microglia and GFAP-positive astrocytes in the cortex, 3 sections with the same reference position were selected from each mouse and were counted by under high power lens (× 400) using a computerized image analysis system. In each section, 5 visual fields were randomly chosen to count GFAP and Iba1 positive cells.\(^1\)

**Western blotting**

The Western blotting test was performed as previously described.\(^4\) The mouse cortical tissues were homogenized in RIPA buffer and centrifuged at 16099 \(\times g\) for 30 min. Supernatant was collected and total protein concentration was quantified by using the Bicinchoninic Acid protein assay kit. Twelve microgram proteins samples were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis bis-Tris gels and then transferred to PVDF membranes. Non-specific binding sites on the membrane were blocked with 5% milk in in TBS containing 0.1% Tween 20 for 1 h at room temperature. The membranes containing the protein were incubated with anti-GFAP (1 : 1000; Abcam, Cambridge, MA, USA) and anti-Iba1 (1 : 1000; Abcam, Cambridge, MA, USA) and mouse anti-β-actin (0.04 μg/mL; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C. After washing with TBST, the membrane was incubated with horseradish peroxidase conjugated anti-rabbit or anti-mouse (1 : 1000; Abcam, Cambridge, MA, USA) and anti-Iba1 (1 : 1000; Santa Cruz, CA, USA) for 1 h at room temperature. The membranes were washed again with TBST, and the immunoreactive bands were visualized with super enhanced chemiluminescence reagent (Applygen Technologies Inc., Beijing, China).

**Enzyme-linked immunosorbent assay (ELISA)**

The dissected cerebral tissues were weighed and solubilized in phosphate buffer solution to a final concentration of 100 mg tissue/mL. The frozen lesioned side of the cortex was mechanically homogenized using 1 mL syringe. The supernatant was collected after centrifugation. Levels of TNF-\(\alpha\), IL-17, and IL-6 in the mouse cerebral tissues were measured by ELISA kits (KeyGen Biotech Inc., Nanjing, China) according to the manufacturer’s instructions. Absorbance was recorded at 450 nm using a 96-well plate reader. The inflammatory mediator measurements were expressed as picogram per milliliter (pg/mL).

**Statistical analysis**

All data were analyzed by SPSS (version 17.0; SPSS, Inc., Chicago, IL, USA) software. Experimental data were expressed as mean ± standard deviation (\(x \pm s\)). Two-way analysis of variance (ANOVA) with repeated measures was used for analyzing data from the Morris water maze test. Other statistical tests were performed using one-way ANOVAs followed by the least significant difference post hoc test. \(P < 0.05\) was considered statistically significant.

**RESULTS**

**Effect of EA on learning and memory in SAMP8 mice**

The MWM test was performed to evaluate effects of EA on cognitive function in the SAMP8 mice. In the place navigation (hidden platform) tests, the SAMP8 mice spent more time searching for the hidden platform than the SAMR1 normal controls (\(P > 0.05\), Figure 1A). The mean escape latency of the SAMP8 mice treated with EA or memantine significantly decreased compared with that of SAMP8 mice (\(P < 0.05\)). In the probe trial on the last day of testing, the SAMP8 mice passed the former platform location significantly fewer times than the SAMR1 mice in the normal control group (\(P < 0.05\)), and such deficit was ameliorated by EA and memantine treatment (\(P < 0.05\), Figure 1B). Moreover, as shown in Figure 1C, in the probe trial, the SAMP8 mice swam randomly in the tank without knowing the target location, whereas the SAMR1 mice, EA-treated the SAMP8 mice, and memantine-treated SAMP8 mice, searched preferentially for the target quadrant, suggesting the improved memory. However, there was no significant difference in the hidden platform and probe trial tests between the EA and memantine groups (\(P > 0.05\), Figure 1A, B). Findings from these behavioral tests indicated that EA-treated SAMP8 mice performed better than untreated the SAMP8 mice in spatial learning and memory function.

**Effect of EA on astrocyte activation in the cortex of SAMP8 mice brain**

GFAP is a sensitive marker of astrocyte activation. To investigate the effect of EA on astrocyte activation in the SAMP8 mice, we used immunohistochemical staining to examine changes of GFAP-specific markers in the cortex of the SAMP8 mice. As shown in Figure 2A, numerous GFAP-immunostained astrocytes were evident in the cortex of the SAMP8 mice in the model group. The staining of the cells was darker, and the number and thickness of neurites increased compared with the normal control group. When treated with EA or memantine, the number of astrocytes was significantly reduced and the staining was weak. The statistical results showed that the SAMP8 mice in the model group contained 2.3 times more activated astrocytes than the normal control mice (Figure 2B) (\(P < 0.05\)). EA or memantine treatment significantly attenuated the increase in the number of activated astrocytes (Figure 2B) (\(P < 0.05\)). These results suggest that EA can inhibit the inflammatory responses in the cortex of the SAMP8 mice in a manner similar to that of memantine. Next, protein expression of GFAP was assessed using Western blot analysis of the cortex of the SAMP8
Effect of EA on microglial activation in the cortex of SAMP8 mice

As macrophages in the central nervous system, microglial cells play an important role in inflammation and the immune response in the brain. Iba1 is a sensitive marker of the microglial activation. Therefore, we examined expression and distribution of microglial cell in the cortex of mouse brain using Iba1 immunohistochemical staining (Figure 3A). As shown in Figure 3A, B, levels of Iba1-immunoreactive microglia with distinct cell bodies were clearly increased in the model group compared to the SAMP1 mice. Administration of EA or memantine for 8 weeks prevented the increase in Iba1 immunoreactivity ($P < 0.05$). Protein expression of Iba1 in the cortex of SAMP8 mice was assessed using Western blot analysis. EA treatment and memantine administration attenuated Iba1 protein levels (Figure 3C, D) ($P < 0.05$). There was no clear difference between the EA and memantine groups in the Iba1 protein levels in the cortex of the SAMP8 mice.

Effect of EA on pro-inflammatory cytokine protein levels in glial cells in the cortex of SAMP8 mice

To investigate whether EA treatment inhibits production of pro-inflammatory cytokines in the cortex of the SAMP8 mice, levels of TNF-$\alpha$, IL-17, and IL-6 were analyzed by ELISA. As shown in Figure 4A-C, levels of TNF-$\alpha$, IL-17, and IL-6 were significantly increased in the cortex of the model group compared with the normal control group. The EA treatment resulted in a marked decrease in release of TNF-$\alpha$, IL-17, and IL-6 compared with the model group ($P < 0.05$). The memantine treatment decreased levels of TNF-$\alpha$ and IL-17, but not the IL-6 level compared with the model group. These results suggest that EA treatment effectively suppresses pro-inflammatory cytokine production.

DISCUSSION

Acupuncture is a complex therapeutic system and has become increasingly popular worldwide. AD is a prevalent neurodegenerative disease characterized by loss of memory and cognitive ability.16 EA is an effective method for treatment of AD that can effectively improve cognitive function among patients and animals with AD.16-18 In the current study, the SAMP8 mouse model of AD was used to investigate effects of EA treatment at Baihui (GV 20), Shenshu (BL 23), and Taixi (KI 3) points as these points are involved in the interaction between kidney and brain. Our results showed that EA treatment could improve spatial learning and memory impairment in the SAMP8 mice. Furthermore, we have shown that EA treatment at Baihui (GV 20), Shenshu (BL 23), and Taixi (KI 3) points decreased ac-
Figure 2 Effect of EA on astrocyte activation in the cortex of SAMP8 mice
A: immunohistochemical staining of GFAP (a sensitive marker of astrocyte activation) showing astrocyte activation in the cortex of the sham, model, EA, and memantine groups (40×). Scale bar = 20 μm; B: the number of GFAP-positive astrocytes in brain cortex was expressed as the ratio in the tested group to that in the normal control group; C: expression of GFAP protein was assessed by Western blot, and β-actin was used as a loading control; D: ratio of GFAP vs β-actin normalized to the sham group. The EA group was received EA administration daily for 15 min over a period of 2 months. The memantine group was treated with 20 mg/kg memantine for 2 months. The sham and model groups were grasped for the same amount of time and with the same extent of strength as mice in the EA group. EA: electroacupuncture; SAMP8: senescence-accelerated mouse prone 8; GFAP: glial fibrillary acidic protein. All values are mean ± standard deviation (n = 6). *P < 0.05 versus the sham group; †P < 0.05 versus the model group.

Figure 3 Effect of EA on microglial activation in the cortex of SAMP8 mice
A: immunohistochemical staining for Iba1 (a sensitive marker of microglial activation) showing microglial activation in the cortex of the sham, model, EA, and memantine groups (40×). Scale bar = 20 μm; B: the number of Iba1-positive microglia in brain cortex was expressed as the ratio in the tested group to that in the normal control group; C: expression of Iba1 protein was assessed by Western blot, and β-actin was used as a loading control; D: ratio of Iba1 vs β-actin normalized to the sham group. The EA group was received EA administration daily for 15 min over a period of 2 months. The memantine group was treated with 20 mg/kg memantine for 2 months. The sham and model groups were grasped for the same amount of time and with the same extent of strength as mice in the EA group. EA: electroacupuncture; SAMP8: senescence-accelerated mouse prone 8. All values are mean ± standard deviation (n = 6). *P < 0.05 versus the sham group; †P < 0.05 versus the model group.
tivation of microglia and astrocytes and decreased levels of inflammatory factors including TNF-α, IL-6, and IL-17 in the cortex of SAMP8 mice. These findings suggest that EA treatment based on the interaction between kidney and brain is effective for treatment of AD in animal models.

AD is considered an encephalopathy in Chinese medicine, which asserts that "kidney storing essence, essence generating marrow". The brain is the sea of marrow, which stores the cerebral spirit and dominates all life activities of the human body. Thus, generating brain marrow depends on the constant filling and nutrition of kidney essence. "Deficiency of kidney essence and lack of marrow sea" has been recognized by many TCM scholars as the pathological basis of AD. Based on this theory, the pathogenesis of AD is considered as consumption and deficiency of kidney-essence and incoordination between the brain and kidney. In this study, we selected 3 acupoints, Baihui (GV20), Shenshu (BL23), and Taixi (KI3), which have been proven to be able to tonify the kidney, as the acupuncture prescription. We observed the effects of the corresponding EA treatment on the spatial learning and memory impairment among the SAMP8 mice.

It has been reported that the SAMP8 mouse is a spontaneous animal model of AD. They present progressive cognitive decline and AD-like pathological changes compared with the age-matched SAMR1 mice, and differences between groups are pronounced at age of 7 months old. Moreover, many studies have used 7- to 8-month-old SAMP8 mice to investigate effects of EA and drug treatment on AD. Thus, in our study, we used this animal model at age of 8 months old to assess effects of EA in treating AD.

Acupoints, which are closely related with kidney, have been widely reported to be effective in improving learning and memory in AD animal models. However, few studies have examined treatment effects of acupoints for tonifying kidney in AD. One of the most common tests used to assess spatial learning and memory in rodents is the MWM. In our study, MWM testing revealed that the 8-month-old SAMP8 mice showed significant learning and memory deficits. In contrast, we found that after EA treatment, learning and memory ability of the SAMP8 mice was improved compared with the untreated SAMP8 mice, suggesting that EA based on the interaction between kidney and brain can improve cognitive ability in an AD animal model.

AD is characterized pathologically by deposition of Aβ deposition in the brain, which leads to neuronal damage and learning and memory decline. Numerous reactive microglia and astrocytes are often observed surrounding senile plaques and known to be major sources of neuroinflammation. It has been reported that Aβ accumulation in the brain can activate microglia and astrocytes and induce generation of pro-inflammatory cytokines, such as TNF-α, IL-17, and IL-6, leading to cognitive decline in AD. SAMP8 mice undergo a moderate level of systemic and brain inflammation as an element of their precocious senescence. Many studies have shown that reactive microglia and astrocytes appear to play significant roles in inflammatory responses and subsequent impairment of memory in SAMP8 mice. Our results demonstrated that EA based on the interaction between kidney and brain could reduce astrocyte and microglial reactivity and decrease generation of pro-inflammatory cytokines, TNF-α, IL-6, and IL-17, compared with levels in the model group. These findings suggest that EA treatment based on the interaction between kidney and brain in SAMP8 mice may be partially mediated by inhibiting activity of glial cells and decreasing expression of inflammatory factors.
mice. Future studies will determine the molecular mechanism through which EA treatment based on the interaction between kidney and brain improves cognitive ability by inhibiting the inflammatory response in AD.

REFERENCES


