The neuroprotection of electro-acupuncture via the PGC-1α/TFAM pathway in transient focal cerebral ischemia rats

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Abstract: ATP depletion is one of the pathological bases in cerebral ischemia. Electro-acupuncture (EA) is widely used in clinical practice for ischemia. However, the mechanism of EA remains unclear. The purpose of this study was to investigate whether EA could activate the AMPK/PGC-1α/TFAM signaling pathway and, consequently, increase the preservation of ATP in rats with ischemia. In this study, 48 rats were randomly divided into four groups as a sham-operation control group (sham group), a middle cerebral artery occlusion group (MCAO group), an EA group, and an EA group blocked by the AMPK inhibitor compound C (EA + CC group) (N = 12/group). The rats of the EA group and EA + CC group received the EA treatment for 7 days. The rats that belonged in the two remaining groups were only grasped in the same condition. Then, their brain tissues were collected for further detection. When compared with other groups, EA significantly reduced neurological deficits score and increased motor function. The cerebral infarction volume was significantly reduced in the EA group according to TTC staining. With western blot, we found that EA improved the ratio of p-AMPKα/AMPKα (P < 0.05), however, there is no difference between the MCAO group and sham group (P > 0.05). In addition, EA also increased the expression of PGC-1α and TFAM (all P < 0.05). By Elisa, we observed that EA increased the preservation of ATP (P < 0.05) and mitochondrial respiratory enzymes, including Complex I (P < 0.05), Complex IV (P < 0.05), but not Complex III (P > 0.05). In summary, we conclude that EA may protect against ischemic damage in MCAO rats, improve the preservation of ATP and mitochondrial respiratory enzymes. This effect may be positively regulated by the activation of the PGC-1α/TFAM signaling pathway.

Introduction

Stroke is currently the second cause of adult death worldwide (Naghavi et al., 2017) and the leading cause of disability (Johnson et al., 2019). Ischemic stroke, which accounts for 85% of all strokes (Gu et al., 2014), often leads to infarctions in the cortex, consequently causes neurological deficits and motor dysfunction. Acupuncture is a classical method in traditional Chinese medicine (TCM) and is widely applied in clinical practice for stroke patients. With that being said, Electro-acupuncture (EA) is a unique technique, featured as an electrical stimulation that is added to traditional acupuncture (Wu et al., 2017). Furthermore, clinical studies have reported that EA treatment is an effective and safe therapy for patients with stroke (Witt et al., 2009; Yu et al., 2019). However, the molecular mechanism of EA for stroke is still unclear.

As we have known, the brain is a high energy-consuming organ that greatly depends on adenosine triphosphate (ATP), which is responsible for supplying energy to maintain brain function. Once ATP depletion occurs, cell damage in the brain can lead to cell death (Fagundes et al., 2018; Giannone et al., 2012). Hence, it can consequently lead to both neurological deficit and motor dysfunction. ATP synthesis is correlated with mitochondrial respiratory chain (MRC), which is composed of NADH dehydrogenase (Complex I), succinate dehydrogenase (Complex II), cytochrome c reductase (Complex III), and cytochrome c oxidase (Complex IV) (Brown et al., 2010; Sousa et al., 2018). A previous study (Olivia et al., 2003) has found that the inhibition of mitochondrial respiratory chain Complexes I-III
discovered that EA could increase the Na⁺-K⁺-ATPase activity and adenosine levels in cerebral ischemia rats. Thus, current evidence indicates that EA treatment has the potential to improve ATP release after stroke.

As mentioned above, we proposed that EA treatment may increase ATP release through the activation of the AMPK/PGC-1α/TFAM signaling pathway in rats with ischemic stroke. Ultimately, it may improve both neurological deficit and motor dysfunction. To verify the hypothesis and to investigate the mechanism of EA treatment, we performed this experiment using an animal model of occlusion of the middle cerebral artery (MCAO). Besides, we observed the effect of EA on neurological deficit score, behavioral assessment, infarct volume, ATP and MRC level, and protein expression of AMPK, p-AMPK, PGC-1α, and TFAM.

Materials and Methods

Reagents and chemicals
Stainless acupuncture needles were prepared by Hua-Tuo Medical Appliance Factory, Suzhou, China. Electroacupuncture apparatus were purchased from Nanjing, China. The suture used in the middle cerebral artery occlusion/reperfusion (MCAO/R) was purchased from Guangzhou Duling Company, China. The primary reagents and chemicals were listed in Tab. 1. The primary and secondary antibodies used in this study were listed in Tab. 2.

Experimental animals and groups
48 male Sprague-Dawley rats [6–8 weeks, 230–250 g, certificate No. SXCK (Zhe) 2019-002] were housed in standard cages under controlled temperature (24 ± 2°C) and a 12 h light/dark cycle (light on 09:00–21:00). Food and water were free to obtain. Based on the random number tables, all the rats were divided into four groups (N = 12/group): Sham-operated control group (sham group), MCAO group, MCAO + EA (EA group), MCAO + EA + compound C group (EA + CC group). All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine.

Preparation of compound C
Compound C (AMPK inhibitor; 10 mg/kg, 0.4 mL/200 g) was dissolved into 10% DMSO and divided into aliquots which were stored at −20°C until use. Aliquots of compound C were administered intraperitoneally to rats in the EA + CC group at the onset of ischemia to inhibit AMPK activation (Yang et al., 2016).

Surgery procedure
Briefly, rats were anesthetized by 10% chloral hydrate (250 mg/kg). After making a ventral cervical midline incision, a nylon suture (diameter: 0.26 mm) was gently introduced from the left common carotid artery (CCA) into the internal carotid artery (ICA), consequently blocking the origin of the middle cerebral artery. The incision was stitched timely. After 1.5 h, the suture was withdrawn to achieve reperfusion. Then, all rats were placed in an environment at room temperature, and oral food intake was allowed. The CCA, ICA, and external carotid artery (ECA) of sham-operated rats were peeled off, but the bloodstream was not blocked. During the surgery, we used a body temperature keeping pad to maintain the body temperature of rats at 37°C.

Experimental treatment
Rats of the EA group and EA + CC group received EA treatment at 48 h after reperfusion. The Stainless

| No. | Reagents and chemicals | Company |
|-----|------------------------|---------|
| 1   | 10% chloral hydrate    | Shanghai Zhanyun Chemical Co., Ltd., Shanghai, China |
| 2   | nylon suture           | Guangzhou Duling Co., Ltd., Shanghai, China |
| 3   | Stainless acupuncture needles | Suzhou Hua Tuo Medical Appliance Factory, Suzhou, China |
| 4   | Electroacupuncture apparatus | HANS-200, Nanjing, China |
| 5   | MRCC I Elisa Kit       | Enzyme Immunoassay Industry of Jiangsu Co., Ltd., China |
| 6   | MRCC III Elisa Kit     | Enzyme Immunoassay Industry of Jiangsu Co., Ltd., China |
| 7   | MRCC IV Elisa Kit      | Enzyme Immunoassay Industry of Jiangsu Co., Ltd., China |
| 8   | ATP Elisa Kit          | Enzyme Immunoassay Industry of Jiangsu Co., Ltd., China |

Note: MRCC: mitochondrial respiratory chain complex.
acupuncture needles (0.3 mm in diameter) were inserted into Qu chi (LI11), Shou san li (LI110), Zu san li (ST36), and Feng long (ST40) on the right limbs at 2–3 mm depth. EA apparatus was connected after acupuncture. Besides, EA treatment was given once a day for continuous 7 days, 30 min per time. EA stimulation using an interrupted wave and low frequency (1–20 Hz) was given to rats to induce the slight tremor of their limbs. Rats of the sham group and MCAO group were only grasped in the same condition. Rats were fixed using cloths during acupuncture.

**Evaluation of the neurological deficit scores**

Neurological evaluation was performed by the same researcher at 48 h after reperfusion and the 7th day after treatment, who was blinded to the group assignment. In this experiment, we adopted the Longa test and modified neurological severity scoring (mNSS) to examine the neurological deficit. The Longa test is a five-point scale (Jiang et al., 2016): Grade 0, symptoms without neurological impairment (normal); Grade 1, inextensibility of its right forepaw when lifting the rats’ tail (mild); Grade 2, circling to the right side while walking (moderate); Grade 3, walking hard and leaning to the right (severe); and Grade 4, cannot walk spontaneously (very severe). In addition, we also employed mNSS to evaluate the neurological deficit scores. The mNSS scale (Bieber et al., 2019; Sha et al., 2019) ranges from 0 (normal) to 18 (maximal deficit). The details of the mNSS test were shown in Appendix 1.

**Evaluation of the motor function**

**Slope test**

We used the slope test to assess the coordination ability and rapid-reaction capability. The rats were placed upside down on a 45° inclined plane, and the time of turning their heads upward >135° was recorded. The results were calculated in seconds. The longer time indicated poorer reaction ability and coordination ability (Fig. 1 (A₁–A₃)).

**Suspension test**

We used the suspension test to assess the muscle strength of the upper limbs in rats. The longer duration time indicated the stronger grasping ability of the upper limbs. Briedly, the rats were allowed to grasp a horizontal cylindrical tool (0.5 cm in diameter) with their front legs, and the height leaving from the ground was 4.5 cm. The time when the rats fell from the tool was recorded. A 5-point scale was adopted in the test as follows: 0, <10 s; 2, 10–30 s; 3, 30 s – 2 min; 4, 2–5 min; 5, >5 min (Fig. 1 (B¹–B³)).

**Balance beam test**

We used an elevated beam (1.5 m length, 25 mm width, and 15 cm height) to assess the gross motor function and balance ability of rats. Before the first test, rats received pre-trained to be familiar with the test. A 5-point scale was adopted in the test as follows (Liang et al., 2018): 0, the rat is able to keep balance and walk on the beam using its unaffected limb preferentially; 2, the rat is able to keep balance and walk on the beam mostly relying on the unaffected limb; 3, the rat is not able to keep balance on the beam once moved; 4, the rat fall off the beam immediately (Fig. 1 (C¹–C³)).

**Measurement of cerebral infarct area**

Three rats from each group were sacrificed at 48 h after reperfusion and 7 days after EA treatment, respectively. All brains were taken quickly and frozen in a refrigerator at −20°C for 20 min. Then, the brain was cut into 5 slices (2 mm thickness) from the frontal extreme to the pillow pole with a sharp blade. In photophobic condition, the brain specimens were stained with 2% 2,3,5-triphenyl tetrazolium chloride (TTC) at 37°C for 20 min and fixed with 4% paraformaldehyde. The normal brain tissue stained red, while infarcted tissue unstained (white). The total volumes of both contralateral and ipsilateral cortex were measured, and the infarct percentage was calculated as % contralateral structure.

**Enzyme-linked immunosorbent assay (ELISA) for mitochondrial respiratory enzymes and ATP**

Peri-infarct cortical tissue samples were resected from the MCAO group, EA group, and EA + CC group, using the method detailed by a previous study (Mätlik et al., 2014). The same position was obtained from the sham group. Brain tissue was obtained from the cortex of the rats prior to centrifugation at 1,000 × g for 10 min at 4°C. Ischemic brain tissue was cut into 30 mg and collected in 270 μL of phosphate-buffered saline (PBS). After homogenization by ultrasonic cell crusher (30 s, on ice), the tissue was centrifuged (14,000 × g, 4°C, 10 min) to collect the

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**TABLE 2**

The primary and secondary antibodies

| No. | Primary                                         | Company                      | Dilution |
|-----|------------------------------------------------|------------------------------|----------|
| 1   | Phosphor-AMPKa (Thr172) Rabbit mAb              | Cell Signaling Technology    | 1:1000   |
| 2   | AMPK alpha Mouse Monoclonal Antibody (66536-1-Ig)| Proteintech                  | 1:1000   |
| 3   | PGC1α Mouse Monoclonal antibody (66369-1-Ig)   | Proteintech                  | 1:1000   |
| 4   | TFAM Rabbit Polyclonal antibody (22586-1-AP)    | Proteintech                  | 1:3000   |
| 5   | Beta Actin Mouse Monoclonal antibody(66009-1-Ig)| Proteintech                  | 1:5000   |
| 6   | HRP-conjugated Affinipur Goat Anti-Mouse IgG(H+L) (SA00001-1) | Proteintech                  | 1:10000  |
| 7   | HRP-conjugated Affinipur Goat Anti-Rabbit IgG(H+L) (SA00001-1) | Proteintech                  | 1:10000  |
supernatant. The titers of AC, cAMP, and PKA were measured using Elisa, and the results were expressed as optical density (OD) units, according to previously published methods (35). The OD of each well was immediately determined using a microplate reader (Bio-Rad) set at 450 nm.

Western blot analysis
We collected brain tissues in the peri-infarct cortical region from the MCAO group, EA group, and EA + CC group, and the same position from the sham group for western blot analysis. The brain tissue was homogenized by an ultrasonic cell crusher (30 s, on ice) and centrifuged (14,000 × g, 4°C, 10 min) to collect the supernatant. BCA protein assay kit was used to assess the protein content. 50 μg protein extracted from each sample were separated by SDS-polyacrylamide gels electrophoresis (SDS–PAGE) and transferred to PVDF membranes. After blockade with 5% nonfat milk for 2 h at room temperature, the membranes were incubated overnight at 4°C with a primary detection antibody. The dilution ratio of the primary detection antibody was listed in Tab. 2. Then, HRP-conjugated secondary antibody was added to the blots and incubated for 1 h at room temperature; protein bands were examined with Image Lab image acquisition and analysis software (Bio-Rad, Hercules, CA, USA). Gray values of proteins from each sample were shown as the densitometric ratio of proteins to β-actin.

Statistical analysis
SPSS statistical package (Version 20.0) was utilized to perform the statistical analysis and GraphPad Prism 8.0 for figures. All data were presented as the mean ± standard deviation. Comparisons among multiple groups were analyzed with one-way ANOVA, followed by LSD (equal variances assumed) or Dunnett’s T3 (equal variances not assumed) test for post hoc analysis. A P-value of less than 0.05 was considered statistically different. The flow chart of this experiment was shown in Fig. 2.

Results

EA treatment reduced neurological deficits
Before treatment, we observed that compared with the sham group, the neurological deficits score increased in the MCAO group, EA group, and EA + CC group (Longa test, all P < 0.05; mNSS test, all P < 0.05) after 48 h of focal cerebral ischemia, and no difference among the three groups (all p > 0.05). The results indicated that the model of focal cerebral ischemia was successfully established. After treatment, EA significantly reduced neurological deficit scores (Longa test, P < 0.05; mNSS test, P < 0.05). There was no difference between the EA group and EA + CC group (P > 0.05) in the Longa test, while a statistical difference was observed in the mNSS test (P < 0.05). Details are shown in Fig. 3. These results demonstrated that EA was able to improve the neurological deficits in transient focal cerebral ischemic rats.

EA treatment increased motor function
According to the results of the balance beam test, after 48 h focal cerebral ischemia, rats in the MCAO group, EA group, and EA + CC group showed poorer balance ability than sham group (all P < 0.05). After treatment, compared with the MCAO group, EA treatment significantly improved the motor function of MCAO rats (P < 0.05) (Fig. 4A). We got the same results from the Slope test (Fig. 4B). However, there was no significant difference between groups based on the results of the suspension test (P > 0.05) (Fig. 4C). These results indicated that rats with focal cerebral ischemia exhibited motor disorder, and EA had the potential advantage to improve the coordination ability, rapid-reaction capability, and balance ability in MCAO rats.
EA treatment attenuated the infarct volumes of the rats’ brains
As shown in Fig. 5, at 48 h after reperfusion, there was no sign of
cerebral injury in the sham group, while obvious cerebral
infarction with white staining was obtained in all rats of the
MCAO group, EA group, and EA + CC group (all P < 0.05).
No difference was existed among the MCAO group, EA
group, and EA + CC group (all P > 0.05). These results
indicated that the MCAO model was successfully established.
At 7 days after intervention, infarct volumes in the MCAO
group were 47.93 ± 6.92%. In contrast, treating with
EA significantly reduced infarct volume to 5.67 ± 3.62%
(P < 0.05). The infarct volume in EA + CC group decreased
to 16.19 ± 5.26% (P < 0.05). However, we also noted that
there is a marked decrease in cerebral infarct volumes in
the MCAO group (from 77.8 ± 11.33 to 47.93 ± 6.92). The
phenomenon may be associated with the tendency of self-
healing with the prolongation of ischemia time.

EA treatment increased the preservation of ATP and
mitochondrial respiratory enzymes
As shown in Fig. 6, the concentration of mitochondrial
respiratory enzymes, including Complex I, Complex III,
and Complex V, and the content of ATP were downregulated in MCAO rats (all $P < 0.05$). Compared to the MCAO group, the EA group had a significant increase of the Complex I, Complex V, and ATP levels (all $P < 0.05$), but not Complex III ($P > 0.05$). The concentration of these enzymes and the ATP content decreased in the EA + CC group, and there was a significant difference between the EA group and EA + CC group ($P < 0.05$).

All these results showed that ATP fall did exist in MCAO rats, and EA treatment was able to improve the preservation of ATP and mitochondrial respiratory enzymes.

As shown in Fig. 7, compared with the sham group, the ratio of p-AMPKα/AMPKα, the expression level of PGC-1α and TFAM was downregulated in the MCAO group (p-AMPKα/AMPKα, $P > 0.05$; PGC-1α, $P < 0.05$; TFAM, $P < 0.05$). EA has a significant advantage to increase these indicators ($P < 0.05$), while compound C had an opposite influence on the ratio of p-AMPKα/AMPKα, the expression level of PGC-1α and TFAM ($P < 0.05$). These results demonstrated that EA treatment could regulate the PGC-1α/TFAM pathway in focal cerebral ischemia.
Discussion

EA is popularly used in the treatment of cerebral ischemia. In clinical practice, EA is commonly performed with the acupoints of Quchi (LI11) and Zusanli (ST36) in patients with stroke (Chen et al., 2020; Cho et al., 2013). Several experimental studies have shown that the application of EA on Quchi (LI11) and Zusanli (ST36) acupoints, acted as a neuroprotective role in cerebral ischemic rats (Tao et al., 2016; Wu et al., 2017). In addition, some data mining studies (Zhang et al., 2018) suggested that the most frequently used meridian is the Yangming meridian. Thus, in this study, we chose Quchi (LI11), Zusanli (ST36), Shousanli (LI10), and Fenglong (ST40) and evaluated the effects of EA treatment in MCAO rats.

Firstly, our study showed that EA treatment could decrease the infarct volume, reduce the neurological deficit, and improve motor function in MCAO rats. Our findings were consistent with several other studies. Sha et al. (2019) and Liu and Lai (2006) reported that EA was able to decrease the infarct volume in MCAO rats. The improvement of motor function induced by EA treatment has been confirmed by animal experiments (Xu et al., 2015), clinical study (Wang et al., 2020b), and meta-analysis (Cai et al., 2017). However, we noted that the infarct volume also decreased in the MCAO group; the reason may be related to the self-healing capability after reperfusion in MCAO rats. Li et al. (2021) observed that the infarct volume of the MCAO group decreased 14 days after reperfusion, and they reported spontaneous recovery existed in the brain lesions of the MCAO rats. Liu and Lai (2006) also observed that infarct volumes of MCAO rats presented a natural reduction between 3 and 7 days after reperfusion.

Secondly, we found that the ATP level, the MRCC concentration, and protein expression of PGC-1α and TFAM significantly decreased in MCAO rats. The results indicated that the ATP depletion caused by the inactivation of the PGC-1α/TFAM pathway may be one of the vital factors for focal cerebral ischemia. When cerebral ischemia occurs, a series of complex pathological events called the ischemic cascade (Brouns and Deyn, 2006; Hossmann, 2006) such as energy failure, neurotoxicity, peri-infarct depolarization, and programmed cell death, may happen. During this process, the energy failure caused by ATP depletion was an initiator of brain damage and ultimately lead to cell death (Frenguelli, 2019). Both Olivia et al. (2003) and Zhao et al. (2013) reported that the occlusion of the middle cerebral artery caused a great reduction of the ATP levels in MCAO rats. Additionally, MRCC, which is comprised of five complexes, could transport electrons to form a proton motive force and further regulate the ATP synthesis (Nagao et al., 2020). A robust increase in the content of the de-active form of Complex I (Stepanova et al., 2019) and Complex IV was observed in ischemic rats (Racay et al., 2009). PGC-1α, as a vital role in the regulation of energy metabolism in tissues/organs (Finck and Kelly, 2006; Handschin and Spiegelman, 2006; St-Pierre et al., 2006), could directly/indirectly upregulate the TFAM. The PGC-1α/TFAM is a major upstream signaling pathway of MRCC (Fu et al., 2016; Yang et al., 2018). Wang et al. (2020a) found that low expression of PGC-1α and TFAM protein led to a reduction of ATP, Complex I and V in MCAO rats. Geng et al. (2017) reported that poor expression of PGC-1α decreased the ATP content, and PGC-1α may be a therapeutic target for the treatment of cerebral ischemia/reperfusion injury.

Thirdly, we discovered that the over-expression of PGC-1α and TFAM induced by EA may improve the preservation of ATP, Complex I, and Complex IV. Some researchers have proposed that preventing ATP depletion may be a neuroprotective strategy in brain ischemia. Our study confirmed this proposition and was consistent with the results of several studies. Researchers reported that the improvement of ATP and MRCC protected neurons in ischemia rats (Dai et al., 2017; Galeffi et al., 2010; Olivia et al., 2003). In addition, our results elucidated that EA activated the PGC-1α/TFAM signaling pathway in ischemic rats. Numerous studies have demonstrated that PGC-1α is able to protect against cerebral ischemia. Xu et al. (2018) reported that the activation of PGC-1α could reduce the cerebral infarction volume and improve the neurological deficit in MCAO rats. Wang et al. (2016) found that over-expression of PGC-1α and TFAM induced by carbon monoxide (CO) could promote ATP release and increase brain mitochondrial biogenesis in global cerebral ischemia. Therefore, the activation of the PGC-1α/TFAM signaling pathway might be a potential therapeutic target for ischemia. Liu et al. (2019) reported that EA significantly upregulated the expression of PGC-1α and TFAM in heart tissue of myocardial ischemia rats. Xie (2016) also observed that the high expression of PGC-1α and TFAM protein could

![FIGURE 6. Effect of EA treatment on the concentration of mitochondrial respiratory enzymes and ATP release. The level of Complex I, Complex III, Complex IV, and ATP in brain tissue were measured using the ELISA test after the 7-day intervention (A–D). Data were expressed as mean ± SD (N = 3). *p < 0.05. MRCC: mitochondrial respiratory chain complex.](image-url)
be induced by EA in myocardial ischemia rats. Our study found EA upregulated the expression of PGC-1α and TFAM in MCAO rats, indicated that EA may act as a neuroprotective role through activation of the PGC-1α/TFAM signaling pathway.

However, we noted that no statistical difference between the MCAO group and sham group in the ratio of p-AMPK/AMPKα. While the behavioral results showed that the EA + CC group could alleviate neurological deficits and motor function. Probably, some other molecule regulates the PGC-1α/TEAM pathway, and AMPK may be a concomitant consequence of EA in ischemia rather than a mechanistic cause.

Overall, our results revealed that EA could reduce infarct volume and neurological deficits, improve the motor function in focal cerebral ischemia rats via promoting the preservation of ATP and mitochondrial respiratory enzymes. This effect may be related to the activation of the PGC-1α/TFAM pathway.

There were also some limitations in this study. Firstly, the sample size of the TTC staining, ELISA test, and Western blot was small. Secondly, the behavioral assessment of rats was limited by the subjective judgment of the researchers. More objective assessment tools are required to reduce the assessment bias, such as the catwalk system (Caballero-Garrido et al., 2017) or the homecage scan monitoring system (Salem et al., 2016). Thirdly, a more intuitive device could be utilized to estimate whether the model was established, such as laser-Doppler flowmetry (LDF) (Harada et al., 2005). Fourthly, intraventricular injection is optimum for further research study. Fifthly, EA’s placebo effect should be taken into consideration, and sham acupuncture should be added as a comparison.

Conclusion

Our results suggested that EA could protect against ischemic damage in MCAO rats, improve the preservation of ATP and mitochondrial respiratory enzymes. Thus, this effect may be positively regulated by the activation of the PGC-1α/TFAM signaling pathway.

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Availability of Data and Materials: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.


**Authors’ Contributions:** The authors confirm contribution to the paper as follows: study conception and design: Luping Yang, Yijing Jiang; the experiment: Luping Yang, Xiaochuan Ye, Yongmei You, Ling Lin, Jing Lian, analysis and interpretation of results: Luping Yang, Juan Li; draft manuscript preparation: Luping Yang; manuscript revision: Shanli Yang, Xiehua Xue. All authors reviewed the results and approved the final version of the manuscript.

**Ethics Approval:** The study was approved by the Ethics Committee of Fujian University of Traditional Chinese Medicine on May 10, 2019. The paper involving rats, their care of the laboratory animals complied with Guidance Suggestions for the Care and Use of Laboratory Animals 2006 administered by Ministry of Science and Technology, China.

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**Conflicts of Interest:** The authors declare that they have no competing interests.

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### Appendix 1

The details of mNSS test

| Context                        | Details                                      | Points |
|--------------------------------|----------------------------------------------|--------|
| **Motor tests**                | Raising rat by the tail                      |        |
|                                | Flexion of forelimb                          | 1      |
|                                | Flexion of hindlimb                          | 1      |
|                                | Head moved >10° to vertical axis within 30 s | 1      |
|                                | Placing rat on the floor (normal = 0; maximum = 3): | 0–3     |
|                                | 0: Normal walk                               |        |
|                                | 1: Inability to walk straight                |        |
|                                | 2: Circling toward the paretic side          |        |
|                                | 3: Fall down to the paretic side             |        |
| **Sensory tests**              | 1: Placing test (visual and tactile test).    | 0–2    |
|                                | 2: Proprioceptive test (deep sensation, pushing the paw against the table edge to stimulate limb muscles). |        |
| **Beam balance tests**         | (normal = 0; maximum = 6):                   | 0–6    |
|                                | 0: Balances with steady posture              |        |
|                                | 1: Grasps side of beam                       |        |
|                                | 2: Hugs the beam and one limb falls down from the beam |        |
|                                | 3: Hugs the beam and two limbs fall down from the beam, or spins on beam (>60 s) |        |
|                                | 4: Attempts to balance on the beam but falls off (>40 s) |        |
|                                | 5: Attempts to balance on the beam but falls off (>20 s) |        |
|                                | 6: Falls off: No attempt to balance or hang on to the beam (<20 s) |        |
| **Reflexes absent and abnormal movements** | Pinna reflex (head shake when touching the auditory meatus) | 1 |
|                                | Corneal reflex (eye blink when lightly touching the cornea with cotton) | 1 |
|                                | Startle reflex (motor response to a brief noise from snapping a clipboard paper) | 1 |
|                                | Seizures, myoclonus, myodystonia             | 1      |
| **Maximum points**             |                                              | 0–18   |