JTCM Journal of Traditional Chinese Medicine

Online Submissions: http://www.journaltcm.com
ISSN 0255-2922
info@journaltcm.com

Original Article

Electroacupuncture ameliorates blood-brain barrier disruption after ischemic stroke through histone acetylation regulation at the matrix metalloproteinase 9 and tissue inhibitor of metalloproteinase 2 genes

CHEN Yonglin, OUYANG Ling, MENG Lingling, WU Bufan, PENG Rou, LIU Sitong, HOU Dan, WANG Yaling, JING Xinyue, LU Shengfeng, FU Shuping

CHEN Yonglin, OUYANG Ling, MENG Lingling, WU Bufan, PENG Rou, LIU Sitong, HOU Dan, WANG Yaling, JING Xinyue, LU Shengfeng, FU Shuping, Key Laboratory of Acupuncture and Medicine Research of Ministry of Education, Nanjing University of Chinese Medicine, Nanjing 210023, China

Supported by the National Natural Science Foundation of China: the Role of Intestinal Flora-Treg/γδT Cell-IL-17 Signaling in the Neuroprotective Effect of Electroacupuncture on Ischemic Brain Injury (No. 81774403), the Natural Science Foundation of the Jiangsu Province of China: Study on the Mechanism of Acupuncture Anti-stroke Immune Inflammatory Response Based on Intestinal Treg/γδT Cell-IL-17 Signaling Pathway (No. BK20171492), the Postgraduate Scientific Research Innovation Practice Project of the Jiangsu Province of China: a Study based on the Butyric Acid-HDAC-Foxp3 Pathway to Explore the Regulatory Effect of Acupuncture on Intestinal Treg in Rats with Stroke (No. KYCX21_1715), a Study on the Anti-brain Injury of Electroacupuncture Based on Intestinal Microbiota-Treg/γδT Cell-IL-17 Pathway (No. KYCX21_1716), and the Key University Science Research Project of Jiangsu Province: the Role of Preactivation of the Treg Immune Response in the Mechanism of Anti-stroke Neuroinflammatory Response in Acupuncture Pretreatment (No. 22KJA360003)

Correspondence to: Prof. FU Shuping, Key Laboratory of Acupuncture and Medicine Research of Ministry of Education, Nanjing University of Chinese Medicine, Nanjing, 210023, China. shupingfu@njucm.edu.cn; Prof. LU Shengfeng, Key Laboratory of Acupuncture and Medicine Research of Ministry of Education, Nanjing University of Chinese Medicine, Nanjing, 210023, China. lushengfeng@njucm.edu.cn
Telephone: +86-18761894980
DOI: 10.19852/j.cnki.jtcm.20240610.004
Received: February 22, 2023
Accepted: July 20, 2023
Available online: June 10, 2024

Abstract

OBJECTIVE: To explore whether the regulation of matrix metalloproteinase 9 (MMP-9)/ tissue inhibitors of MMPs (TIMPs) gene expression through histone acetylation is a possible mechanism by which electroacupuncture (EA) protects blood-brain barrier (BBB) integrity in a middle cerebral artery occlusion (MCAO) rat model.

METHODS: Male Sprague-Dawley rats were divided into four groups: the sham group, the MCAO group, the MCAO + EA (MEA) group, and the MCAO + EA + HAT inhibitor (HATi) group. The MCAO model was generated by blocking the middle cerebral artery. EA was applied to Baihui (GV20). Samples were collected 1 or 3 d after reperfusion. Neurological function scores and Evans blue extravasation were employed to evaluate the poststroke injury. The effect of EA on MMP-9/TIMPs gene expression was assessed by real-time fluorescence quantitative polymerase chain reaction (RT-qPCR) and chromatin immunoprecipitation (ChIP).

RESULTS: Our results showed that EA treatment prominently improved neurological function and ameliorated BBB disruption. The RT-qPCR assay showed that EA reduced the expression of MMP-9 and promoted TIMP-2 mRNA expression, but HATi reversed these effects of EA. In addition, ChIP results revealed that EA decreased the enrichment of H3K9ace/H3K27ace at MMP-9 promoters and notably stimulated the recruitment of H3K9ace/H3K27ace at TIMP-2 promoter.

CONCLUSION: EA treatment at Baihui (GV20) regulates the transcription of MMP-9 and TIMP-2 through histone acetylation modification in the acute stage of stroke, which preserves the structural integrity of the BBB in MCAO rats. These findings suggested that the histone acetylation-mediated transcriptional activity of target genes may be a crucial mechanism of EA treatment in stroke.

Keywords: blood-brain barrier; matrix metalloproteinase 9; tissue inhibitor of metalloproteinases; electroacupuncture; histone acetylation

1. INTRODUCTION

Stroke, caused by the interruption of blood flow in the brain area, will lead to death or permanent neurological dysfunction, which is one of the main causes of death and disability in adult worldwide and results in heavy health and economic burdens globally. Meanwhile, ischemic stroke is the most common type of stroke.[1] The destruction of the blood-brain barrier (BBB) plays a significant role in the occurrence and development of

ischemic stroke. The BBB is composed of brain microvascular endothelial cells, pericytes, astrocytes, neuronal processes, perivascular microglia, and the basal lamina.[2] The BBB affects the communication between central nervous system (CNS) cells and peripheral cells. Its barrier protection function contributes to maintaining the microenvironmental homeostasis of the CNS. After the interruption of cerebral blood flow, oxidative stress, apoptosis, and the inflammatory response induced by hypoxia and ischemia lead to the activation of matrix metalloproteinases (MMPs) and the degradation of tight junctions (TJs), which destroy the permeability of the BBB. Peripheral immune cells, chemicals, and fluid spread into the brain parenchyma across the impaired BBB and aggravate tissue damage.[3]

MMPs are key substances that induce BBB injury after cerebral ischemia and reperfusion. For example, MMP-9 has been the focus of studies on BBB breakdown after stroke.[2] In patients with ischemic stroke, the degree of BBB damage is related to the level of MMP-9.[2] Research has found that increased serum MMP-9 levels in patients with acute ischemic stroke are associated with higher risk of mortality and severe disability.[4] Another clinical study confirmed that infarct tissue has increased MMP-9 content, which is related to neutrophil infiltration and microglial activation.[5] A further study revealed that MMP-9-positive neutrophils were highly expressed surrounding cerebral microvessels, which was associated with basal lamina type IV collagen degradation and bleeding tendency, suggesting that MMP-9 was involved in the destruction of the BBB and hemorrhagic complications after stroke.[6] On the other hand, for the experimental stroke models, a large number of research showed that MMPs can open the BBB by degrading TJs such as Claudin-5 and occludin.[7, 8] In MMP-9 knockout mice, the degradation of BBB components is obviously alleviated, and ischemic injury is also reduced.[9] MMPs inhibitors significantly maintain the integrity of BBB by inhibiting the activity of MMP-9.[10] Short-term early inhibition of MMPs promotes the expression of tight junction protein and neurovascular remodeling, which contribute to the restructuring of the BBB and the reduction of brain injury.[11] The broad spectrum MMP inhibitor, BB-1101, prevents degradation of the TJs and reduces the BBB opening by inhibiting MMPs activity.[8] The selective gelatinase inhibitor inhibits MMP-9 activity, leading to the attenuation of neuronal laminin degradation and protecting neurons from cerebral ischemia.[12] Tissue inhibitors of MMPs (TIMPs), as endogenous inhibitors of MMPs, contribute to the control of MMP activity.[13] A case-control study suggests that TIMP-2 polymorphisms is associated with a lower risk of large artery atherosclerotic stroke.[14] And many preclinical studies indicated that increasing the expression of TIMP-1 or TIMP-2 contributes to the protection of the BBB.[13,15] As MMP-9 levels are increased after stroke, the expression of TIMP-1 is altered in a time-, region-, and cell-dependent manner.[16] TIMP-1 and MMP-9 transcriptional activities were both upregulated in ischemic hemispheres and TIMP-1$^{-/-}$ mice had higher MMP-9 protein expression and activity than WT mice. In addition, TIMP-1$^{-/-}$ and TIMP-2$^{-/-}$ mice showed a significant increase in Evans blue extravasation and a decrease in zonula occludens-1 expression compared with WT mice.[17] All these findings suggest a closely relationship between TIMPs and BBB damage.

Acupuncture, a kind of classical Traditional Chinese Medicine therapy, has a long history in the treatment of stroke. Combined with acupuncture and electrical stimulation, electroacupuncture (EA) is extensively used in experimental research and clinical treatment of ischemic stroke.[18] Previous studies have indicated that EA effectively alleviates BBB disruption following ischemic stroke.[18-20] Accompanied by a reduced infarct area and neurological deficits in a rat model of MCAO, MMP-9 mRNA and protein expression decrease with EA treatment.[21-23] These findings indicate that the neuroprotective role of EA against ischemia/reperfusion (I/R) injury is associated with MMP-9 expression. In stroke and many other disease models, it has been found that EA can regulate the mRNA and protein expression of MMP9 and TIMPs.[21,24,25] Numerous studies have confirmed that EA has a significant regulatory effect on gene transcription activity in the acute stage of stroke.[26-29] Single or multiple EA treatments can significantly regulate the transcription level of neurotrophic factors, chemokines, inflammatory factors, apoptosis, and tight junction related genes in the ischemic hemisphere.[30,31] However, the exact mechanisms by which EA regulates the transcription activity of target genes have not been completely clarified and remain to be explored.

Histone lysine acetylation is an important epigenetic mechanism regulating gene expression in eukaryotic cells. The acetyl level of histones is usually regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs).[32] Studies have shown that the acetylation level of histone H3 in ischemic brain tissue is significantly reduced and the activity of HDACs is increased in a variety of cerebral ischemia model animals.[33,34] The use of HDAC inhibitors or HAT can effectively block the decrease of histone acetylation level in ischemic brain tissue and play an anti-brain injury role by regulating the transcription activity of genes related to oxidative stress, apoptosis, inflammation, BBB breakdown, and angiogenesis.[35-38] In previous studies, we discovered that EA treatment upregulated VEGF expression by augmenting H3K9ace occupancy at the VEGF promoter region and promoted angiogenesis after myocardial ischemia.[39] We also confirmed that EA decreased the HDAC activity of rats with cerebral ischemia.[40] Xu *et al* [41] also reported that EA increased the enrichment of H4K16ace on the promoter of Beclin1, which is an autophagic marker, and alleviated I/R injury. These results indicated that the protective effect of EA is associated with the histone acetylation pathway.

In this study, we applied EA treatment on Baihui (GV20) in rats with middle cerebral artery occlusion (MCAO) and observed the correlation between EA, MMP-

9/TIMPs transcriptional activity, and histone acetylation to investigate whether the protective effects of EA on the BBB are mediated by histone acetylation modification of the target gene promoter.

2. MATERIALS AND METHODS

2.1. Experimental animals and grouping

Adult male specific pathogen-free (SPF) Sprague-Dawley rats (7-8 weeks old, 250-280 g), provided by the experimental animal center of Nanjing University of Chinese Medicine, were used in this experiment [animal license: SCXK (JING) 2019-0010]. Animals were housed under a 12-hour light cycle and had free access to food and water. After adaptive feeding for one week, the rats were divided by the random number method into the following four experimental groups: the sham operation group (sham, n = 18), the middle cerebral artery occlusion group (MCAO, n = 18), the MCAO + EA group (MEA, n = 18), and the MCAO + EA + HAT inhibitor group (HATi, n = 11). All rats were fasted (with free access to water) for 24 h before the surgical operation. All the operations, including surgery, EA treatment, and inhibitor intervention, were performed in the morning. The study was performed following the guidelines of the Care and Use of Laboratory Animals published by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the Nanjing University of Chinese Medicine (ethics approval number: 201911A018).

2.2. *In vivo* MCAO

The rat MCAO model was established to induce cerebral ischemia-reperfusion injury as described previously.[42,43] During the operation, the temperature of the rats was kept at 37 ℃ with the heating pad. Rats were deeply anesthetized by inhalation of 5% isoflurane and maintained under anesthesia with 2% isoflurane (in a mixture of 70% N_2O and 30% O_2). After disinfection, an incision was made in the middle of the neck, and the muscle tissue was bluntly separated. The left common carotid artery was separated and clamped. A 3/0 monofilament nylon suture was inserted into the left external carotid artery, entered the anterior cerebral artery through the internal carotid artery, and finally blocked the middle cerebral artery. After 2 h of ischemia, the nylon suture was removed to induce reperfusion. In the sham group, the same procedure was performed but without suture insertion. After the operation, the rats were transferred to cages with clean padding and given adequate feed and water, and the ambient temperature was maintained at 25 ℃.

2.3. EA treatment

Rats in the MEA group and the HATi group were subjected to EA treatment at the Baihui (GV20), which is located at the line midpoint joining the auricular apices. They received EA treatment once a day after I/R injury. The rats that were sacrificed 1 or 3 d after stroke received 2 or 4 EA treatments respectively. When the cerebral blood flow was recovered, the first EA intervention was performed. The last treatment was applied two hours before they were executed. Animals received isoflurane anesthesia. The handles of the two needles were wrapped with adhesive tape respectively, and then the two needles were bound tightly together. Two acupuncture needles were inserted into Baihui (GV20), which was connected to Han's electroacupuncture instrument (Nanjing Jisheng Medical Technology, Nanjing, China). The strength was 1.0 mA for 30 min each time, and the frequency was 2/15 Hz. The rats of the sham group and MCAO group were only anesthetized with light isoflurane in the same way for 30 min when the MEA group received treatment.

2.4. HATi intervention

When EA stimulation was started after withdraw of the suture, rats in the HATi group were intraperitoneally injected with Histone Acetyltransferase Inhibitor Ⅱ simutanously (Millipore, Darmstadt, Germany) at a concentration of 1 mg/mL and a dosage of 4 mg/kg. Each rat was injected once.

2.5. Neurobehavioral evaluation

The neurobehavioral evaluation was performed 1 or 3 d after reperfusion according to the Longa neurologic score: a score of 0 indicated no neurological deficits; a score of 1 indicated failure to fully extend the right forepaw; a score of 2 circled to the right; a score of 3 fell to the opposite side; a score of 4 was unable to walk independently and had a depressed level of consciousness; and a score of 5 died. Rats with scores of 2 and 3 were included in the current study.

2.6. Evans blue (EB) extravasation

EB extravasation was carried out 1 or 3 d after I/R injury. The rats were anesthetized 1 hour before sacrifice. Evans blue solution (2% in saline, 3 mL/kg) was injected into the femoral vein, and the dye was allowed to circulate for 1 hour. Then, they were perfused with saline from the heart until clarified liquid flowed out from the right auricle, and the brain was collected on ice. Next, each hemisphere was weighed and homogenized in formamide (3 mL/600 mg). After 24 h of incubation at 60 ℃, the samples were centrifuged, and the supernatant was aspirated. The absorbance at 632 nm was measured by a microplate reader to evaluate the permeability of the BBB.

2.7. Real-time quantitative polymerase chain reaction (RT-qPCR)

Rats were transcardially perfused with cold saline, and the brain was collected 24 h after reperfusion. TRIzol (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA of the ischemic brain. Then, the RNA was reverse-transcribed by a Revert Aid First Strand cDNA Synthesis kit (Thermo, Waltham, MA, USA). The two-step PCR cycling protocol was performed with SYBR

GREEN PCR Master Mix (QIAGEN, Hilden, Germany) according to the manufacturer's recommendation. The relative expression of target genes was analyzed by the $2^{-\Delta\Delta CT}$ method, with GAPDH as the internal reference gene. The primer sequences were as follows: MMP-9, 5'-GCATCTGTATGGTCGTGGCT-3' (forward), and 5'-TGCAGTGGGACACATAGTGG-3' (reverse); TIMP-1, 5'-CGAGACCACCTTATACC-AGCG-3' (forward), and 5'-CAGGAAGCTGCAGG-CAGTGAT-3' (reverse); TIMP-2, 5'-GCTGCGAGTG-CAAGATCACA-3' (forward), and 5'-CGCGCAAGA-ACCATCACTTC-3' (reverse); and GAPDH, 5'-GGCACAGTCAAGGCTGAGAATG-3' (forward), and 5'-ATGGTGGTGAAGACGCCAGTA-3' (reverse).

2.8. Chromatin immunoprecipitation (ChIP) assay

A ChIP assay was performed to determine histone modifications in target gene promoter regions. Using 150 mg ischemic hemispheric tissue, the steps of the ChIP kit (Cell Signaling Technology, Danvers, MA, USA) instructions were followed. In brief, first, DNA-protein cross-linking experiments were performed, followed by nuclear extraction and chromatin fragmentation to obtain protein-chromatin lysates. Chromatin immune-oprecipitation was performed with the corresponding antibodies (normal rabbit IgG, H3K9ace rabbit mAb, H3K27ace rabbit Ab, Cell Signaling Technology, Danvers, MA, USA) and Protein G magnetic beads. The chromatin was then de-crosslinked and purified. Finally, the target genes bound to H3K9ace or H3K27ace were amplified by qPCR. The primer sequences were as follows: MMP-9: −21 bp-− 177 bp: 5'-CCAGAA-GCTTCGGTTCTCAC-3' (forward) and 5'-AGCCCTTCCCAACTTTCACT-3' (reverse); MMP-9: − 678 bp - − 886 bp: 5'-AGCCAAGACCCCAG-TCTAGT-3' (forward) and 5'-AGGCTGGCTAACAT-CGTTCC-3' (reverse); TIMP-1: − 271 bp - − 549 bp: 5'-TAAGCCCCTGAAGCC-TTGTG- 3' (forward) and 5'-CAACGCTCACCACA-CCATTC-3' (reverse); TIMP-1: −580 bp -−822 bp: 5'-ACAGACAGA-GCCCAGGTAGT-3' (forward) and 5'-TAAGCCCC-TGAAGCCTTGTG-3' (reverse); TIMP-2: − 248 bp - − 425 bp: 5'-GCCCATCAGGAATAA-CCCGT-3' (forward) and 5'-AGAACCCCGGCTTTT-GTTGT-3' (reverse); TIMP-2: −34 bp - −150 bp: 5'-CCTCGGAGCGCAATAAAACG-3' (forward) and 5'-ATGGTGGTGAAGACGCCAGTA-3' (reverse).

The relative enrichment of H3K9ace/H3K27ace in the promoter region of target genes in each group was compared by percentage, that is, percent input = 2% × $2^{[c\ (T)\ 2\%\ input\ sample-c\ (T)\ IP\ sample]}$.

2.9. Statistical analysis

The data is presented in the form of the mean ± standard deviation (*SD*). All statistical analyses were performed using Prism 8.0.2 (GraphPad Software, Boston, MA, USA). Data were subjected to the normality test, and then group comparisons were determined by one-way analysis of variance. Bonferroni's multiple comparisons test was used when the variances were homogeneous, and Tamhane's T2 multiple comparisons test for non-homogeneous variances. Use the Kruskal-Wallis test for data with a nonnormal distribution. Differences were considered statistically significant when $P < 0.05$.

3. RESULTS

3.1. EA at Baihui (GV20) reduced the neurological function score and BBB permeability in ischemical stroke rats

A schematic diagram of the experimental procedures is shown in Figure 1A. In the sham group, rats received sham surgery. Other rats received MCAO surgery to induce ischemia. Animals received isoflurane anesthesia during operation. After 2 h of ischemia, the nylon suture was removed to induce reperfusion. Some rats with a Longa neurologic score from 2 to 3 were included and divided into the MCAO, MEA, and HATi groups. The rats in the MEA and HATi groups underwent EA treatments at the "Baihui (GV20)" point once a day after stroke. The HATi group received HATi intervention after reperfusion. Rats were sacrificed after 1 or 3 d of reperfusion.

To investigate the protective effect of EA on cerebral injury induced by I/R, we first measured the neurological function score and BBB permeability. Longa neurologic score was used to evaluate neurological function. The neurological function score increased significantly 1 and 3 d after stroke, while EA reduced the score evidently (Figures 1B, 1C). EB extravasation was used to evaluate BBB permeability. The exudation of EB increased because the BBB was damaged after ischemia (Figure 1D). This result showed that, compared with the sham group, I/R injury markedly increased the EB content of brain tissue at 24 h and 3 d. However, EB penetration in the MEA group was noticeably less than that in the MCAO group ($P < 0.01$) (Figures 1E, 1F). In addition, rats given EA plus HATi intervention had higher neurological function score and EB penetration than rats in the MEA group.

3.2. EA effectively regulates the expressions of MMP-9 and TIMP-1/2 Genes in the MCAO rats ischemic hemisphere

We tested the mRNA expression of MMP-9 (Figure 2A), TIMP-1 (Figure 2B), and TIMP-2 (Figure 2C) in the ischemic hemisphere. The RT-qPCR results suggested that I/R injury significantly increased mRNA expression of MMP-9, TIMP-1, and TIMP-2. EA intervention prominently decreased mRNA expression of MMP-9 and TIMP-1 but enhanced the increase of expression in TIMP-2. Compared to the MEA group, cotreatment with EA and HATi prominently reversed the tendency of the mRNA levels of MMP-9, TIMP-1, and TIMP-2. EA Markedly Regulated H3K9ace Occupancy at Target Gene Promoters.

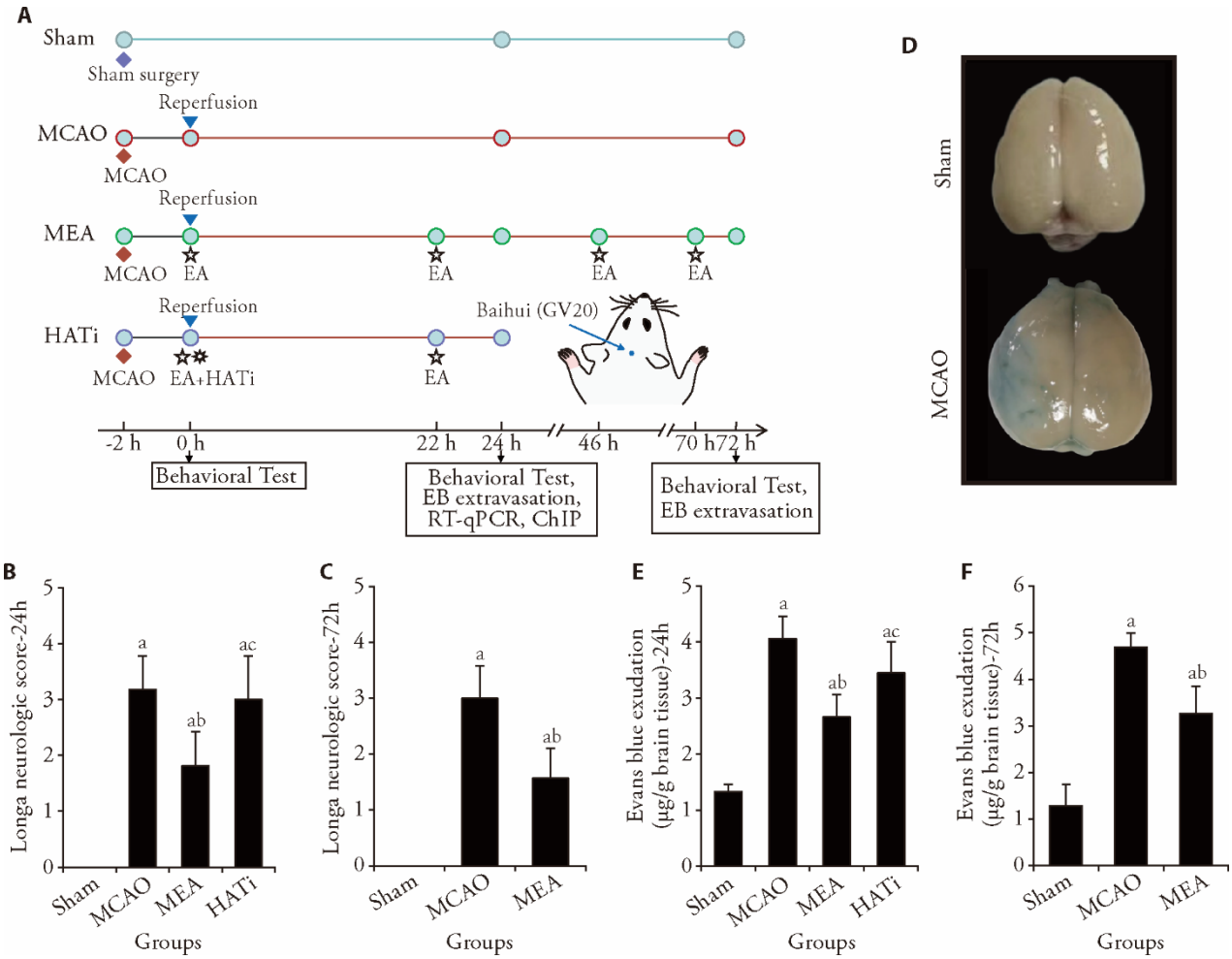

Figure 1 EA treatment reduced cerebral damage after I/R injury in rats

A: the experimental timeline; B: Longa neurologic score 1 d after ischemia, n = 11; C: Longa neurologic score 3 d after ischemia, n = 7; D: representative photograph of Evans blue extravasation in the brains of rats 24 h after I/R injury. The blue area shows penetrating Evans blue, signifying BBB destruction; E: quantitative analysis of Evans blue penetration in each group 1 d after ischemic stroke, n = 7; F: Evans blue exudation 3 d after ischemia, n = 4. Sham group: sham operated; MCAO group: MCAO operated; MEA group: MCAO rats received 3 d of EA treatment; HATi group: MCAO rats received 3 d of EA treatment and a single 4 mg/kg intraperitoneal injection of HATi. I/R: ischemia/reperfusion; MCAO: middle cerebral artery occlusion; EA: electroacupuncture; HATi: histone acetyltransferase inhibitor; EB: Evans blue; RT-qPCR: real-time quantitative polymerase chain reaction; ChIP: chromatin immunoprecipitation; BBB: blood-brain barrier. Data were analyzed by one-way analysis of variance and are represented as the mean ± standard deviation. $^{a}P < 0.05$ *vs* the sham group, $^{b}P < 0.05$ the MCAO group *vs* the MEA group, $^{c}P < 0.05$ the MEA group *vs* the HATi group.

ChIP assay results indicated that, compared with the sham group, H3K9ace occupancy at the −21 bp - −177 bp or −678 bp - −886 bp region of MMP-9 was augmented prominently in the MCAO group. EA treatment decreased the enrichment of acetylated H3K9 at these two sites compared with MCAO ($P < 0.01$). The application of HATi increased H3K9ace occupancy at both regions compared with the MEA group (Figure 3A, 3B).

We also selected two regions of TIMP-1 promoters. There was no significant difference in acetylation level between groups at the −271 bp - −549 bp region (Figure 3C). At the −580 bp - −822 bp region, I/R injury reduced the acetylation level compared with sham surgery. EA treatment reversed this decline, and HATi partially blocked the effect of EA (Figure 3D).

For the binding of H3K9ace to the two regions of the TIMP-2 promoter, at the −248 bp - −425 bp region, the acetylation level was reduced after I/R injury and EA treatment had no obvious effect on H3K9ace enrichment in this region compared with the MCAO group (Figure 3E). At the −34 bp - −150 bp region, no amplification products of the H3K9ace-DNA fragment could be detected after I/R injury, but significant enhancement of acetylation was detected in the MEA group ($P < 0.01$). The enrichment of H3K9ace in the MEA group increased more than 200 times compared with that in the sham group. Similarly, HATi treatment partially blocked the effect of EA ($P < 0.01$) (Figure 3F).

3.3. EA Notably Regulated H3K27ace Occupancy at Target Gene Promoters

The result of H3K27ace-DNA fragment amplification also revealed that H3K27ace occupancy at the −21 bp - −177 bp region and the −678 bp - −886 bp region of MMP-9 was prominently augmented in rats after MCAO.

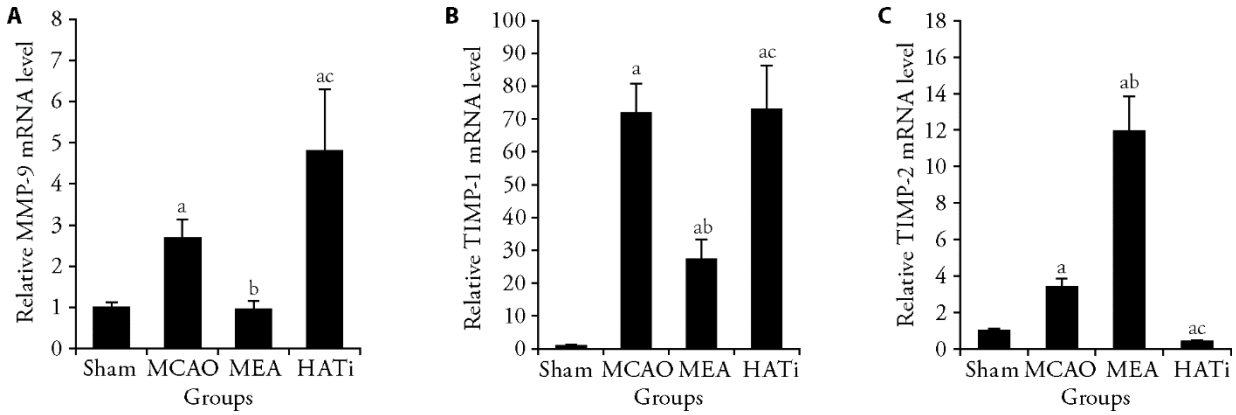

Figure 2 EA treatment regulated the expression of MMP-9 and TIMP-1/2 after MCAO

A: RT-qPCR shows the relative mRNA levels of MMP-9 in ischemic brain tissues 24 h after I/R injury, n = 4; B: RT-qPCR shows the relative mRNA levels of TIMP-1 in ischemic brain tissues 24 h after ischemia/reperfusion injury, n = 4; C: RT-qPCR shows the relative mRNA levels of TIMP-2 in ischemic brain tissues 24 h after I/R injury, n = 4. Sham group: sham operated; MCAO group: MCAO operated; MEA group: MCAO rats received 3 d of EA treatment; HATi group: MCAO rats received 3 d of EA treatment and a single 4 mg/kg intraperitoneal injection of HATi. MMP-9: matrix metalloproteinase 9; TIMP-1/2: tissue inhibitor of metalloproteinase 1/2; MCAO: middle cerebral artery occlusion; EA: electroacupuncture; HATi: histone acetyltransferase inhibitor; RT-qPCR: real-time quantitative polymerase chain reaction. Data were analyzed by one-way analysis of variance and are represented as the mean ± standard deviation. $^{a}P < 0.05$, *vs* the sham group; $^{b}P < 0.05$, the MCAO group *vs* the MEA group; $^{c}P < 0.05$, the MEA group *vs* the HATi group.

EA treatment decreased the enrichment of acetylation at these two sites compared with the I/R condition, and this effect of EA treatment could be further expanded by HATi at the −21 bp - −177 bp region. Although HATi tended to reduce H3K27ace enrichment at the −678 bp - −886 bp region of the MMP-9 promoter, the difference from the MEA group was not statistically meaningful (Figures 4A, 4B).

For the Timp-1 gene, the MCAO group had a lower acetylation level of H3K27 at the TIMP-1 promoter than the sham group. However, EA treatment notably increased H3K27ace occupancy. At the −580 bp - −822 bp region, the percentage of H3K27ace-enriched TIMP-1 DNA fragments was almost twice as high as that in the MCAO group. In addition, the application of HATi partly reduced the level of H3K27 acetylation at both regions (Figures 4C, 4D).

For the TIMP-2 gene, the binding of H3K27ace to the −248 bp - −425 bp region was weakened in the MCAO, MEA, and HATi groups, compared with the sham group. However, there was no significant difference among the groups (Figure 4E). At the −34 bp - −150 bp region, no amplification products of the H3K27ace-DNA fragment could be detected in the MCAO group. In contrast, a significant enhancement of acetylation was detected in the MEA group. The effect of EA was weakened when combined with HATi intervention (Figure 4F). Additionally, our results also showed that the occupancy levels of H3K27ace at the MMP-9, TIMP-1, and TIMP-2 promoters were significantly lower than those of H3K9ace in each group (Figures 3, 4).

4. DISCUSSION

The occurrence and progression of brain injury after ischemic stroke are caused by various factors, among which the damage of the BBB and the increase of permeability play an important role.[44] The BBB is the physical barrier between the blood and the CNS and can restrict substances and cells in the peripheral circulation from accessing the brain. With the interruption of cerebral blood flow, the transmission of oxygen and glucose is damaged, which results in an imbalance in brain metabolism. Then, a series of neurochemical and molecular events, including ionic imbalances, reactive oxygen species generation, the release of inflammatory cytokines, increase in MMP activity, and neuronal death, have a role in BBB disruption.[45-47] Soon after, substances in peripheral blood permeate the damaged BBB, causing brain edema, hemorrhagic transformation, and increased mortality.[2] Several studies have found that BBB permeability peaks in the acute/subacute stage of stroke. MRI analysis showed that BBB permeability and permeable BBB volume 24 h after I/R injury were significantly higher than those in the sham control, accompanied by progressive edema accumulation.[48] In the next 72-96 h, the neuroinflammatory reaction further ruptures the BBB, leading to the second peak of its permeability.[49] In this study, we used Evans blue extravasation to evaluate BBB leakage. Our result is in favor of ischemic stroke inducing the disruption of BBB integrity 1 and 3 d after I/R injury, which is consistent with published work.[48,49]

As an alternative and complementary therapy for stroke, EA is recommended by the World Health Organization.[50] Previous studies have revealed that EA can promote neurogenesis and astrogliosis,[51] increase BDNF expression,[52] decrease apoptosis,[53] and attenuate inflammation,[54] all of which contribute to reducing brain injury after stroke. Baihui (GV20) is a classic acupoint for stroke treatment. A meta-analysis showed significant effects of Baihui (GV20)-based acupuncture for improving infarct volume and neurological function in experimental ischemic stroke.[55] In rats with I/R injury,

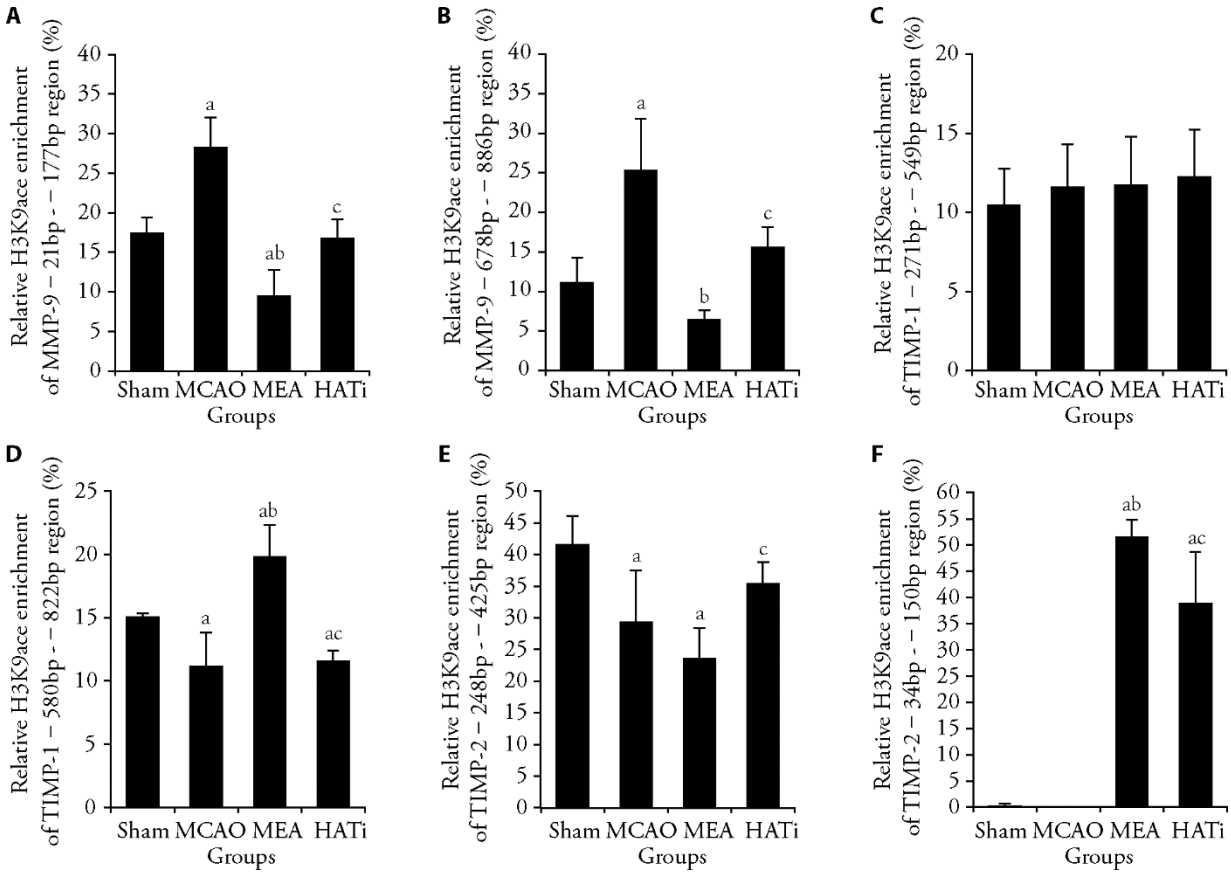

Figure 3 EA treatment regulated H3K9ace occupancy at target gene promoters

A: ChIP analysis of the enrichment of acetylated H3K9 on promoters of MMP-9 −21 bp - −177 bp region, *n* = 3 or 4; B: ChIP analysis of the enrichment of acetylated H3K9 on promoters of MMP-9 −678 bp - −886 bp region, *n* = 3 or 4; C: ChIP analysis of the enrichment of acetylated H3K9 on promoters of TIMP-1 −271 bp - −549 bp region, *n* = 3 or 4; D: ChIP analysis of the enrichment of acetylated H3K9 on promoters of TIMP-1 −580 bp - −822 bp region, *n* = 3 or 4. E: ChIP analysis of the enrichment of acetylated H3K9 on promoters of TIMP-2 −248 bp - −425 bp region, *n* = 3 or 4. F: ChIP analysis of the enrichment of acetylated H3K9 on promoters of TIMP-2 −34 bp - −150 bp region, *n* = 3 or 4. Sham group: sham operated; MCAO group: MCAO operated; MEA group: MCAO rats received 3 d of EA treatment; HATi group: MCAO rats received 3 d of EA treatment and a single 4 mg/kg intraperitoneal injection of HATi. MCAO: middle cerebral artery occlusion; EA: electroacupuncture; HATi: histone acetyltransferase inhibitor; H3K9ace: histone H3 lysine 9 acetylation; MMP-9: matrix metalloproteinase 9; TIMP-1: tissue inhibitor of metalloproteinase 1; TIMP-2: tissue inhibitor of metalloproteinase 2; ChIP: chromatin immunoprecipitation. Data were analyzed by one-way analysis of variance and are represented as the mean ± standard deviation. $^{a}P < 0.05$ *vs* the sham group, $^{b}P < 0.05$ the MCAO group *vs* the MEA group, $^{c}P < 0.05$ the MEA group *vs* the HATi group.

EA at Baihui (GV20) and Zusanli (ST36) protects BBB integrity by upregulating TJ protein expression.[30] EA at Baihui (GV20) and Shenting (GV24) inhibited BBB breakdown and reduced MMP-2/9 expression in I/R-injured rats.[22] In our previous study, EA at Baihui (GV20) improved cerebral infarction volume of MCAO rats in acute stage.[40] In this study, our results also showed that EA at Baihui (GV20) has an obviously protective effect on the BBB and improves neurobehavioral function after stroke.

Next, we want to explore the possible mechanism of the protective effect of EA on BBB. MMP-9 concentration is an intense biomarker of BBB destruction in stroke. MMP-9 can mediate the degradation of TJ protein, which form the endothelial barrier. Basement membranes are also vulnerable to attack by MMP-9.[7] Previous research has found that MMP-9 siRNA could preserve BBB integrity and improve neurological function.[56] In a separate study, treatment with a broad-spectrum MMP inhibitor, BB-94, also protected against brain damage in MCAO rats due to the prevention of BBB opening.[7] GM6001, another MMP inhibitor, was also reported to enhance TJ protein formation and markedly reduce brain tissue damage in ischemic rats.[11] These results indicated that MMP inhibition is a potential therapeutic target for stroke. TIMPs, natural inhibitors of MMPs *in vivo*, also play a prominent role during the injury and repair processes after cerebral ischemia.[57] The TIMP family has four homologous members, TIMP-1 to TIMP-4, each of which has different binding specificities. TIMP-1 has a strong inhibitory effect on MMP-9, while TIMP-2 preferentially cleaves MMP-2, which can also inhibit other MMPs.[58] TIMP-1-deficient mice showed increased MMP-9 activity and BBB injury 24 h after I/R injury, suggesting that TIMP-1 has a neuroprotective function in the early stage of stroke.[59] An experimental stroke study showed that hypothermia increased TIMP-2 expression while decreasing MMP-2 and MMP-9 activity, which contributed to the BBB protective effect of hypothermia.[60] Multiple clinical trials have found that

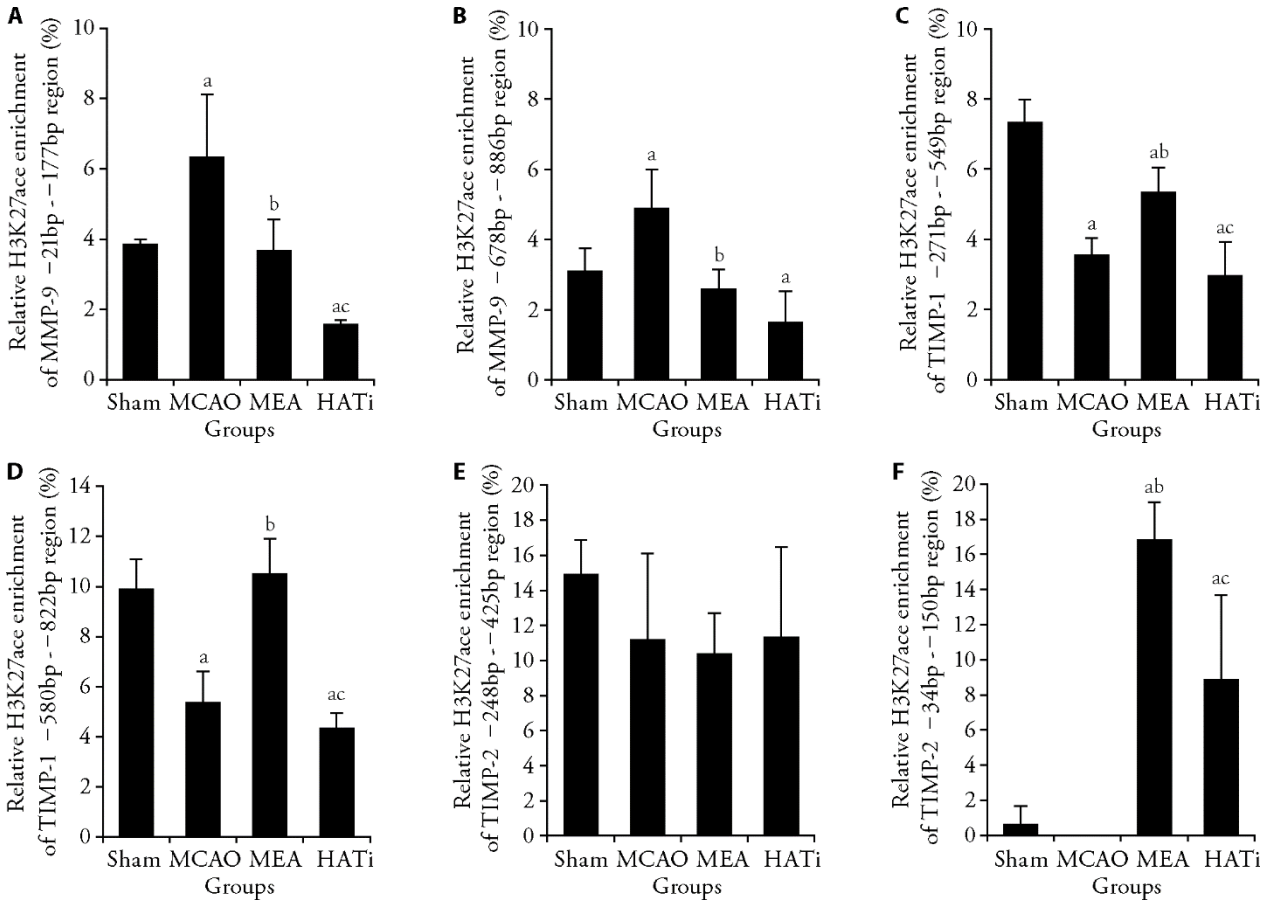

Figure 4 EA treatment regulated H3K27ace occupancy at target gene promoters

A: ChIP analysis of the enrichment of acetylated H3K27 on promoters of MMP-9 −21 bp - −177 bp region, *n* = 3 or 4; B: ChIP analysis of the enrichment of acetylated H3K27 on promoters of MMP-9 −678 bp - −886 bp region, *n* = 3 or 4; C: ChIP analysis of the enrichment of acetylated H3K27 on promoters of TIMP-1 −271 bp - −549 bp region, *n* = 3 or 4; D: ChIP analysis of the enrichment of acetylated H3K27 on promoters of TIMP-1 −580 bp - −822 bp region, *n* = 3 or 4; E: ChIP analysis of the enrichment of acetylated H3K27 on promoters of TIMP-2 −248 bp - −425 bp region, *n* = 3 or 4. F: ChIP analysis of the enrichment of acetylated H3K27 on promoters of TIMP-2 −34 bp - −150 bp region, *n* = 3 or 4. Sham group: sham operated; MCAO group: MCAO operated; MEA group: MCAO rats received 3 d of EA treatment; HATi group: MCAO rats received 3 d of EA treatment and a single 4 mg/kg intraperitoneal injection of HATi. MCAO: middle cerebral artery occlusion; EA: electroacupuncture; HATi: histone acetyltransferase inhibitor; H3K27ace: histone H3 lysine 27 acetylation; MMP-9: matrix metalloproteinase 9; TIMP-1: tissue inhibitor of metalloproteinase 1; TIMP-2: tissue inhibitor of metalloproteinase 2; ChIP: chromatin immunoprecipitation. Data were analyzed by one-way analysis of variance and are represented as the mean ± standard deviation. $^{a}P < 0.05$ *vs* the sham group, $^{b}P < 0.05$ the MCAO group *vs* the MEA group, $^{c}P < 0.05$ the MEA group *vs* the HATi group.

the concentration in the serum of patients with acute stroke or the expression in infarcted brain tissue of MMPs and TIMPs are increased.[61,62] Consistently, our results demonstrated that the expression of MMP-9, TIMP-1, and TIMP-2 mRNA was significantly stronger 24 h after reperfusion. The increase in TIMPs may be a mechanism of body self-protection. In addition, we found that EA could abate the expression of MMP-9 mRNA and boost the transcriptional activity of TIMP-2 mRNA, which may be the target of EA-mediated BBB protection. Although many studies, including ours, support that EA plays a protective role after stroke by regulating the transcriptional activity of MMP9 and TIMPs, the mechanism of EA regulates gene expression is still unclear.

Of note, our study found that HATi can weaken the brain protective effect of EA and reverse the effect of EA on gene transcription regulation. HATs can catalyze histone lysine N-acetylation, which affects genome organization and function. The opening of chromatin structure and activation of gene transcription are related to histone acetylation.[63] In general, excessive acetylation leads to increased gene expression associated with activation of gene transcription, while low acetylation means inhibition of gene expression. It was reported that valproic acid (VPA), a HDAC inhibitor, alleviated MCAO-induced BBB disruption and brain edema. Meanwhile, VPA blocked the reduction in acetylated histone H3 levels and attenuated the elevation of MMP-9 and the degradation of TJs after MCAO. In addition, sodium butyrate, another HDAC inhibitor, imitated the effects of VPA.[64] To explore whether EA exerts its effect through the acetylated histone mechanism, we detected the enrichment of H3K9ace and H3K27ace at the MMP-9, TIMP-1, and TIMP-2 gene promoters. Both H3K9 and H3K27 are common sites of histone acetylation. H3K9ace mainly modifies the promoter region of the target gene, while H3K27ace mostly binds to the

enhancer region.[65, 66] The results of our study indicated that the binding strength of H3K9ace to DNA fragments was significantly higher than that of H3K27ace in the upstream promoter region of most target genes. We also found that H3K9ace/H3K27ace at the two regions of the MMP-9 promoter both increased after stroke and EA treatment decreased the enrichment of acetylation at these sites. However, at the －34 bp - －150 bp region of TIMP-2, EA at Baihui (GV20) notably increased the acetylation level. In addition, most of our data indicated that the regulatory effect of EA on histone acetylation modifications in the MMP-9 and TIMP-2 promoter regions can be reversed by HATi. These results showed the same trend as the transcription levels of the target genes. All these data confirmed that EA at Baihui (GV20) regulates MMP-9 and TIMP2 transcriptional activity directly through histone acetylation. We confirmed that histone acetylation is an important mechanism for EA to regulate gene transcription.

Notably, the acetylation levels of H3K9 and H3K27 at the two regions of the TIMP-1 promoter did not show the same upward trend as the expression of mRNA after I/R injury. At the same time, EA did not enhance the transcriptional activity of TIMP-1, although EA treatment enhanced H3K9ace and H3K27ace occupancy at the －580 bp - －822 bp region. Studies have confirmed that histone methylation modification[67] or microRNA[68] also involved in the regulation of TIMP-1 transcription. Histone acetylation at these two sites in our study may not be the main regulatory pathway of TIMP-1 transcription. As an endogenous inhibitor of MMP-9, TIMP-1 expression levels are usually associated with MMP-9. Therefore, the decrease in TIMP-1 mRNA levels after EA may be associated with the decrease in MMP-9 expression. The detailed mechanism needs further study.

There are some limitations of this study that should be noted. We have noticed that MMP-9 has a biphasic effect after stroke. Early (day 1) MMP-9 inhibition can mitigate brain injury, but treatment at 7 d after stroke increased ischemic brain injury and impaired functional recovery at 14 d.[69, 70] TIMPs, as MMP inhibitors, may have short-term beneficial but long-term harmful effects. In this study, we focused on the regulatory effects of EA on MMP-9 and TIMP-1/2 during the acute phase of stroke. However, further research at different stages of stroke will be more conducive to a comprehensive understanding of the mechanism of brain protection produced by EA.

In summary, our data indicate that EA at Baihui (GV20) reduces BBB disruption by regulating the expression of MMP-9 and TIMP-2 in the acute stage of MCAO rats, which is mediated through H3K9/H3K27 acetylation modification directly at the target gene promoter (supplement Figure 1). This study suggests that histone modification plays an important role in the protective effect of EA in ischemic brain injury, and can become a target of EA or medicine therapy after cerebral ischemia.

5. SUPPORTING INFORMATION

Supporting data to this article can be found online at http://journaltcm.cn.

6. REFERENCES

1. Collaborators GBDS. Global, regional, and national burden of stroke, 1990-2016: a systematic analysis for the global burden of disease study 2016. Lancet Neurol 2019; 18: 439-58.
2. Yang C, Hawkins KE, Dore S, Candelario-Jalil E. Neuroinflammatory mechanisms of blood-brain barrier damage in ischemic stroke. Am J Physiol Cell Physiol 2019; 316: 135-53.
3. Keaney J, Campbell M. The dynamic blood-brain barrier. FEBS J 2015; 282: 4067-79.
4. Zhong C, Yang J, Xu T, et al. Serum matrix metalloproteinase-9 levels and prognosis of acute ischemic stroke. Neurology 2017; 89: 805-12.
5. Rosell A, Ortega-Aznar A, Alvarez-Sabin J, et al. Increased brain expression of matrix metalloproteinase-9 after ischemic and hemorrhagic human stroke. Stroke 2006; 37: 1399-406.
6. Rosell A, Cuadrado E, Ortega-Aznar A, Hernandez-Guillamon M, Lo EH, Montaner J. MMP-9-positive neutrophil infiltration is associated to blood-brain barrier breakdown and basal lamina type IV collagen degradation during hemorrhagic transformation after human ischemic stroke. Stroke 2008; 39: 1121-6.
7. Rosenberg GA, Yang Y. Vasogenic edema due to tight junction disruption by matrix metalloproteinases in cerebral ischemia. Neurosurg Focus 2007; 22: 1-9.
8. Yang Y, Estrada EY, Thompson JF, Liu W, Rosenberg GA. Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat. J Cereb Blood Flow Metab 2007; 27: 697-709.
9. Asahi M, Wang X, Mori T, et al. Effects of matrix metalloproteinase-9 gene knock-out on the proteolysis of blood-brain barrier and white matter components after cerebral ischemia. J Neurosci 2001; 21: 7724-32.
10. Bauer AT, Burgers HF, Rabie T, Marti HH. Matrix metalloproteinase-9 mediates hypoxia-induced vascular leakage in the brain *via* tight junction rearrangement. J Cereb Blood Flow Metab 2010; 30: 837-48.
11. Yang Y, Thompson JF, Taheri S, et al. Early inhibition of MMP activity in ischemic rat brain promotes expression of tight junction proteins and angiogenesis during recovery. J Cereb Blood Flow Metab 2013; 33: 1104-14.
12. Cui J, Chen S, Zhang C, et al. Inhibition of MMP-9 by a selective gelatinase inhibitor protects neurovasculature from embolic focal cerebral ischemia. Mol Neurodegener 2012; 7: 1-21.
13. Krizanac-Bengez L, Hossain M, Fazio V, Mayberg M, Janigro D. Loss of flow induces leukocyte-mediated MMP/TIMP imbalance in dynamic *in vitro* blood-brain barrier model: role of pro-inflammatory cytokines. Am J Physiol Cell Physiol 2006; 291: 740-9.
14. Guo T, Hao H, Zhou L, Zhou F, Yu D. Association of SNPs in the TIMP-2 gene and large artery atherosclerotic stroke in southern Chinese Han population. Oncotarget 2018; 9: 4698-706.
15. Wei H, Wang S, Zhen L, et al. Resveratrol attenuates the blood-brain barrier dysfunction by regulation of the MMP-9/TIMP-1 balance after cerebral ischemia reperfusion in rats. J Mol Neurosci 2015; 55: 872-9.
16. Rivera S, Ogier C, Jourquin J, et al. Gelatinase B and TIMP-1 are regulated in a cell- and time-dependent manner in association with neuronal death and glial reactivity after global forebrain ischemia. Eur J Neurosci 2002; 15: 19-32.
17. Fujimoto M, Takagi Y, Aoki T, et al. Tissue inhibitor of metalloproteinases protect blood-brain barrier disruption in focal cerebral ischemia. J Cereb Blood Flow Metab 2008; 28: 1674-85.
18. Huang YG, Tao W, Yang SB, Wang JF, Mei ZG, Feng ZT. Autophagy: novel insights into therapeutic target of

electroacupuncture against cerebral ischemia/ reperfusion injury. Neural Regen Res 2019; 14: 954-61.
19. Jung YS, Lee SW, Park JH, Seo HB, Choi BT, Shin HK. Electroacupuncture preconditioning reduces ROS generation with NOX4 down-regulation and ameliorates blood-brain barrier disruption after ischemic stroke. J Biomed Sci 2016; 23: 1-32.
20. Xu H, Zhang Y, Sun H, Chen S, Wang F. Effects of acupuncture at GV20 and ST36 on the expression of matrix metalloproteinase 2, aquaporin 4, and aquaporin 9 in rats subjected to cerebral ischemia/reperfusion injury. PLoS One 2014; 9: e97488.
21. Ma R, Yuan B, Du J, et al. Electroacupuncture alleviates nerve injury after cerebra ischemia in rats through inhibiting cell apoptosis and changing the balance of MMP-9/TIMP-1 expression. Neurosci Lett 2016; 633: 158-64.
22. Lin R, Yu K, Li X, et al. Electroacupuncture ameliorates post-stroke learning and memory through minimizing ultrastructural brain damage and inhibiting the expression of MMP-2 and MMP-9 in cerebral ischemia-reperfusion injured rats. Mol Med Rep 2016; 14: 225-33.
23. Zhang XC, Gu YH, Xu WT, et al. Early electroacupuncture extends the rtpa time window to 6 h in a male rat model of embolic stroke *via* the ERK1/2-MMP9 pathway. Neural Plasticity 2020; 2020: 1-15.
24. Jiang X, Tian Y, Xu L, et al. Inhibition of triple-negative breast cancer tumor growth by electroacupuncture with encircled needling and its mechanisms in a mice xenograft model. Int J Med Sci 2019; 16: 1642-51.
25. Bao F, Sun H, Wu ZH, Wang DH, Zhang YX. Effect of acupuncture on expression of matrix metalloproteinase and tissue inhibitor in cartilage of rats with knee osteoarthritis. Zhong Guo Zhen Jiu 2011; 31: 241-6.
26. Ren L, Wang YK, Fang YN, Zhang AW, Li XL. Effect of electroacupuncture therapy on the expression of Na(v)1.1 and Na(v)1.6 in rat after acute cerebral ischemia. Neurol Res 2010; 32: 1110-6.
27. Zhang JJ, Du YH, Li J, Yang LH, Chen LL, Zha QP. Effect of electroacupuncture on neurological function and Wnt signaling pathway in ischemic brain tissue of cerebral infarction rats. Zhen Ci Yan Jiu 2020; 45: 202-8.
28. Li MX, Fu ZT, Liu Q, Song ZS, Zhang H, Tang W. Effect of electroacupuncture of different acupoint groups on cerebral cortical neurovascular unit and PI3K/Akt signaling in rats with ischemic stroke. Zhen Ci Yan Jiu 2021; 46: 921-8.
29. Yang LH, Du YH, Li J. Effect of electroacupuncture on expression of apelin-APJ system of cerebral vascular endothelial cell in rats with cerebral infarction. Zhen Ci Yan Jiu 2017; 42: 9-13.
30. Zhang YM, Xu H, Sun H, Chen SH, Wang FM. Electroacupuncture treatment improves neurological function associated with regulation of tight junction proteins in rats with cerebral ischemia reperfusion injury. Evid Based Complement Alternat Med 2014; 2014: 989340.
31. Wang C, Yang F, Liu X, Liu M, Zheng Y, Guo J. Neurotrophic signaling factors in brain ischemia/reperfusion rats: differential modulation pattern between single-time and multiple electroacupuncture stimulation. Evid Based Complement Alternat Med 2014; 2014: 625050.
32. Tang J, Zhuang S. Histone acetylation and DNA methylation in ischemia/reperfusion injury. Clin Sci (Lond) 2019; 133: 597-609.
33. Demyanenko S, Uzdensky A. Epigenetic alterations induced by photothrombotic stroke in the rat cerebral cortex: deacetylation of histone h3, upregulation of histone deacetylases and histone acetyltransferases. Int J Mol Sci 2019; 20: 2882.
34. Gao Y, Ya B, Li X, Guo Y, Yin H. Myricitrin ameliorates cognitive deficits in MCAO cerebral stroke rats via histone acetylation-induced alterations of brain-derived neurotrophic factor. Mol Cell Biochem 2021; 476: 609-17.
35. Cao W, Feng Z, Zhu D, et al. The role of PGK1 in promoting ischemia/reperfusion injury-induced microglial M1 polarization and inflammation by regulating glycolysis. Neuromolecular Med 2023; 25: 301-11.
36. Wang Z, Tsai LK, Munasinghe J, et al. Chronic valproate treatment enhances postischemic angiogenesis and promotes functional recovery in a rat model of ischemic stroke. Stroke 2012; 43: 2430-6.
37. Patnala R, Arumugam TV, Gupta N, Dheen ST. HDAC inhibitor sodium butyrate-mediated epigenetic regulation enhances neuroprotective function of microglia during ischemic stroke. Mol Neurobiol 2017; 54: 6391-411.
38. Demyanenko S, Dzreyan V, Sharifulina S. Histone deacetylases and their isoform-specific inhibitors in ischemic stroke. Biomedicines 2021; 9: 1445.
39. Fu SP, He SY, Xu B, et al. Acupuncture promotes angiogenesis after myocardial ischemia through H3K9 acetylation regulation at VEGF gene. PLoS One 2014; 9: e94604.
40. Fu SP, Gong L, Li XX, Wang YL, Yuan J, Lu SF. Effects of acupuncture at "baihui" (GV20) on histone deacetylases expression in ischemia-reperfusion injury model rats. J Tradit Chin Med 2020; 61: 146-51.
41. Xu SY, Lv HQ, Li WQ, Hong H, Peng YJ, Zhu BM. Electroacupuncture alleviates cerebral ischemia/reperfusion injury in rats by histone H4 lysine 16 acetylation-mediated autophagy. Front Psychiatry 2020; 11: 576539.
42. Fu S, Gu Y, Jiang JQ, et al. Calycosin-7-O-beta-D-glucoside regulates nitric oxide /caveolin-1/matrix metalloproteinases pathway and protects blood-brain barrier integrity in experimental cerebral ischemia-reperfusion injury. J Ethnopharmacol 2014; 155: 692-701.
43. Fu S, Yu M, Xu H, et al. Genome-wide transcription analysis of electroacupuncture precondition-induced ischemic tolerance on SD rat with ischemia-reperfusion injury. Front Genet 2021; 12: 719201.
44. Bernardo-Castro S, Sousa JA, Bras A, et al. Pathophysiology of blood-brain barrier permeability throughout the different stages of ischemic stroke and its implication on hemorrhagic transformation and recovery. Front Neurol 2020; 11: 594672.
45. Thompson JW, Narayanan SV, Koronowski KB, Morris-Blanco K, Dave KR, Perez-Pinzon MA. Signaling pathways leading to ischemic mitochondrial neuroprotection. J Bioenerg Biomembr 2015; 47: 101-10.
46. Sahota P, Savitz SI. Investigational therapies for ischemic stroke: neuroprotection and neurorecovery. Neurotherapeutics 2011; 8: 434-51.
47. Liu P, Zhang R, Liu D, et al. Time-course investigation of blood-brain barrier permeability and tight junction protein changes in a rat model of permanent focal ischemia. J Physiol Sci 2018; 68: 121-7.
48. Pillai DR, Dittmar MS, Baldaranov D, et al. Cerebral ischemia-reperfusion injury in rats--a 3 T MRI study on biphasic blood-brain barrier opening and the dynamics of edema formation. J Cereb Blood Flow Metab 2009; 29: 1846-55.
49. Huang Y, Chen S, Luo Y, Han Z. Crosstalk between inflammation and the BBB in stroke. Curr Neuropharmacol 2020; 18: 1227-1236.
50. Chavez L, Huang SS, MacDonald I, Lin JG, Lee YC, Chen YH. Mechanisms of acupuncture therapy in ischemic stroke rehabilitation: a literature review of basic studies. Int J Mol Sci 2017; 18: 2270.
51. Young-Wook P, Gi Yoon H, Min Jae K, Seo-Yeon L, Byung Tae C, Hwa Kyoung S. 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. J Tradit Chin Med 2019; 39: 833-41.
52. Li SS, Hua XY, Zheng MX, et al. Electroacupuncture treatment improves motor function and neurological outcomes after cerebral ischemia/reperfusion injury. Neural Regen Res 2022; 17: 1545-55.
53. Chen B, Lin WQ, Li ZF, et al. Electroacupuncture attenuates ischemic brain injury and cellular apoptosis *via* mitochondrial translocation of cofilin. Chin J Integr Med 2021; 27: 705-12.
54. Liu R, Xu NG, Yi W, Ji C. Electroacupuncture attenuates inflammation after ischemic stroke by inhibiting NF-kappaB-mediated activation of microglia. Evid Based Complement Alternat Med 2020; 2020: 8163052.

55. Wang WW, Xie CL, Lu L, Zheng GQ. A systematic review and Meta-analysis of Baihui (GV20)-based scalp acupuncture in experimental ischemic stroke. Sci Rep 2014; 4: 3981.
56. Hu Q, Chen C, Khatibi NH, et al. Lentivirus-mediated transfer of MMP-9 shRNA provides neuroprotection following focal ischemic brain injury in rats. Brain Res 2011; 1367: 347-59.
57. Cunningham LA, Wetzel M, Rosenberg GA. Multiple roles for MMPs and TIMPs in cerebral ischemia. Glia 2005; 50: 329-39.
58. Kaczorowska A, Miekus N, Stefanowicz J, Adamkiewicz-Drozynska E. Selected matrix metalloproteinases (MMP-2, MMP-7) and their inhibitor (TIMP-2) in adult and pediatric cancer. Diagnostics (Basel) 2020; 10: 547.
59. Adibhatla RM, Hatcher JF. Tissue plasminogen activator (tPA) and matrix metalloproteinases in the pathogenesis of stroke: therapeutic strategies. CNS Neurol Disord Drug Targets 2008; 7: 243-53.
60. Lee JE, Yoon YJ, Moseley ME, Yenari MA. Reduction in levels of matrix metalloproteinases and increased expression of tissue inhibitor of metalloproteinase-2 in response to mild hypothermia therapy in experimental stroke. J Neurosurg 2005; 103: 289-97.
61. Palm F, Pussinen PJ, Safer A, et al. Serum matrix metalloproteinase-8, tissue inhibitor of metalloproteinase and myeloperoxidase in ischemic stroke. Atherosclerosis 2018; 271: 9-14.
62. Cuadrado E, Rosell A, Penalba A, et al. Vascular MMP-9/TIMP-2 and neuronal MMP-10 up-regulation in human brain after stroke: a combined laser microdissection and protein array study. J Proteome Res 2009; 8: 3191-7.
63. Voss AK, Thomas T. Histone lysine and genomic targets of histone acetyltransferases in mammals. Bioessays 2018; 40: e1800078.
64. Wang Z, Leng Y, Tsai LK, Leeds P, Chuang DM. Valproic acid attenuates blood-brain barrier disruption in a rat model of transient focal cerebral ischemia: the roles of HDAC and MMP-9 inhibition. J Cereb Blood Flow Metab 2011; 31: 52-7.
65. Yang L, Yu Y, Tian G, Deng H, Yu B. H3K9ac modification was involved in doxorubicin induced apoptosis by regulating Pik3ca transcription in H9C2 cells. Life Sci 2021; 284: 119107.
66. Creyghton MP, Cheng AW, Welstead GG, et al. Histone H3K27ac separates active from poised enhancers and predicts developmental state. Proc Natl Acad Sci U S A 2010; 107: 21931-6.
67. Zhang W, Sun W, Qin Y, et al. Knockdown of KDM1A suppresses tumour migration and invasion by epigenetically regulating the TIMP1/MMP9 pathway in papillary thyroid cancer. J Cell Mol Med 2019; 23: 4933-44.
68. Wang J, Gao Y, Ma M, et al. Effect of miR-21 on renal fibrosis by regulating MMP-9 and TIMP1 in kk-ay diabetic nephropathy mice. Cell Biochem Biophys 2013; 67: 537-46.
69. Zhao BQ, Wang S, Kim HY, et al. Role of matrix metalloproteinases in delayed cortical responses after stroke. Nat Med 2006; 12: 441-5.
70. Rosenberg GA, Estrada EY, Dencoff JE. Matrix metalloproteinases and TIMPs are associated with blood-brain barrier opening after reperfusion in rat brain. Stroke 1998; 29: 2189-95.