

## Original Article

# Neuroprotective effects of electroacupuncture on Ren and Du vessels through anti-inflammation in transient focal cerebral ischemia rats

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**Abstract:** The present study was designed, from the perspective of inflammatory reaction, to uncover the potential neuroprotective effect of electroacupuncture on Ren and Du vessels in transient focal cerebral ischemia in middle cerebral artery occlusion (MCAO) rats. In total, 192 rats were randomly assigned to four groups, sham operation group (S), model group (M), Du vessel group (D), together with Ren and Du vessels group (RD), with 48 for each. Each group was further divided into six subgroups of 12, 24, 48, 72, 96, and 144 h after model establishing. We performed immunohistochemistry to detect dynamic expressions of pro-inflammatory mediators interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and matrix metalloproteinase-9 (MMP-9) in affected brain tissues. It showed the lowest levels of all these four indexes in sham group. Expressions of IL-1 $\beta$ , IL-6 and MMP-9 in the rest three groups peaked at 24 h, 24 h and 48 h after model establishing, respectively. Levels in the other two groups were lower by contrast with model group, and they were lower in RD group than those in D group ( $P < 0.05$ ). MCP-1 expressions in model group peaked at 48 h, while at 12 h in D group and RD group. They were remarkably lower in the other two groups in comparison to model group ( $P < 0.05$ ). At most time points, levels in RD group were lower than those in D group ( $P < 0.05$ ). Our findings demonstrated neuroprotective effect of electroacupuncture on Ren and Du vessels on cerebral ischemia reperfusion injury.

**Keywords:** Transient focal cerebral ischemia, inflammatory reaction, immunohistochemistry, electroacupuncture

## Introduction

With rapid demographic and epidemiological changes in the past few decades, stroke has become the second leading cause of death worldwide, and about 16.9 million people suffer from it each year, with characteristics of huge differences between high and low income countries [1]. Globally, more than two thirds of stroke deaths occur in developing countries [2]. As a developing country with the largest population in the world, China is facing increasing burden brought about by this problem, with features of high incidence, prevalence and mortality [3]. Currently, stroke remains the major cause of death in China, and about 1.6 million people lose their lives yearly [4, 5]. According to the American Heart Association, ischemic

stroke accounts for over 80% of all stroke cases [6].

Cerebral ischemia triggers a series of complex pathophysiological changes, involving production of oxygen free radicals, toxicity of excitatory amino acid, inflammation injury, chondriosome injury, calcium overloading, as well as cell apoptosis [7]. Neuroimmune interactions take an important role in cerebral ischemia reperfusion injury, because nervous and immune systems are tightly integrated and cooperate in local and systemic reflexes restoring homeostasis in response to tissue injury and infection, enabling bidirectional communication through cytokines and neurotransmitters [8]. As a result, reperfusion injury post ischemia might exacerbate focal cerebral damage due to numerous inflam-

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matory mediators synthesizing and releasing. In view of this, more attention should be pay in inflammation inhibiting to reduce cerebral ischemia reperfusion injury (CIRI).

In clinical practice, acupuncture has long been proved to be an effective approach to stroke. Although accumulating experimental evidence has dominated the potential mechanisms of acupuncture for cerebral ischemia [9, 10], few reports are available to highlight the unique effect of Ren and Du vessels now that they play an important and irreplaceable part among all the meridians in the whole body. In our previous studies, we have highly valued the mechanism of electroacupuncture on Ren and Du vessels in an induced middle cerebral artery occlusion (MCAO) rat model. Our findings reveal that electroacupuncture on these two vessels could improve neurogenesis and angiogenesis [11, 12]. Furthermore, we hypothesize that inhibiting inflammatory injury might be another clue to illustrate the mechanism of electroacupuncture on Ren and Du vessels against CIRI.

We try to investigate how electroacupuncture on Ren and Du vessels influences the dynamic changes of relevant inflammatory cytokines, so as to further understand the neuroprotective effect on transient focal cerebral ischemic injury. In this research, the authors conducted immunohistochemistry to observe expressions of inflammatory mediators in the first six days in transient focal cerebral ischemia rats, including interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and matrix metalloproteinase-9 (MMP-9).

## Materials and methods

### *Reagents and instruments*

Rabbit anti-rat IL-1 $\beta$  and IL-6 polyclonal antibody were provided by Bioss Biotech Co., Ltd. (Beijing, China). Rabbit anti-rat MCP-1 and MMP-9 polyclonal antibody were provided by Boster Biotech Co., Ltd. (Wuhan, China). SP rabbit & mouse HRP kit was provided by Com Win Biotech Co., Ltd. (Beijing, China). Occluding sutures (3600A) were purchased from Jialing Biotech Co., Ltd. (Guangzhou, China). A stereo microscope was purchased from Finial Science and Technology Co., Ltd. (Shenzhen, China). Acupuncture needles were purchased from Suzhou Acupuncture & Moxibustion Appliance

Co., Ltd. (Suzhou, China). Electroacupuncture apparatus were purchased from Suzhou Medical Appliance Factory (Suzhou, China).

### *Animals and groups*

In total, 192 adult male SD rats weighing 250-280 g were provided by Laboratory Animal Center of Guangzhou University of Chinese Medicine [license No. SCXK (Yue) 2013-0034]. All these rats of specific pathogen-free grade were housed in a specific pathogen-free grade laboratory [license No. SCXK (Yue) 2013-0001]. Experimental protocols in the present research were approved by Animal Experiment Ethics Committees of Guangzhou University of Chinese Medicine, Guangzhou, China (permit No. S20140057).

All rats were randomly assigned to four groups: sham operation group (S), model group (M), Du vessel group (D), together with Ren and Du vessels group (RD). Each group was further divided into six subgroups of 12, 24, 48, 72, 96, and 144 h after reperfusion time, with 8 rats in each time point.

### *Establishment of MCAO model*

We established MCAO model in accordance with Longa method [13]. Rats were anesthetized with 10% chloral hydrate by intraperitoneal injection of 0.35 mL/100 g, and the surgery was conducted under a stereo microscope. Briefly, an incision about 2 cm was made on the midline of its neck after regular disinfection. Next, the right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed and slightly separated, preventing surrounding arteries and nerves from being damaged. The branches of ECA were electrocoagulated. An occluding suture with a round and blunted tip coated with silica gel was inserted into ICA from a small incision made on ECA. The suture was inserted approximately 18-20 mm to block blood supply of MCA, with a slight resistance felt when reaching the origin of MCA. Two hours later, the suture was finally pulled out and the cut was stitched. Rats were kept warm at about 37°C during the surgery and permitted free access to food and water after analepsia. Rats in sham operation group underwent the same surgical procedures without inserting a suture.

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Neurological test was an index to evaluate whether the model is successful or not. We gave scores as described by Bederson [14]: 0 point, failure to observe any symptoms of neurological deficits when lifting a rat by its tail; 1 point, failure to unfold the left forelimb fully; 2 points, circling to the left; 3 points, falling to the left; 4 points, unable to walk spontaneously. Rats with scores of both 0 and 4 points were excluded from this experiment.

### *Treatment*

The acupoints Dazhui (DU 14), Baihui (DU 20), and Shuigou (DU 26) on Du vessel were selected for D group. Guanyuan (RN 4), Qihai (RN 6), Chengjiang (RN 24), as well as those three points for D group were applied in RD group. The location of all these six points was described in a textbook [15]. Needles of 0.30×25 mm were used to insert about 1 mm on DU 26 and RN 24 vertically, approximately 5 mm toward Yintang (EX-HN3) on DU 20 at an angle of 0°, and about 5 mm on DU 14, RN 4 and RN 6 vertically. All acupoints, except DU 26 and RN 24, were connected to an electroacupuncture apparatus for 20 min, using continuous wave at a frequency of 15 Hz.

Rats in both D group and RD group received the first treatment right after MCAO model was successfully established, and the last treatment was 3 h before being sacrificed. Treatments were given once a day, 1-7 times in total for different subgroups respectively. Treatment was not given to those rats in both S group and M group.

### *Immunohistochemistry*

Rats were narcotized by intraperitoneal injection at six different time points. Brains were quickly removed and fixed in 4% paraformaldehyde at room temperature overnight and then made into paraffin blocks after dehydration. We prepared consecutive right brain coronal slices at thickness of 4 μm. Specimens were dewaxed and antigens underwent heat repairing. Sections were washed in 0.05 mol/L phosphate buffer saline (PBS) for 3 times and incubated with rabbit anti-rat IL-1β, IL-6, MCP-1 and MMP-9 polyclonal antibody (1:200) at room temperature for 48 h, respectively. Then, they were incubated with secondary antiserum at room temperature for 2 h after being washing

in PBS for 3 times. Subsequently, sections were incubated with streptavidin at room temperature for 30 min after being washed in PBS for 3 times. Finally, they were incubated with diaminobenzidine for 5 min before being washed in PBS for 3 times. Each section was photographed at five different areas to detect optical density (OD) of positive products marked with brown dots through Image-Pro Plus 6.0 software (Media Cybernetics, Warrendale, USA).

### *Statistical analysis*

All data were analyzed by SPSS 22.0 software (Chicago, IL, USA) and presented as mean ± standard deviation. Differences among groups were conducted by one-way analysis of variance (ANOVA) followed by LSD t-test for multiple comparisons. All *P*-values were two-tailed and *P*<0.05 was considered to be statistically significant.

## **Results**

### *Expressions of IL-1β in brain tissues*

Levels of IL-1β in the right brain tissues in all groups appeared a single peak at 24 h after model establishing. It manifested the lowest expressions of IL-1β in sham group, which were noticeably lower than those in model group at the same time points (*P*<0.05). At the same time points, levels in both D group and RD group were lower by contrast with model group (*P*<0.05); expressions in RD group were lower in comparison to D group (*P*<0.05). Furthermore, IL-1β expressions in RD group did not show remarkable increasing in early stage of 12 h; while they were reduced to similar levels to sham group in late period of 96 h and 144 h, and the differences between these two groups were not statistically significant (**Table 1; Figure 1**).

### *Expressions of IL-6 in brain tissues*

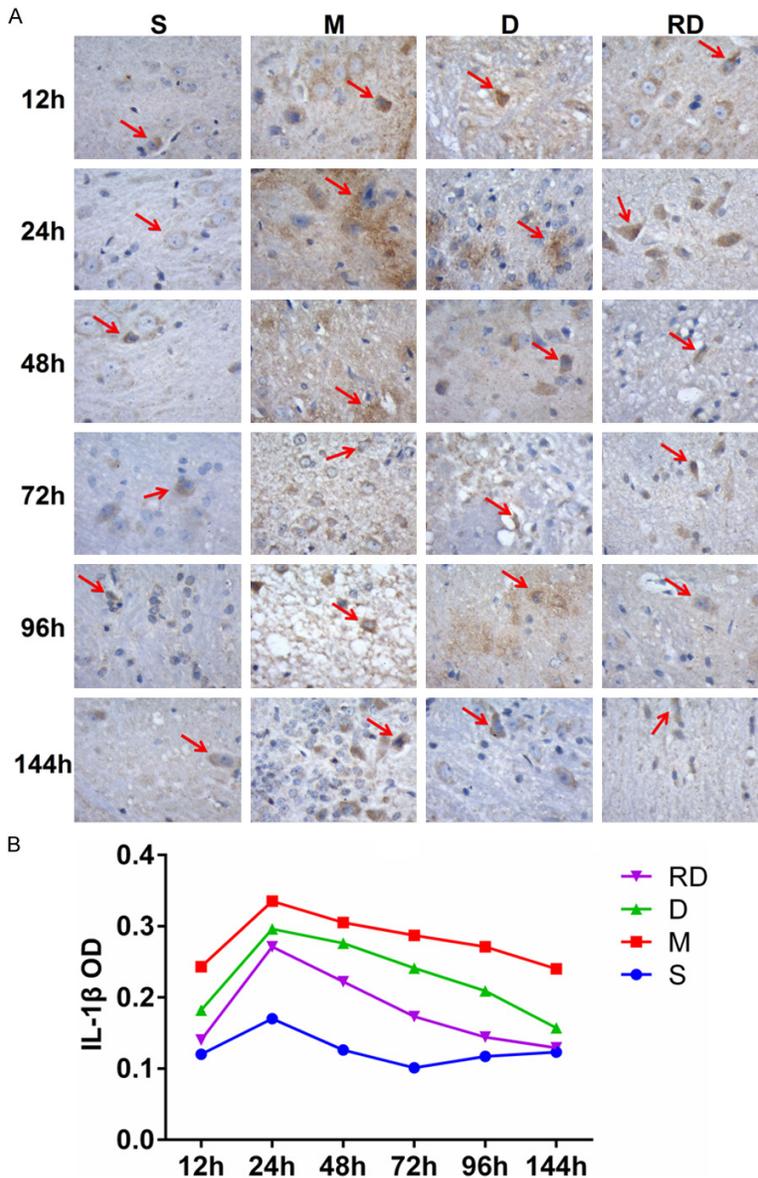
Expressions of IL-6 in all groups appeared a single peak at 24 h after model establishing. It indicated the lowest expressions in sham group, which were noticeably lower by contrast with model group at different time points (*P*<0.05). Levels in both D group and RD group were in between the other two groups. After 48 h, IL-6 expressions in D group were lower than those in model group at the same time points

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**Table 1.** Expressions of IL-1 $\beta$  in the right brain tissues at different time points in each group ( $\bar{x} \pm s$ )

Group	n	12 h	24 h	48 h	72 h	96 h	144 h
Sham	48	0.120 $\pm$ 0.015	0.170 $\pm$ 0.018	0.126 $\pm$ 0.021	0.101 $\pm$ 0.014	0.117 $\pm$ 0.012	0.123 $\pm$ 0.018
Model	48	0.243 $\pm$ 0.028 <sup>Δ</sup>	0.335 $\pm$ 0.034 <sup>Δ</sup>	0.305 $\pm$ 0.007 <sup>Δ</sup>	0.287 $\pm$ 0.006 <sup>Δ</sup>	0.271 $\pm$ 0.006 <sup>Δ</sup>	0.240 $\pm$ 0.009 <sup>Δ</sup>
D	48	0.182 $\pm$ 0.016 <sup>Δ#</sup>	0.296 $\pm$ 0.011 <sup>Δ#</sup>	0.276 $\pm$ 0.011 <sup>Δ#</sup>	0.241 $\pm$ 0.007 <sup>Δ#</sup>	0.209 $\pm$ 0.009 <sup>Δ#</sup>	0.157 $\pm$ 0.009 <sup>Δ#</sup>
RD	48	0.140 $\pm$ 0.007 <sup>Δ#,*</sup>	0.271 $\pm$ 0.010 <sup>Δ#,*</sup>	0.222 $\pm$ 0.016 <sup>Δ#,*</sup>	0.173 $\pm$ 0.008 <sup>Δ#,*</sup>	0.144 $\pm$ 0.008 <sup>Δ#,*</sup>	0.129 $\pm$ 0.006 <sup>Δ#,*</sup>

Notes: <sup>Δ</sup> $P < 0.05$ , compared with sham group; <sup>#</sup> $P < 0.05$ , compared with model group; <sup>\*</sup> $P < 0.05$ , compared with D group.



**Figure 1.** Expressions of IL-1 $\beta$  in the right brain tissues at different time points in each group. A. Photographs of immunohistochemical staining at different time points from all groups (magnification 200 $\times$ ). Red arrows indicated positive cells manifested as brown dots. B. Expression trend of OD values in the right brain tissue in all groups. Sham group (S), model group (M), Du vessel group (D), Ren and Du vessels group (RD) at 12 h, 24 h, 48 h, 96 h and 144 h.

( $P < 0.05$ ). In RD group, expressions at all six time points were remarkably lower in comparison to both D group and model group ( $P < 0.05$ ), slight higher than those in sham group at most of the time points ( $P < 0.05$ ), and were similar to sham group at 144 h (Table 2; Figure 2).

### Expressions of MCP-1 in brain tissues

Levels of MCP-1 in sham group and model group peaked at 48 h, while they peaked at 12 h in D group and RD group. At all the time points, it showed considerably high levels of MCP-1 in model group while low in sham group, and expressions in sham group were far fewer than those in model group ( $P < 0.05$ ). MCP-1 expressions in D group and RD group were remarkably lower in comparison to model group ( $P < 0.05$ ). At 24 h, 72 h, 96 h and 144 h, levels in RD group were lower in comparison to D group with statistical significance ( $P < 0.05$ ). After 72 h, MCP-1 content in RD group was similar to sham group, and differences between these two groups were not statistically significant (Table 3; Figure 3).

### Expressions of MMP-9 in brain tissues

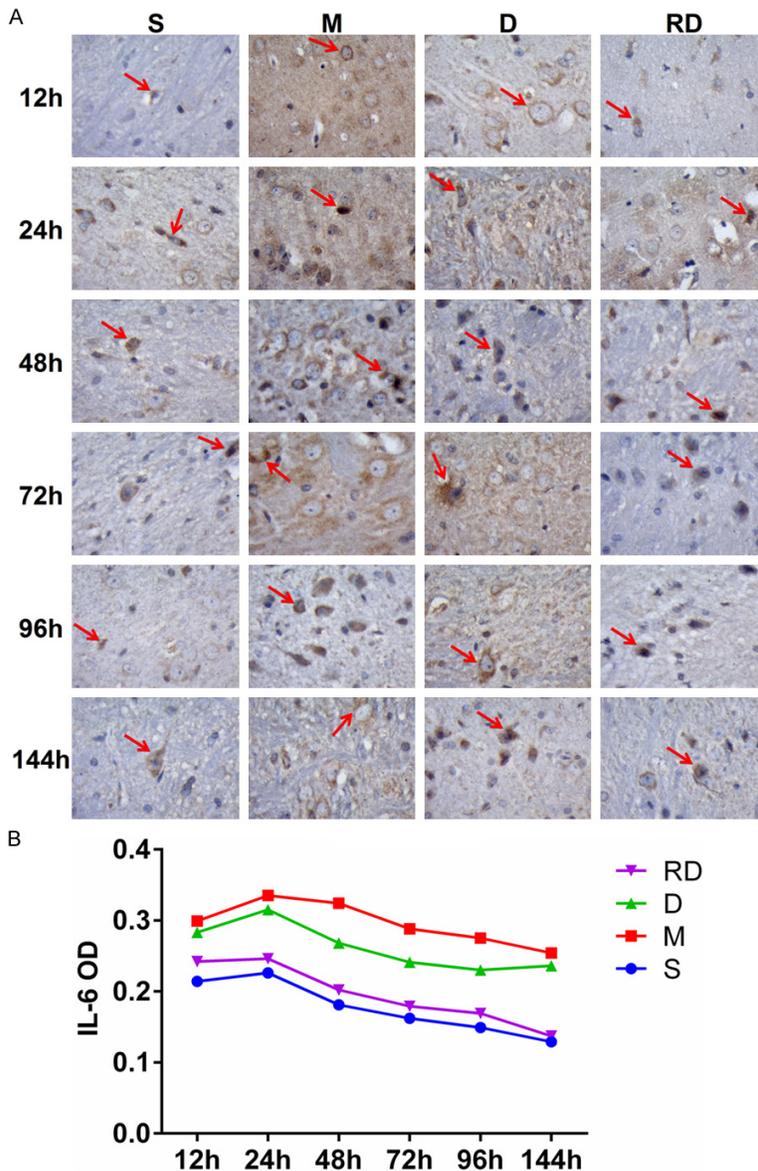
In sham group, it manifested considerably low expressions

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**Table 2.** Expressions of IL-6 in the right brain tissues at different time points in each group ( $\bar{x} \pm s$ )

Group	n	12 h	24 h	48 h	72 h	96 h	144 h
Sham	48	0.214±0.016	0.226±0.017	0.181±0.012	0.162±0.010	0.149±0.008	0.129±0.014
Model	48	0.299±0.013 <sup>Δ</sup>	0.335±0.023 <sup>Δ</sup>	0.324±0.023 <sup>Δ</sup>	0.288±0.015 <sup>Δ</sup>	0.275±0.009 <sup>Δ</sup>	0.254±0.009 <sup>Δ</sup>
D	48	0.283±0.016 <sup>Δ</sup>	0.315±0.009 <sup>Δ</sup>	0.268±0.021 <sup>Δ#</sup>	0.241±0.023 <sup>Δ#</sup>	0.230±0.012 <sup>Δ#</sup>	0.236±0.009 <sup>Δ#</sup>
RD	48	0.242±0.020 <sup>Δ#,*</sup>	0.246±0.053 <sup>#,*</sup>	0.202±0.013 <sup>Δ#,*</sup>	0.179±0.013 <sup>Δ#,*</sup>	0.169±0.010 <sup>Δ#,*</sup>	0.137±0.020 <sup>#,*</sup>

Notes: <sup>Δ</sup> $P < 0.05$ , compared with sham group; <sup>#</sup> $P < 0.05$ , compared with model group; <sup>\*</sup> $P < 0.05$ , compared with D group.



**Figure 2.** Expressions of IL-6 in the right brain tissues at different time points in each group. A. Photographs of immunohistochemical staining at different time points from all groups (magnification 200 $\times$ ). Red arrows indicated positive cells manifested as brown dots. B. Expression trend of OD values in the right brain tissue in all groups. Sham group (S), model group (M), Du vessel group (D), Ren and Du vessels group (RD) at 12 h, 24 h, 48 h, 96 h and 144 h.

of MMP-9 in a horizontal manner. Levels in the rest three groups appeared a single peak at 48 h. Compared with model group, expressions in sham group were far lower at all the time points ( $P < 0.05$ ). In D group and RD group, expressions were all lower, in a remarkable manner at most time points, than those in model group ( $P < 0.05$ ). By contrast with D group, expressions beginning at 24 h were fewer in RD group with statistical significance ( $P < 0.05$ ). At 144 h, levels in both RD group and sham group were similar without statistical significance (Table 4; Figure 4).

### Discussion

In this study, we specifically focused on the perspective of neuroimmune physiology and pathology, and firstly tried to investigate the anti-inflammatory impact of electroacupuncture on Ren and Du vessels against CIRI, through observing dynamic changes of relevant inflammatory mediators. Our data manifested that IL-1 $\beta$ , IL-6, MCP-1, and MMP-9 expressions in affected brain tissues were significantly inhibited in transient focal ischemia rats.

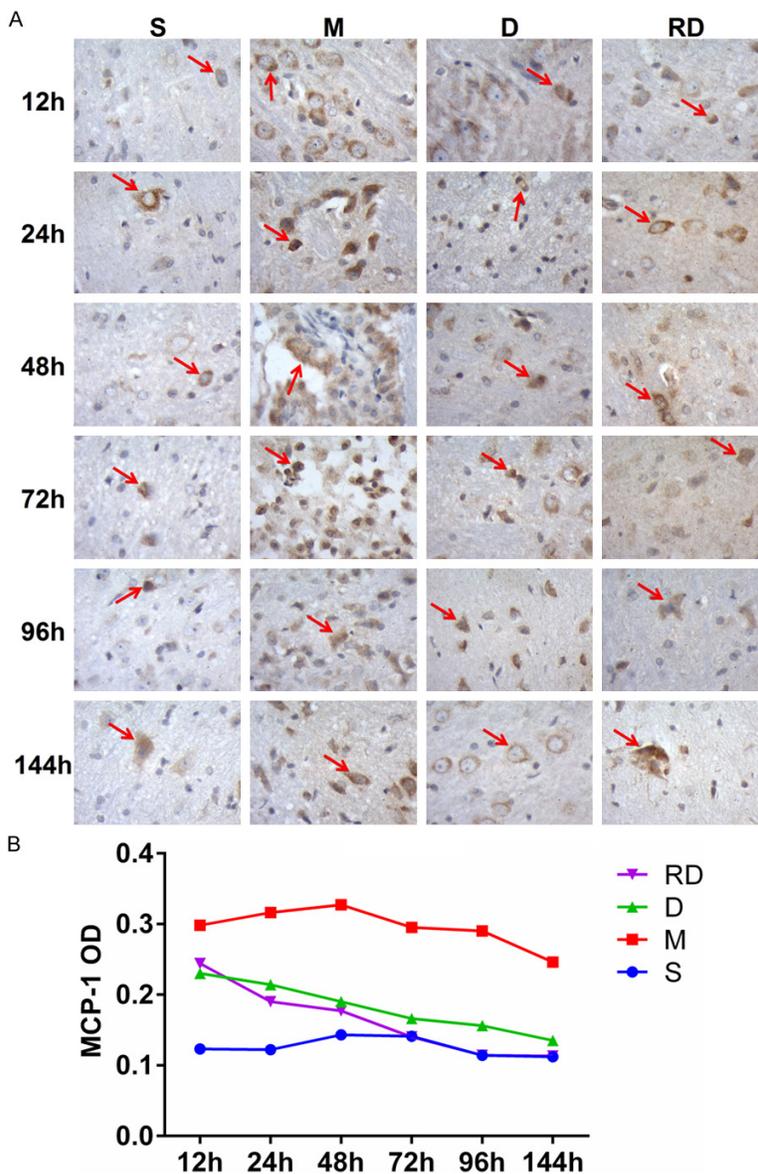
Cerebral ischemia is categorized as stroke, based on the theory of traditional Chinese medicine (TCM), and the path-

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**Table 3.** Expressions of MCP-1 in the right brain tissues at different time points in each group ( $\bar{x} \pm s$ )

Group	n	12 h	24 h	48 h	72 h	96 h	144 h
Sham	48	0.123±0.022	0.122±0.018	0.143±0.011	0.141±0.015	0.114±0.018	0.112±0.015
Model	48	0.298±0.028 <sup>Δ</sup>	0.316±0.023 <sup>Δ</sup>	0.327±0.020 <sup>Δ</sup>	0.295±0.029 <sup>Δ</sup>	0.290±0.022 <sup>Δ</sup>	0.246±0.019 <sup>Δ</sup>
D	48	0.230±0.007 <sup>Δ, #</sup>	0.214±0.022 <sup>Δ, #</sup>	0.190±0.021 <sup>Δ, #</sup>	0.166±0.012 <sup>Δ, #</sup>	0.156±0.010 <sup>Δ, #</sup>	0.135±0.011 <sup>Δ, #</sup>
RD	48	0.244±0.024 <sup>Δ, #</sup>	0.190±0.011 <sup>Δ, #, ☆</sup>	0.177±0.013 <sup>Δ, #</sup>	0.140±0.011 <sup>Δ, #, ☆</sup>	0.114±0.017 <sup>Δ, #, ☆</sup>	0.113±0.009 <sup>Δ, #, ☆</sup>

Notes: <sup>Δ</sup>*P*<0.05, compared with sham group; <sup>#</sup>*P*<0.05, compared with model group; <sup>☆</sup>*P*<0.05, compared with D group.



**Figure 3.** Expressions of MCP-1 in the right brain tissues at different time points in each group. A. Photographs of immunohistochemical staining at different time points from all groups (magnification 200×). Red arrows indicated positive cells manifested as brown dots. B. Expression trend of OD values in the right brain tissue in all groups. Sham group (S), model group (M), Du vessel group (D), Ren and Du vessels group (RD) at 12 h, 24 h, 48 h, 96 h and 144 h.

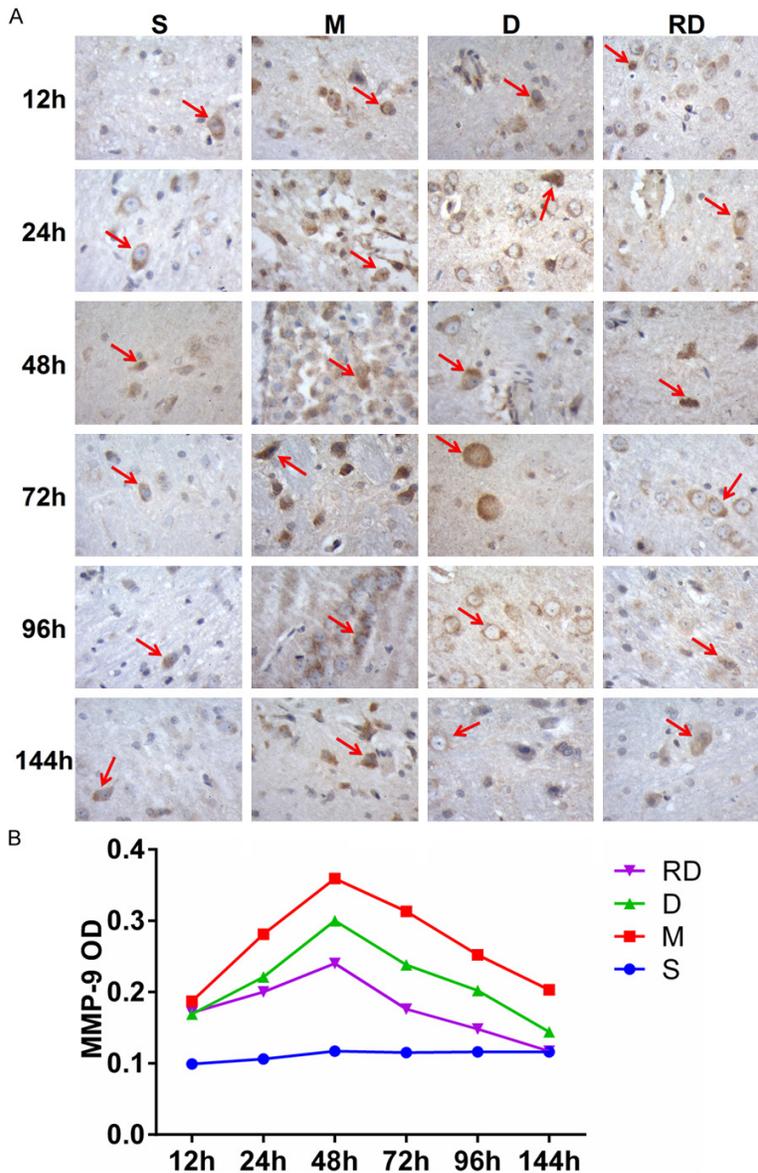
ogenic characteristics could be concluded by imbalance between yin and yang, qi deficiency and blood stagnation, as well as obstruction of phlegm and stasis inside the human body. In the long history of TCM, acupuncture has gained its fame for outstanding therapeutic effect on numerous diseases both in ancient and modern times, specialized in neurological disorders. According to available literature and clinical reports, Ren and Du vessels play irreplaceable parts among all twelve regular and eight extraordinary meridians, well known as sea of yin meridians for Ren vessel and sea of yang meridians for Du vessel, respectively. Thus, selecting these two vessels are responsible for regulating yin and yang in the whole body. In spite of this, few researches on combination of these two vessels are conducted to discuss the underlying mechanisms of acupuncture on experimental ischemic stroke. In present studies, acupoints DU 14 and DU 20 on Du vessel, as well as Zusanli (ST36) on stomach meridian are most frequently applied [10, 16]. It can't be denied that these acupoints are essential for ischemic stroke, however, a more important reason why combination of these two vessels failing to be applied

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**Table 4.** Expressions of MMP-9 in the right brain tissues at different time points in each group ( $\bar{x} \pm s$ )

Group	n	12 h	24 h	48 h	72 h	96 h	144 h
Sham	48	0.099±0.013	0.106±0.012	0.117±0.011	0.115±0.012	0.116±0.013	0.116±0.019
Model	48	0.187±0.016 <sup>Δ</sup>	0.281±0.021 <sup>Δ</sup>	0.359±0.013 <sup>Δ</sup>	0.313±0.016 <sup>Δ</sup>	0.252±0.008 <sup>Δ</sup>	0.203±0.010 <sup>Δ</sup>
D	48	0.169±0.012 <sup>Δ#</sup>	0.221±0.011 <sup>Δ#</sup>	0.300±0.020 <sup>Δ#</sup>	0.238±0.014 <sup>Δ#</sup>	0.202±0.013 <sup>Δ#</sup>	0.144±0.008 <sup>Δ#</sup>

Notes: <sup>Δ</sup>*P*<0.05, compared with sham group; <sup>#</sup>*P*<0.05, compared with model group; <sup>\*</sup>*P*<0.05, compared with D group.



**Figure 4.** Expressions of MMP-9 in the right brain tissues at different time points in each group. A. Photographs of immunohistochemical staining at different time points from all groups (magnification 200×). Red arrows indicated positive cells manifested as brown dots. B. Expression trend of OD values in the right brain tissue in all groups. Sham group (S), model group (M), Du vessel group (D), Ren and Du vessels group (RD) at 12 h, 24 h, 48 h, 96 h and 144 h.

could be increased difficulties in experiment. Taking two different positions for treatment requires much more time, especially for those large sample and long period ones. All the acupoints in this research have been investigated in our previous studies [17, 18], and we have proved the protective effect of electroacupuncture on Ren and Du vessels on CIRI rats. Furthermore, electroacupuncture maintains continuous stimulation on acupoints, and it has greater effects on neuroblast plasticity than acupuncture alone [19]. Besides, a previous study shows that 15 and 30 Hz electroacupuncture intervention plays a protective role in cerebral ischemic injury [20].

Immunity and inflammation are an integral part of the pathogenic processes triggered by ischemia and reperfusion [21]. After onset of cerebral ischemia, a cascade of inflammatory events is initiated through activation of resident cells and recruitment of circulating leucocytes. Inflammatory mediators such as cytokines, chemokines, adhesion molecules, matrix metalloproteinases, nitric oxide, and reactive oxygen species are released, exacerbating cell death and eventually leading to dysfunction of the blood-brain barrier [22]. In the early phase of cerebral isch-

emia, activated microglia and macrophage can secrete TNF- $\alpha$  and IL-1 $\beta$ , which have been primarily involved in the effects of inflammation on brain function downstream actors [23]. Firstly, TNF- $\alpha$  is able to stimulate secretion of IL-1 $\beta$ , furthermore, they can also induce synthesis of other proinflammatory cytokines, such as IL-6, chemokines, and matrix metalloproteinases [24]. IL-1 $\beta$  and IL-6, serving as proinflammatory cytokines [25], could exacerbate brain edema or even induce death of brain cells by the means of recruiting leucocytes, resulting in more severe brain injury. What's more, IL-6 has especially been highlighted as an indicator to reflect the severity of inflammation in stroke lesions [26]. In the later stage, however, IL-6 promotes post-stroke angiogenesis and thereby affords long-term histological and functional protection [27]. Previous studies have proved that IL-1 receptor antagonist reduced ischemic brain damage [28], and reduced the expressions of IL-6 and MMP-9 as well [29].

In this present study, low expressions of IL-1 $\beta$  and IL-6 in shame group are shown. In the early phase of cerebral ischemia reperfusion injury, levels of IL-1 $\beta$  and IL-6 in model group peaked at 24 h, and declined gradually in the middle and late phase. What's more, trend of changes in these two cytokines is similar. The parallel changes suggest that brain injury occurs after cerebral ischemia/reperfusion, and most severe outcome appear in the early stage, gradually the severity reduces as time extending, which are in accordance with previous report [30]. However, we did not found protective impact of IL-6 on brain injury, mainly owing to short time of observation only lasted for 6 days. After treatments, expressions of IL-1 $\beta$  and IL-6 in both D group and RD group were remarkably inhibited in comparison to model group, and lower in RD group than in D group. More specifically, expressions of IL-1 $\beta$  in RD group were inhibited as early as 12 h, and declined to similar levels with shame group in the late phase. Expressions of IL-6 in RD group were significantly down-regulated only slightly higher than those in shame group. As a result, we consider that electroacupuncture on Ren and Du vessels or Du vessel alone plays a neuroprotective part in cerebral ischemia reperfusion injury rats though inhibiting expressions of IL-1 $\beta$  and IL-6 to alleviate inflammation, and electroacupuncture on Ren and Du vessels received much better outcome.

When cerebral ischemia reperfusion happens, MCP-1 enhances acute inflammatory response through cytokine production, altered BBB permeability, and immune cell recruitment contributing to ischemia-induced damage in the brain [24]. It has been shown increased expressions of MCP-1 in experimental ischemic brain tissues, and down-regulation of it could be an effective way to reduce cerebral ischemia injury [31]. In the present study, MCP-1 secretion increased in ischemic brain tissues, thus, we approve that increased levels are closely related exacerbated injury, which is in consistent with previous report. Our data suggested low levels in sham group; MCP-1 expressions were considerably declined after treatment by contrast with model group. Furthermore, they are much lower in RD group than in D group. At the middle and late phase, MCP-1 levels are similar to shame group. Thus, electroacupuncture on Ren and Du vessels might alleviates brain impairment by the way of reducing MCP-1 secretion.

When cerebral ischemia happens, MMP-9 activation could lead to blood-brain barrier breakdown and matrix proteolysis, facilitating leukocyte extravasation [21]. It's reported that inhibiting MMP-9 expression displayed function of nerve protection in experimental focal ischemia reperfusion [32]. In the present study, MMP-9 manifests in a horizontal manner. They are remarkably reduced both in D group and RD group in comparison to model group, with lower levels in RD group. At late period of 144 h, MMP-9 level in RD group is similar to sham group. The results indicate that electroacupuncture on Ren and Du vessels reduces cerebral damage by restoring blood-brain barrier.

Important discoveries were revealed by this study, though, there are also limitations. Cerebral injury is both local and systemic outcome in response to neuroimmune interactions. In this study, we only detected expressions of relevant inflammatory cytokines in ischemic brain tissues, however, whether these cytokines participate in neuroinflammatory regulation or not and to what extent they are affected in the healthy side of the brain remain to be known, and further studies still need to be conducted in the future.

### Conclusion

In summary, we firstly report that electroacupuncture on Ren and Du vessels plays a neuro-

protective part in transient focal cerebral ischemia rats through anti-inflammation. This reveals a potential mechanism of co-application of Ren and Du vessels and is further proved to be an effective approach to cerebral ischemia in clinical practice.

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## Disclosure of conflict of interest

None.

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