Subacute electroacupuncture at Baihui (GV 20) and Dazhui (GV 14) promotes post-stroke functional recovery via neurogenesis and astrogliosis in a photothrombotic stroke mouse model

Young-Wook Park, Gi Yoon Heo, Min Jae Kim, Seo-Yeon Lee, Byung Tae Choi, Hwa Kyoung Shin

Young-Wook Park, Gi Yoon Heo, Byung Tae Choi, Hwa Kyoung Shin, Division of Meridian and Structural Medicine, School of Korean Medicine, Pusan National University, Yangsan, Gyeongnam 50612, Republic of Korea
Min Jae Kim, Seo-Yeon Lee, Byung Tae Choi, Hwa Kyoung Shin, Department of Korean Medical Science, School of Korean Medicine, Pusan National University, Yangsan, Gyeongnam 50612, Republic of Korea
Min Jae Kim, Seo-Yeon Lee, Byung Tae Choi, Hwa Kyoung Shin, Graduate Training Program of Korean Medicine for Healthy-Aging, Pusan National University, Yangsan, Gyeongnam 50612, Republic of Korea
Min Jae Kim, Seo-Yeon Lee, Byung Tae Choi, Hwa Kyoung Shin, Korean Medical Science Research Center for Healthy-Aging, Pusan National University, Yangsan, Gyeongnam 50612, Republic of Korea
Support by a 2-Year Research Grant of Pusan National University
Correspondence to: Hwa Kyoung Shin, Department of Korean Medical Science, School of Korean Medicine, Pusan National University, Yangsan, Gyeongnam 50612, Republic of Korea. julie@pusan.ac.kr
Telephone: +82-515108476
Accepted: September 21, 2018

Abstract

OBJECTIVE: To investigate the optimal timing and underlying mechanism of electroacupuncture (EA) at Baihui (GV 20) and Dazhui (GV 14) for improved long-term functional recovery after focal cerebral ischemia in a photothrombotic stroke mouse model.

METHODS: Totally 50 adult male C57BL/6J mice were assigned into 5 groups: (a) the control group, sham-operated mice (n = 10); (b) the vehicle group, focal cerebral ischemia induction without EA (n = 10); (c) the acute EA group, mice received EA immediately post-ischemia, followed by once-daily treatments for 7 consecutive days (n = 10); (d) the subacute EA group, mice received EA 4 days post-ischemia, followed by once-daily treatments for 7 consecutive days (n = 10); (e) the delayed EA group. EA stimulation (2 Hz, 2 V for 20 min) was applied to acupuncture points (acupoints), Baihui (GV 20) and Dazhui (GV 14), once a day for 7 consecutive days beginning immediately (acute treatment), 4 d (subacute treatment) and 10 d (delayed treatment) after focal cerebral ischemia in C57BL/6J mice. Behavioral assessments were conducted 21 and 28 d post-ischemia and histopathological analyses were performed 28 days post-ischemia.

RESULTS: The subacute EA treatment at Baihui (GV 20) and Dazhui (GV 14) significantly improved functional recovery compared to the vehicle group 28 d after ischemic brain injury, although brain atrophy was not reduced. The number of NeuN+ and NeuN+/BrdU+ cells as well as GFAP intensity in the ipsilateral cortex were significantly increased in the subacute group compared to the vehicle group 28 d post-ischemia. We concluded that EA stimulation 4 d post-ischemia (subacute treatment) enhanced neurogenesis and astrogliosis, likely contributing to long-term functional recovery following focal cerebral ischemia.

CONCLUSION: Our findings suggest that the timing of the EA therapy at Baihui (GV 20) and Dazhui (GV 14) determines the therapeutic effects in mice with focal cerebral ischemia induced by photothrombotic occlusion.
Keywords: Electroacupuncture; Stroke; Astrocytes; Neurogenesis; Post-stroke remodeling

INTRODUCTION

Stroke is one of the major causes of death and long-term disability worldwide. Serious physical deficits, including motor disability, are common after stroke with important implications on quality of life. The only approved acute drug treatment for ischemic stroke is tissue plasminogen activator (tPA); however, only a small percentage of patients are eligible for tPA, due to the risk of hemorrhage and limited therapeutic time window. Intravascular therapy with a mechanical recanalization device has become increasingly used, albeit with risks of various complications and no clear evidence of benefits. Therefore, it is of critical importance to develop effective treatments and rehabilitation strategies to recover lost function without risk of complications.

Stroke patients mostly rely on rehabilitation, which includes electroacupuncture (EA) treatment. Systemic reviews of stroke rehabilitation only incorporate research conducted in actual clinical settings, however, intervention timings with in RCTs with respect to stroke onset are not routinely reported. Similarly, Cochrane reviews typically draw conclusions as to the efficacy of interventions based on RCTs performed any time after stroke. Normally the first 30 d after stroke are critical for treatment initiation. However, there is no strong evidence for the appropriate timing of EA therapy for acute ischemic stroke. Therefore, a study regarding the optimal timing of this intervention to improve the effectiveness of EA therapy is required.

A systematic review of preclinical studies indicates that EA treatment decreases infarct volume and ameliorates neurological impairment in animal models with acute ischemia. A growing number of studies have shown that EA is an effective rehabilitation strategy for improving clinical outcomes after stroke. The beneficial effects of EA in acute ischemic stroke are also supported by studies in animal models, showing attenuation of ROS generation, inhibition of neuron apoptosis, improvements in cerebral blood flow, and increases in neurotrophic factors including BDNF and SDF-1α. However, the fundamental mechanism are not yet completely understood and studies on the effect of EA on chronic ischemic brain injury are currently insufficient.

Neurorestorative events contribute to functional improvements after stroke. Neurogenesis and gliosis/glial scar formation are components of brain remodeling in the chronic phase after cerebral ischemia. Neurogenesis, induced by stroke, involves proliferation of neural stem and progenitor cells, differentiation of neural progenitor cells, and migration of neuroblasts to ischemic boundaries, where neuroblasts mature and integrate into parenchymal tissues. However, these self-repair mechanism are thought to operate only in the acute phase after stroke, with the number of generated neurons small and their existence transitory. In addition, glial scars formed by reactive gliosis may act as physical and biochemical barriers for neuron regeneration. As EA increases the activation of astrocytes 3 d after ischemic brain injury, the therapy may affect astrocyte proliferation (astrogliosis) and glial scar formation. Thus, it is important to investigate the effects of EA on chronic ischemic brain injury, and resultant brain remodeling.

In this study, we aimed to evaluate the effect of EA treatment at Baihui (GV 20) and Dazhui (GV 14) on long-term functional outcomes in adult male C57BL/6J mice with cerebral ischemia induced by photothrombotic occlusion.

MATERIALS AND METHODS

Animals

All experiments were performed in accordance with the guidelines of the Pusan National University-Institutional Animal Care and Use Committee on ethical procedures and scientific care, following approval by the institutional review board of Pusan National University (approval number: PNU-2015-1041). Adult male C57BL/6J mice (6 weeks, 20-25 g) were housed under diurnal lighting conditions with free access to food and tap water, with a 12-h light/dark cycle. The mice were adapted to these conditions for at least 7 d prior to the experiments, and were assigned to the following 5 groups after collecting baseline measurements: (a) the control group, sham-operated mice (n = 10); (b) the vehicle group, focal cerebral ischemia induction without EA (n = 10); (c) the acute EA group, mice received EA immediately post-ischemia, followed by once-daily treatments for 7 consecutive days (n = 10); (d) the subacute EA group, mice received EA 4 d post-ischemia, followed by once-daily treatments for 7 consecutive days (n = 10); (e) the delayed EA group, mice received EA 10 days post-ischemia, followed by once-daily treatments for 7 consecutive days (n = 10). Computer-generated randomization was conducted by SigmaPlot 11.2 (Systat Software Inc., San Jose, CA, USA) for group allocation.

Experimental ischemic stroke mouse model

Focal cerebral ischemia was induced via photothrombosis, as previously described. Briefly, mice were anesthetized using face mask-delivered 2% isoflurane, and maintained on 1.5 % air with 80 % N2O and 20 % O2. For the surgery, the head of the mouse was fixed in a stereotactic frame (David Kopf Instruments, Tujunga, CA, USA), and the bregma and lambda points were identified following a middle scalp.
incision. A photochemical dye, Rose Bengal (Sigma-Aldrich, St. Louis, MO), was administrated intraperitoneally (10 mg/ml in saline) 5 min prior to illumination. The exposed intact skull was then illuminated with the fiber optic bundle of a KL6000 LED cold light source (Carl Zeiss, Jena, Germany) using a micromanipulator for 15 min. Thereafter, the surgical wound was sutured and the mouse was allowed to recover.

Electroacupuncture therapy
Animals were anesthetized with isoflurane to avoid restraint stress. The transpositional method was used to determine the acupuncture points (acupoints) in mice. In this method, the veterinary acupoints are located by transforming human acupoints onto the animal anatomy. The Baihui (GV 20) acupoint, located at the right midpoint of the parietal bone, and Dazhui (GV 14) acupoint, located on the posterior midline and in the depression below the spinous process of the seventh cervical vertebra, were stimulated as per the standard criteria in mice (Figure 1). Acupuncture needles (0.18630 mm) were inserted into Baihui (GV 20) and Dazhui (GV 14), after which the acupoints were stimulated at an intensity of 2 V and frequency of 2 Hz for 20 min using a Grass S88 electrostimulator (Grass Instrument Co., West Warwick, RI, USA). The delivered current was monitored throughout using a Digital Storage Oscilloscope (Tektronix Inc., Beaverton, OR, USA). The intensity was maintained just below the level inducing visible muscle contraction. Subjects in the non-EA groups received only light isoflurane anesthesia for 20 min.

Bromodeoxyuridine labeling
Bromodeoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.9 % saline and administered intraperitoneally (50 mg/kg). To analyze cell proliferation, all animals received BrdU injections once a day for 5 consecutive days after ischemia.

Behavior tests
Hanging wire test: A hanging wire test to evaluate vestibular motor function was conducted with the mouse placed on a metal wire (length: 45 cm) suspended across 2 upright poles (height: 45 cm). The mouse was scored based on the way they held onto and traversed the wire for 60 s. The score was quantified using a 5-point scale: grade 0, inability to remain on the wire for 30 s; grade 1, failure to hold on to the wire with both the fore-paws and hind-paws; grade 2, grasping the wire with the fore- and hind-paws, but not the tail; grade 3, grasping of the wire using the tail, along with the fore and hind paws; and grade 4, movement along the wire on all 4 paws using the tail.

Cylinder test
The cylinder test was adapted for use in mice to assess forelimb use and rotation asymmetry. Briefly the mouse was placed in a transparent cylinder measuring...
9 cm in diameter and 15 cm tall. After the mouse was placed in the cylinder, forelimb use (at first contact with the wall after rearing and during lateral exploration) was recorded. The final score = (nonimpaired forelimb movement - impaired forelimb movement) / (nonimpaired forelimb movement + impaired forelimb movement + both movement). This test evaluates asymmetrical forelimb use for weight shifting during vertical exploration, and provides higher inter-rater reliability, even with inexperienced raters.

**Rota-rod test**
The rota-rod test was performed to evaluate motor coordination and equilibrium, using a rota-rod apparatus (Panlab S.L.U., Barcelona, Spain). The rota-rod speed was increased from 4 to 40 rpm during the adaptation trials. Thereafter, each mouse was placed on the rotating rod. Five trials were performed per day at a speed of 18 rpm for 3 min. The data are presented as the average of 5 recorded values.

**Determination of brain atrophy**
Brain atrophy was estimated via hematoxylin and eosin (H&E) staining. Mice were anesthetized with sodium thiopental, and perfused with cold PBS followed by 4% paraformaldehyde (PFA), after which the brains were removed. Fixed brains were embedded in paraffin, serially sectioned (5 μm), and stained with H&E. Tissue slides were mounted in the mounting medium (Vector Laboratories, Burlingame, CA, USA), and the contralateral and ipsilateral hemispheres were analyzed with iSolution analysis software (Image & Microscope Technology, Vancouver, Canada).

**Immunohistochemistry**
Mice were anesthetized with sodium thiopental, perfused with cold PBS followed by 4% PFA and the brains were removed and placed into molds with paraffin. The brains were cut at a thickness of 5 μm. After deparaffinization, the brain sections were incubated at 4°C overnight with the following primary antibodies: BrdU (1: 500; OBT0030GAbD, Serotec, Oxford, UK), NeuN (1: 500; MAB377, Milipore Corporation) and GFAP (1: 100; MAB360, Milipore Corporation, Billerica, MA, USA). The sections were then incubated with fluorescent conjugated secondary antibodies (Thermo, Waltham, MA, USA) and DAPI (Invitrogen Corporation, Carlsbad, CA, USA) for 2 h at room temperature. Subsequently, slides were washed and cover-slipped with mounting medium (Vector Laboratories, Inc.). GFAP sections were processed by 3,3-Diaminobenzidine (DAB) staining. Images of each section were captured with a laser scanning confocal microscope (Carl Zeiss, Inc., Jena, Germany), and morphological analyses and quantification of positive cells were conducted using iSolution analysis software (Image & Microscope Technology). The counted cells and intensities were captured from 3 fields per 3 predefined areas per 3 adjacent brain sections from each mouse.

**Statistical analysis**
Values are expressed as mean ± standard error of mean (SEM). Statistical comparisons were performed using the SigmaStat statistical program version 11.2 (Systat Software Inc., Chicago, IL, USA). Data were analyzed using one-way analysis of variance (ANOVA) or one-way repeated ANOVA, followed by Student-Newman-Keuls tests. A P < 0.05 was considered statistically significant.

**RESULTS**

**EA subacute treatment for functional recovery**
To determine whether acute, subacute or delayed EA treatment can promote functional recovery after focal cerebral ischemia, we conducted hanging wire, cylinder, and rota-rod tests 21 and 28 d post-ischemia (Figure 2). The hanging wire test and rota-rod test indicated significant lower values and cylinder test indicated higher values in the vehicle group than in the control group, suggesting that focal ischemic brain injury impaired motor functions 21 and 28 d post-ischemia. In the hanging wire test for vestibular motor function 28 d after focal cerebral ischemia, values in the subacute EA groups were significantly higher than in the vehicle group (Figure 2A). Similarly, in cylinder test 28 d post-ischemia, values in the subacute EA group were significantly lower than in the vehicle group, suggesting improved forelimb use (Figure 2B). In addition, rota-rod test both 21 and 28 d post-ischemia showed that motor coordination was significantly recovered in the subacute EA groups compared to the vehicle group (Figure 2C).

We evaluated whether post-stroke brain atrophy was affected by EA stimulation (Figure 3). Twenty-eight days after cerebral ischemia, apparent atrophy in the ischemic cortex was observed in brain sections stained with hematoxylin-eosin, and no difference was detected according to EA treatment (Figure 3).

**EA subacute treatment for neurogenesis and astroglisis**
We observed the effects of post-ischemic EA stimulation on neuron density changes using NeuN staining. In the boundary zone adjacent to the ischemic core, NeuN− cells were significantly reduced in the vehicle group compared to the control group, and the EA treatment attenuated neuron loss in the boundary zone 28 d after focal cerebral ischemia (Figure 4). To further investigate whether EA treatment influences neurogenesis, we performed double immunostaining with NeuN and BrdU. The number of NeuN+/BrdU− cells was significantly greater in the vehicle group than in the control group, and these counts were even greater in the acute, subacute, and delayed EA groups. In addition, we found that subacute EA significantly increased the number of...
NeuN+/BrdU+ cells in the peri-infarction cortex following ischemia, when EA groups were compared.
with the vehicle group (Figure 4). In addition, subacute EA stimulation increased the density of GFAP-positive astrocytes in the boundary zone (Figure 5).

**DISCUSSION**

In this study, we examined the effect of EA therapy in mice with chronic ischemic brain injury induced by photothrombotic occlusion, and we also studied the mechanism underlying its action, and found that the EA treatment modulated post-stroke remodelling by accelerating neurogenesis and increasing astrocyte proliferation around ischemic tissues. However, the EA treatment did not reduce brain atrophy or lesions 28 d after ischemia. However, the most effective time window for the EA treatment after ischemic stroke is unclear. Treat-
ment timing is important for stroke prognosis, but only a few studies have addressed the optimal start time for rehabilitation after acute ischemia stroke. One study indicated that initiating rehabilitation earlier (i.e. within 5 d) rather than later (i.e. within 30 d provided significantly greater functional recovery. Exposing rats to an enriched environment in combination with daily sessions of reach training therapy following middle cerebral artery occlusion resulted in significant gains in forelimb reaching ability, when rehabilitation was initiated 5 or 14 d, but not 30 d after stroke. However, some studies have shown that early treatment can irreversibly damage tissues and thus delay functional recovery. In our study, we found that when EA was initiated 4 days after focal cerebral ischemia (subacute treatment), better behavioral outcomes were achieved than when EA was initiated earlier (1 d after focal cerebral ischemia; acute treatment) or later (10 d after focal cerebral ischemia; delayed treatment), consistent with previous studies. Thus, the brain is most receptive to rehabilitation 4 to 10 d after stroke.

Statistical analyses of clinical results are indicative of the beneficial effects of EA therapy on stroke. A systematic review of preclinical studies indicates that the EA treatment decreases infarct volume, ameliorates neurological impairment, and plays a neuroprotective role in animal models with acute ischemia. However, in our study, neuroprotection did not restore function in the chronic phase after cerebral ischemia. Neurorestoration, including neurogenesis and gliosis/glial scar formation, contributes to functional improvements after stroke. We found the subacute EA stimulation significantly increased NeuN and NeuN+/BrdU cells to detect neurons and proliferative neurons in the peri-infarction cortex, suggesting that subacute EA enhances newly formed neurons within the peri-infarction region following ischemic brain injury. It is possible that the regeneration of neurons around necrotic tissues resulted in behavioral improvements.

In stroke, cerebral ischemia induces the activation of astrocytes and formation of glial scars in the brain. In the acute phase of ischemic stroke, astrocytes in the peri-infarct region are proliferative, referred to as astrogliosis, and perform both beneficial and harmful functions. Astrogliosis remodels injured tissues and temporarily controls local immune responses; activated astrocytes can upregulate GFAP, an astrogliosis and glial scar iconic protein in the ischemic brain. Excess astrogliosis creates a wall-like structure depicted as a glial scar. Excess astrogliosis and glial scars interfere with neurite outgrowth and neurogenesis. Therefore, inducing appropriate astrogliosis may be an important strategy in the treatment of ischemic stroke. EA stimulation can activate astrocytes in the peri-infarct region, and promote recovery of behavioral dysfunction after ischemia. Accordingly, a significant increase of GFAP staining in the subacute group was observed in our study, suggesting that astrogliosis was promoted, which may have resulted in behavioral improvements.

In conclusion, the subacute EA treatment at Baihui (GV 20) and Dazhui (GV14) attenuates late injury, modulating post-stroke remodeling by accelerating neurogenesis and increasing astrocyte proliferation around the ischemic tissues in the photothrombotic stroke mice.
REFERENCES

18. Wang WW, Xie CL, Lu L, Zheng GQ. A systematic review and meta-analysis of Bailu (GV 20)-based scalp acupun -
35. Liu SY, Hsieh CL, Wei TS, Liu PT, Chang YJ, Li TC. Acupuncture stimulation improves balance function in stroke patients: a single-blinded controlled, randomized


