

# Synergistic effects of electroacupuncture and bone marrow stromal cells transplantation therapy in ischemic stroke

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**Abstract. – OBJECTIVE:** Animal studies and clinical trials demonstrated the effectiveness of a combination of transplanted bone marrow stromal cells (BMSC) and electroacupuncture (EA) treatment in improving neurological deficits. However, the ability of the BMSC-EA treatment to enhance brain repair processes or the neuronal plasticity of BMSC in ischemic stroke model is unclear. The purpose of this study was to investigate the neuroprotective effects and neuronal plasticity of BMSC transplantation combined with EA in ischemic stroke.

**MATERIALS AND METHODS:** A male Sprague-Dawley (SD) rat middle cerebral artery occlusion (MCAO) model was used. Intracerebral transplantation of BMSC, transfected with lentiviral vectors expressing green fluorescent protein (GFP), was performed using a stereotactic apparatus after modeling. MCAO rats were treated with BMSC injection alone or in combination with EA. After the treatment, proliferation and migration of BMSC were observed in different groups by fluorescence microscopy. Quantitative real-time PCR (qRT-PCR), Western blotting, and immunohistochemistry were performed to examine changes in the levels of neuron-specific enolase (NSE) and nestin in the injured striatum.

**RESULTS:** Epifluorescence microscopy revealed that most BMSC in the cerebrum were lysed; few transplanted BMSC survived, and some living cells migrated to areas around the lesion site. NSE was overexpressed in the striatum of MCAO rats, illustrating the neurological deficits caused by cerebral ischemia-reperfusion. The combination of BMSC transplantation and EA attenuated the expression of NSE, indicating nerve injury repair. Although the qRT-PCR results showed that BMSC-EA treatment elevated nestin RNA expression, less robust responses were observed in other tests.

**CONCLUSIONS:** Our results show that the combination treatment significantly improved restoration of neurological deficits in the animal stroke model. However, further studies are required to see if EA could promote the rapid differentiation of BMSC into neural stem cells in the short term.

*Key Words:*

Bone marrow mesenchymal stem cells, Cerebral infarction, Stereotaxic transplantation, Electroacupuncture.

## Introduction

Ischemic stroke is a cerebrovascular event that is caused by blood supply disruption and is associated with high morbidity and mortality rates<sup>1,2</sup>. Existing neuroprotective pharmacotherapy often fails to prevent the death of neurons in the ischemic penumbra or to promote cerebrovascular regeneration and neurogenesis after the acute phase<sup>3</sup>, possibly due to the significant damage caused by the stroke.

The use of mesenchymal stem cells (MSC) for treating stroke has been extensively studied for over two decades. These are pluripotent cells that are easily cultured<sup>4</sup>, able to differentiate into various cell lineages, including neuron-like cells<sup>5,6</sup>, are suitable for safe autologous stem cell transplantation, and provide support and regeneration of damaged tissue<sup>7</sup>.

Bone marrow stromal cells (BMSC) are multipotent MSC that can be safely harvested, induced to differentiate into neurons and endothelial cells and used to reconstitute various damaged tissues

and enhance endogenous neurogenesis, synaptogenesis, and angiogenesis in ischemic brain tissue<sup>8</sup>. BMSC express a broad range of growth factors that promote maintenance and repair of penumbra, decrease the infarction size, and improve functional deficits associated with stroke<sup>9</sup>. However, many issues remain to be resolved, such as extending BMSC survival time, promoting neuroblast migration, and promoting the specific differentiation of BMSC into neuronal cells but not into fat, bone, or cartilage cells<sup>10</sup>. To date, several compounds have shown<sup>11,12</sup> a synergistic effect in combined treatment with MSCs in various models of stroke. The enhancement of BMSC therapy by other treatments not only provides a cumulative effect of dual therapy but also can more effectively target specific organs and diseases<sup>13</sup>.

Electroacupuncture (EA), a combination of acupuncture and electrical stimulation, is widely accepted in clinical practice as a supplementary therapy for ischemic stroke<sup>14</sup>. Studies<sup>15-18</sup> show that EA is able to lower the toxicity of excitatory amino acids<sup>15</sup>, promote the synthesis and release of brain-derived neurotrophic factor and nerve growth factor<sup>16</sup>, increase the number of endogenous mesenchymal stem cell<sup>17</sup>, and promote the survival and differentiation of BMSC in injured spinal cord animal model<sup>18</sup>. However, whether EA can improve the efficacy of BMSC treatment in stroke model remains to be determined.

Several biological markers are commonly used to assess neuronal damage and regeneration. Neuron-specific enolase (NSE) is a glycolytic enzyme and a highly specific marker for neurons and peripheral neuroendocrine cells<sup>19</sup>. NSE is currently considered a reliable indicator of the extent of neuronal damage<sup>20-22</sup>. Serum NSE levels of stroke patients are significantly higher than that of healthy individuals<sup>23-25</sup>. These levels correlate with neurobehavioral outcome and the volume of infarction<sup>20,26</sup>. In animal experiments, cerebral and circulatory NSE levels increased following focal ischemia<sup>27</sup>, and hypothermia followed by rapid rewarming at the ischemic stroke site in rats enhanced the neuronal loss and elevated plasma levels of NSE<sup>28</sup>. NSE concentrations in rat cerebrospinal fluid were verified as a quantitative marker of neuronal damage<sup>29</sup>. NSE level also decreased in the peripheral blood of the disease model upon pretreatment with (S)-3,5-dihydroxyphenylglycine or nicardipine administration<sup>30,31</sup>. Nestin is a protein marker of multipotent neural stem cells (NSC)<sup>32,33</sup>, that together with other structural proteins, participates in cellular

remodeling. During embryogenesis, most nestin-positive cells in early development stages are stem/progenitor populations engaged in active proliferation<sup>34</sup>. However, in adult tissues, nestin is primarily restricted to regions of regeneration. Numerous *in vivo* and *in vitro* studies now rely on nestin expression to track the proliferation, migration, and differentiation of MSC<sup>35,36</sup>.

The aim of this study was to determine whether EA combined with BMSC transplantation could be a viable therapeutic approach to stimulate brain plasticity and functional recovery.

## Materials and Methods

### Animals

Seventy-two healthy, male, specific pathogen-free Sprague-Dawley (SD) rats (age, 8-10 weeks; weight, 240-260 g) were provided by the Changsha Tianqin Experimental Animal Center [certificate of conformity: SCXK (Xiang) 2014-0011]. All the experiments were performed in accordance with the Guidelines for Animal Research of Hainan Medical University. Relevant protocols were submitted to the Hainan Medical College Ethics Committee (No. HYLL-2022-134, Date: 2022-03-24). The experiments were conducted between June 2016 and January 2018 at the Hainan Medical University Animal Laboratory Center.

### Middle Cerebral Artery Occlusion Animal Model

Male SD rats were anesthetized by intraperitoneal administration of 10% Pentobarbital sodium (0.3 ml/100 g, Merck, Darmstadt, Germany; catalog number: 57330). The transient middle cerebral artery occlusion (MCAO) model was induced by intraluminal vascular occlusion in the right middle cerebral artery. The method used to induce ischemia was modified from that of Li et al<sup>37</sup>. Neurological deficits were assessed using a modified neurological function score (NSS)<sup>38</sup>. At 24 hours after reperfusion, neurological deficit scores of 6-12 points were assigned, and MCAO rats were randomly divided into a model group, EA group, BMSC group, and EA+BMSC group (n=12/group). The control groups were a sham operation group, and a healthy control group (n=12/group).

### Recovery and Passage of BMSC and Green Fluorescent Protein (GFP)-Labeled BMSC

BMSC (catalog number: RASMX-01001) and GFP-labeled BMSC (catalog number: RASMX-01101)

were purchased from Cyagen Biosciences Inc. (Guangzhou, China). BMSC and GFP-labeled BMSC were obtained from the bone marrow of SD rats. After adherent culture, GFP-labeled BMSCs were transduced by lentivirus with *GFP* gene to make the cells stably express GFP. BMSC and GFP-labeled BMSC were identified by flow cytometry. The results showed that the bone marrow stromal marker CD90 and CD44 (>70%) and CD34, CD11b and CD45 (<5%) was qualified. The cells were then resuspended in fresh OriCell TM MSC growth medium (Cyagen Biosciences Inc, Santa Clara, CA, USA, RASMIX-90011) and seeded into T25 flasks. Cells were incubated at 37°C in a 5% CO<sub>2</sub> humidified incubator. The next day, the medium was replaced with fresh growth medium (pre-warmed to 37°C). Thereafter, the growth medium was changed every three days. When the cells were approximately 80-90% confluent, they were dissociated with 0.25% trypsin/0.04% EDTA (EDTA-10001) and passaged at a 1:2 dilution. After 2 passages, the cell suspension concentration was adjusted to 5×10<sup>7</sup> mL<sup>-1</sup> BMSC/GFP-labeled BMSC and the MCAO rats were injected intracranially with BMSC/GFP-labeled BMSC at the concentration of 5×10<sup>5</sup>/10 μl. GFP labeled BMSC was used to observe cell differentiation and migration in the brain under fluorescence microscope.

#### **Transplantation of BMSCs into MCAO Rats**

After one week, BMSC were injected intracerebrally into the striatum of the infarcted hemisphere of the animal model using a ZH-Lanxin C/S stereotactic apparatus (Anhui Zhenghua Teaching Experimental Instrument Factory, Anhui, China) at the following coordinates: 0.3 mm posterior to the bregma, 3.00 mm laterally, and 4.0 mm in depth (referring to Paxinos and Watson's rat brain atlas<sup>39</sup>). To avoid affecting the follow-up EA treatment, an incision was made in the cranial midline lateral 3-mm, and the skull was drilled with a dental drill. The BMSC group and EA+BMSC group were injected with 5×10<sup>5</sup> BMSC suspended in 10 μl phosphate buffered saline (PBS) in the right striatum using a micro-pump (Shanghai GAUGE Industry and Trade Co., Ltd., Shanghai, China) over a period of 10 min (5×10<sup>5</sup>/10 μl BMSCs). After the injection, the needles were kept in place for 5 min and then slowly removed. The wound was sealed by bone wax and closed using a medical suture. The rats were not given immunosuppressive drugs or antibiotics postoperatively.

#### **EA Treatment**

The BMSC group was observed without intervention. The EA group and EA+BMSC groups were treated by EA for one week after BMSC implantation. The treatment was initiated at 8:00 each day. The rats were treated at the Baihui (GV 20) (parietal bone) and Dazhui (DU14) acupuncture points (between the seventh cervical vertebra and the first thoracic vertebra, in the middle of the back). The Baihui point was punctured using a 0.5-inch needle, from the front of the head midline to the back with a horizontal displacement of 5 mm. Stabbing obliquely at a 45° angle in the back into the Dazhui (DU14). After the needle insertion, continuous-wave (frequency, 3 Hz; intensity, 1 V) EA stimulation was sustained for 15 min<sup>40</sup>. The treatments were administered for one week. After one week, rats were euthanized by cervical dislocation, and the brains were fixed for further testing.

#### **Fluorescence of GFP-Labeled BMSC**

Rats were anesthetized with 10% Pentobarbital sodium and then thoracotomized. The rats were perfused with 4°C saline (150 mL) rapidly until the liver and lungs became white and then transfused with cold 4% paraformaldehyde. The brains were quickly removed and fixed with cold 4% paraformaldehyde. Brain tissue was dehydrated by sucrose solution gradient, embedded with optimal cutting temperature (OCT), frozen in liquid nitrogen, and stored at -80°C. Frozen sections were prepared (coronal section thickness, 8 μm), and the tissue was dehydrated, fixed, and directly observed and photographed under the Olympus IX51 Inverted Epi-Fluorescence Research Microscope.

#### **Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)**

Expression levels of NSE and nestin were measured by SYBR-Green real-time qRT-PCR in all the groups. NSE, Nestin, and GAPDH (internal reference) primers were purchased from Shanghai Biological Engineering Co., Ltd (Shanghai, China). The SYBR-Green fluorescence quantitative PCR kit was provided by Tiangen Biochemical Technology Co., Ltd (Beijing, China). Total RNA was extracted using the RNAprep Pure Tissue Kit (Tiangen Biochemical Technology Co. Ltd, Beijing, China) according to the manufacturer's instructions. The purity and concentration of extracted total RNA were determined at 260 and

280 nm using an ultraviolet spectrophotometer. The integrity of the total RNA was determined by agarose gel electrophoresis, and the RNA was then transcribed into cDNA. The volume of the PCR reaction was 20  $\mu$ l (10  $\mu$ l SYBR-Green master mix, 0.6  $\mu$ l each of the forward and reverse primers, 1  $\mu$ l of cDNA, 0.4  $\mu$ l of 50  $\times$  ROX Reference Dye, 7.4  $\mu$ l of ddH<sub>2</sub>O). The following parameters were used for the qRT-PCR reaction: 15 min of pre-denaturation (95°C) and 40 cycles of 95°C for 10 s and 62°C for 30 s using a Light Cycler 480 (Roche Automatic launch by machine Mx3005P, Basel, [Switzerland]). At least three independent PCR reactions were performed for each sample [fluorescence quantitative PCR was performed using the Mx3005P system (Stratagene, La Jolla, CA, US)]. Relative target gene expression was calculated using the comparative  $2^{-\Delta\Delta Ct}$  method, and SYBR-Green qRT-PCR was performed. The forward and reverse primers were as follows: NSE FWD: GGAGTTGGATGGGACTGAGA, REV: TGAGCAATGTGGCGATAGAG; Nestin FWD: AGATCGCTCAGATCCTGGAA, REV: AGGTGTCTGCAACCGAGAGT; GAPDH FWD: GACATGCCGCCTGGAGAAAC, REV: AGCCCAGGATGCCCTTTAGT.

#### **Western Blotting for NSE and Nestin Protein Expression in the Striatum**

The specimens from the striatum were added to 400  $\mu$ l of lysis buffer containing phenylmethylsulfonyl fluoride (PMSF). Tissue homogenate was then incubated at 4°C for 30 min, followed by 10,000 g centrifugation for 5 min at 4°C to extract the sample protein. Sample protein concentration was determined by the bicinchoninic acid (BCA) method using fetal bovine serum albumin (BSA) as a standard. The samples were stored at -20°C. Then, 30  $\mu$ g of protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by wet transfer to polyvinylidene fluoride (PVDF) membrane (Sigma-Aldrich Shanghai Trading Co. Ltd, Shanghai, China) and blocked using 3% BSA. The membranes were incubated with the following primary antibodies: NSE mouse monoclonal antibody (1:1,000; Proteintech; catalog number: 66150-1-Ig), nestin mouse monoclonal antibody (1:1,000; Proteintech; catalog number: 66259-1-Ig), and anti-GAPDH mouse monoclonal antibody (internal standard; 1:5,000; Abbkine; catalog number: A01020). The primary antibodies were detected using horseradish peroxidase-conjugated goat

anti-mouse IgG (1:1,000 dilution; servicebio; catalog number: GB23301) and a DAB reaction were used to visualize the protein bands (ZLI-9017, Zhongshan Golden Bridge, Beijing, China). Gray values on the immunoblot were analyzed using Quantity One 1-D Analysis Software version 4.4 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

#### **Fluorescent Immunohistochemistry to Detect NSE and Nestin Expression**

Frozen brain sections were fixed in cold (4°C) acetone for 15 min, rinsed with PBS/0.3% TritonX-100, and incubated for 10 min in normal goat serum to block nonspecific antigen binding. The membranes were incubated with the primary antibody (1:50 NSE mouse monoclonal antibody or 1:100 nestin mouse monoclonal antibody) overnight at 4°C, followed by incubation in 1:100 Cy3- or FITC-conjugated goat anti-mouse IgG secondary antibody (Boster, Wuhan, China) at 37°C for 30 min. The sections were placed on a slide and observed under a fluorescence microscope. As a control, PBS was used instead of the primary antibody, and nonspecific staining was determined following the addition of the conjugated secondary antibody alone.

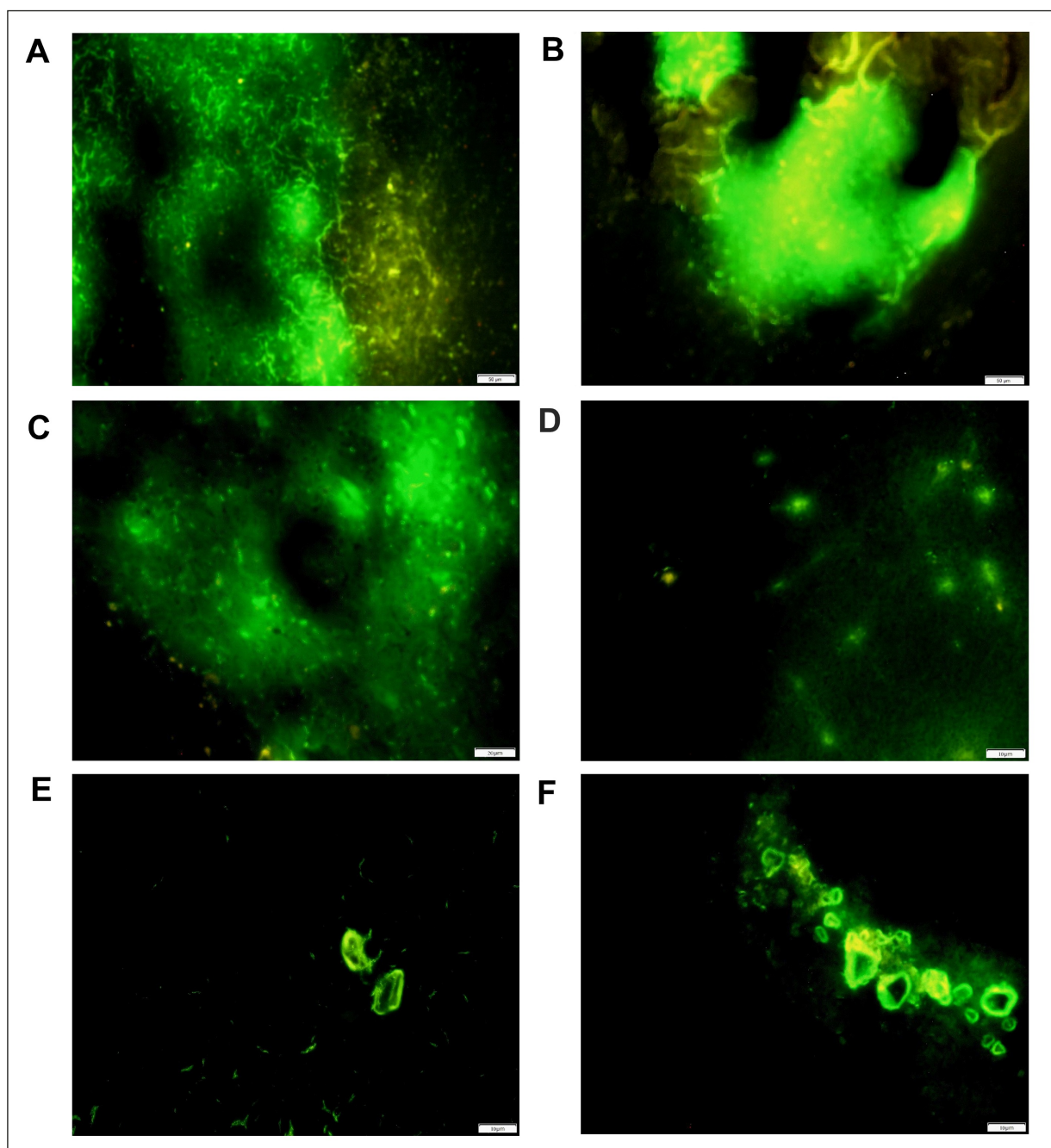
#### **Statistical Analysis**

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Measurement data were expressed as the means  $\pm$  standard deviation ( $\bar{x}\pm s$ ), and the results were analyzed using one-way analysis of variance followed by the LSD-*t* post hoc test for multiple comparisons of each treatment group. Statistical significance was defined as a *p*-value <0.05.

## **Results**

Epifluorescence microscopy revealed that some BMSC survived intracranially, and some living cells migrated to areas around the lesion site. However, most BMSC in the cranium were lysed within a week after transplantation and were absorbed by the surrounding tissues. There were neuronal precursor cells in the BMSC group, and some BMSC were transformed into chondrocytes and adipocytes (Figure 1).

The mRNA levels of NSE were increased in the model groups compared to that of the healthy control group, and the difference between the MCAO group and control group was statistically significant (*p*=0.004, <0.05).



**Figure 1.** Survival, migration, and differentiation of BMSC one week after the implantation. **A**, Implanted GFP-labeled BMSC in the striatum; **B**) Migration of BMSC to the adjacent cortex; **C**) BMSC transformed into different types of cells; **D**) BMSC transformed into neuronal precursor cells; **E**) Fluorescence after partial cell lysis; **F**) Stem cells differentiated into chondrocytes or adipocytes. **A-C**, 50  $\mu\text{m}$ ; **D-F**) 10  $\mu\text{m}$ .

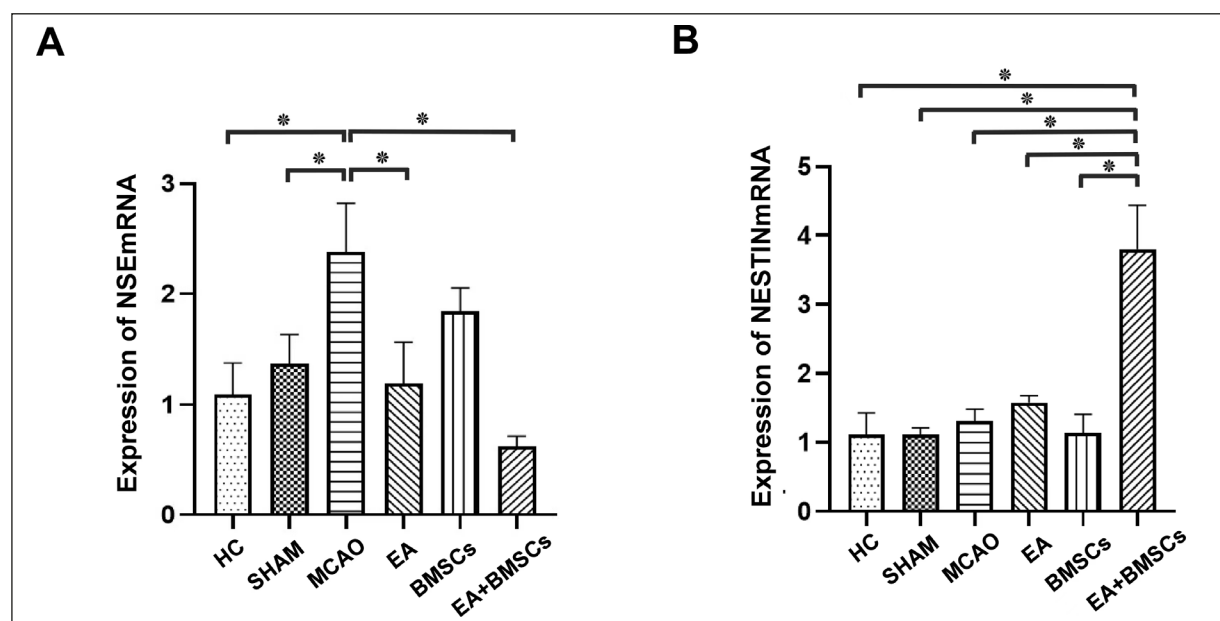
NSE expression in the BMSC group was sustained at a similar elevated level compared to MCAO group ( $p > 0.05$ ). However, NSE expression in the EA group ( $p = 0.012$ ,  $< 0.05$ ) or the EA+BMSC group ( $p = 0.001$ ,  $< 0.05$ ) was significantly lower than that in MCAO group. No significant difference in nestin expression

was observed among the normal control, sham operation, model, EA and BMSCs groups ( $p > 0.05$ ). Levels of nestin expression were significantly different in the EA+BMSC group compared to the model ( $p = 0.000$ ,  $< 0.05$ ) and the BMSC group ( $p = 0.000$ ,  $< 0.05$ ) (Table I and Figure 2).

**Table 1.** NSE and nestin mRNA expression in cerebral infarction rats.

Group	Health control group	Sham operation group	MCAO group	EA group	BMSC group	EA + BMSC group	F	p
NSE	1.089±0.581	1.371±0.514 <sup>b</sup>	2.378±0.888 <sup>a</sup>	1.190±0.731 <sup>b</sup>	1.838±0.424	0.622±0.186 <sup>b</sup>	4.247	0.01
Nestin	1.110±0.636 <sup>c</sup>	1.117±0.186 <sup>c</sup>	1.311±1.575 <sup>c</sup>	1.575±0.206 <sup>c</sup>	1.142±0.526 <sup>c</sup>	3.802±1.273	10.74	0.000

<sup>a</sup>: compared with the health control group, <sup>b</sup>: compared with MCAO, <sup>c</sup>: compared with EA+BMSC. Statistical analysis by one-way ANOVA, pairwise comparisons using LSD-*t* test,  $p < 0.05$ .



**Figure 2.** Comparison of the mRNA expressions of NSE (A) and nestin (B) in each group. Statistical analysis by one-way ANOVA, pairwise comparisons using an LSD-*t* test. \*  $p < 0.05$ .

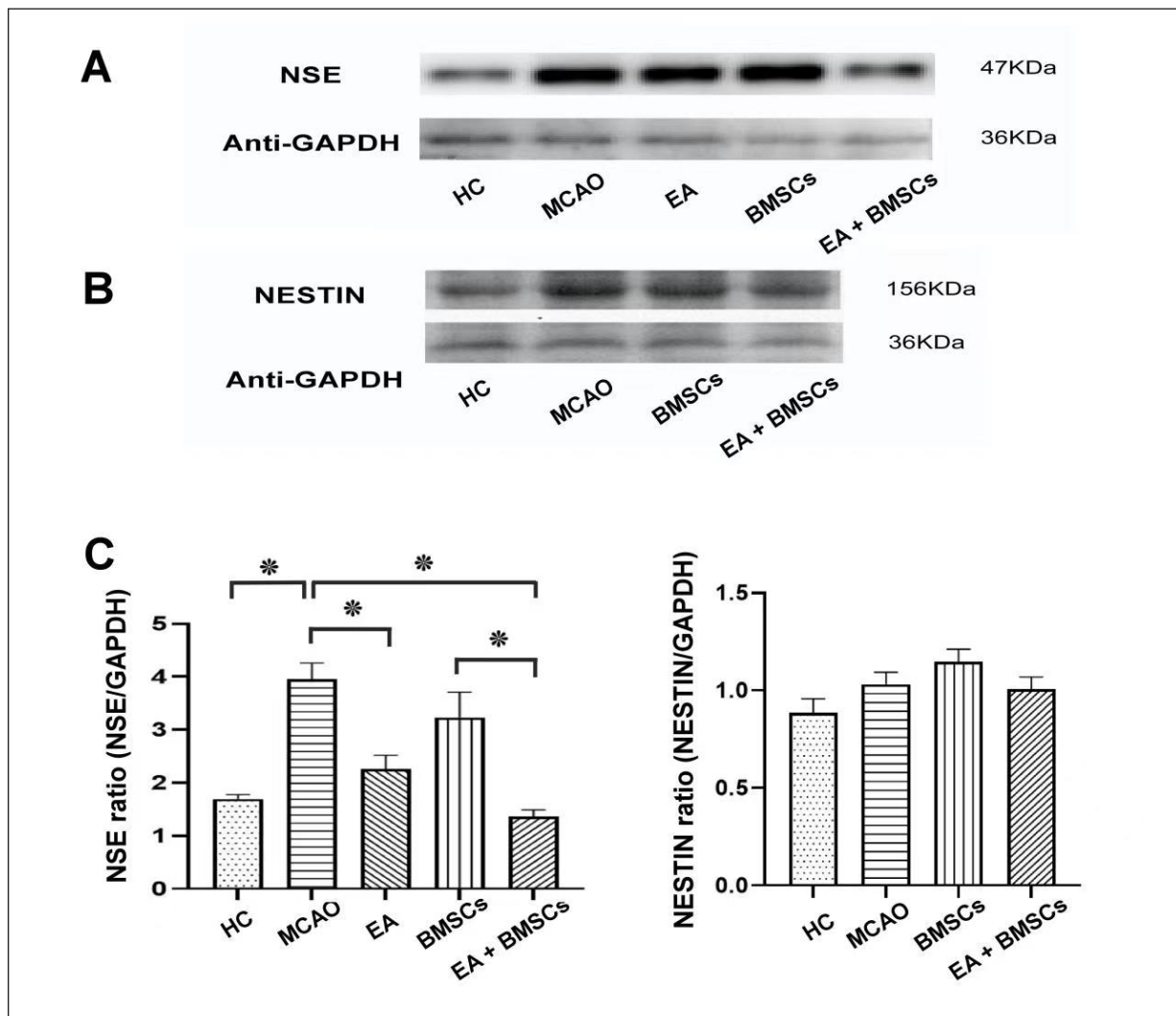
NSE protein expression in the MCAO group was significantly increased compared to that in the healthy control group ( $p = 0.000$ ,  $< 0.05$ ). In the BMSC group, NSE levels were not significantly different from that of the MCAO group ( $p > 0.05$ ). The expression of NSE in the EA group was lower than that in MCAO group ( $p = 0.001$ ,  $< 0.05$ ). The expression of NSE in the EA+BMSC group was lower than that in MCAO group ( $p = 0.000$ ,  $< 0.05$ ), and in BMSC group ( $p = 0.000$ ,  $< 0.05$ ). Nestin expression did not differ among the healthy control, model, BMSCs, and EA+BMSCs groups ( $p > 0.05$ ) (Figure 3).

The immunofluorescence results showed that NSE was strongly expressed in the striatum in the cerebral infarction rat model group. NSE-positive neurons exhibited red fluorescence in the cerebral infarction rat model. Cellular NSE expression was lower in EA and EA+BMSC groups. NSE protein expression in the MCAO

and EA + BMSC groups was consistent with the gene expression analyses (Figure 4). Nestin was not expressed in any of the groups.

## Discussion

BMSC transplantation is regarded as a potential strategy for ischemic stroke treatment due to the capability of BMSC to provide neuroprotection and cell replacement in damaged tissue<sup>41</sup>. Therefore, the acclimatization and survival of BMSC in the brain are paramount since clinical efficacy can only be achieved by viable cells, unlike cells *in vitro* that grow and proliferate for long periods of time<sup>42</sup>. BMSC survival following transplantation has been reported<sup>43,44</sup> to range from several days to almost one year. Our results indicate that some grafted BMSCs survived after one week. While lysed cells were assimilated by the surrounding tissue,



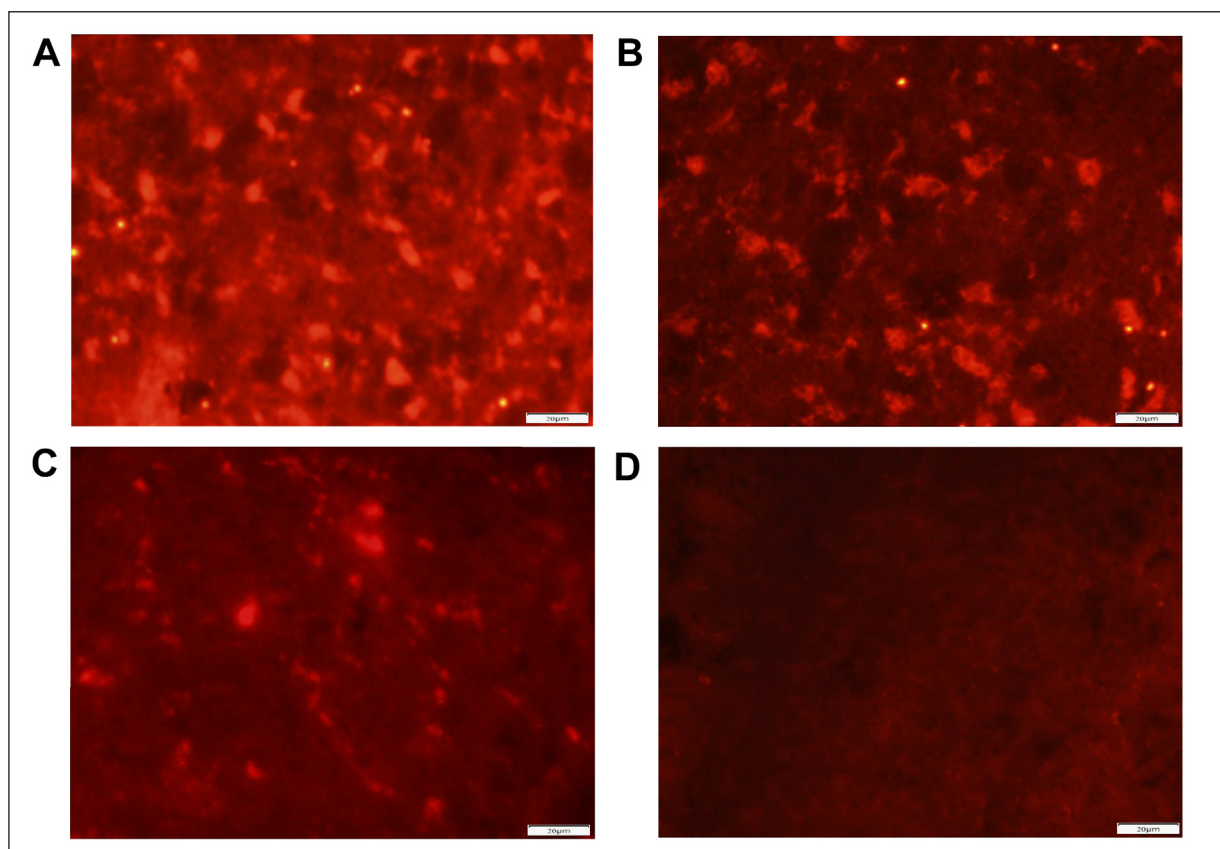
**Figure 3.** Comparison of NSE and nestin protein expression among the control, MCAO, EA, BMSC, and EA+BMSC groups. **A**, Band analysis of NSE protein expression among the control, MCAO, EA, BMSC, and EA+BMSC groups, as detected by Western blotting. **B**, Band analysis of nestin protein expression among the control, MCAO, BMSC, and EA+BMSC groups, as detected by Western blotting. **C**, Expression levels of NSE and nestin protein of different groups were compared. \*  $p < 0.05$ .

active cell proliferation could not be observed in this study due to the cell lysis.

Scholars<sup>45</sup> show that transplantation technique can influence the survival and homing of the grafted stem cells, transplantation of the stem cells can be done intravenously<sup>46</sup>, intra-arterially<sup>47</sup>, intraventricularly<sup>48</sup> and intracerebrally<sup>49</sup>. Cumulative evidence has suggested that cells implanted *via* veins exhibit lower survival than those implanted stereotactically. Vaquero et al<sup>50</sup> showed that direct cell transplantation results in markedly increased cell migration and functional recovery compared to systemic administration. In the present study, intracerebral transplantation was used to promote the survival and homing capacity of BMSC in the

ischemic cortex, even though this technique may carry an increased risk of needlestick injury. We found that in infarct rat model some BMSC migrated from the injection site in the striatum toward the cortex on the side of the lesion<sup>48,51</sup>.

Previous studies<sup>52-56</sup> show that BMSC exert their neuroprotective function<sup>52</sup> through oxidative stress reduction, and anti-inflammatory and antiapoptotic activity<sup>53</sup>, and impact angiogenesis by the paracrine secretion of cytokines or growth factors. Soluble neuroprotective factors, including anti-inflammatory cytokines (IL-10, IL-6)<sup>54</sup>, neurotrophins/growth factors (GDNF, NT-3, NGF, and BDNF)<sup>55,56</sup>, and vascular endothelial growth factor (VEGF)<sup>46</sup>,



**Figure 4.** Immunostaining to detect NSE protein expression in the striatum. **A**, Expression of NSE-positive cells in the striatum of the MCAO model group; **B**) Expression of NSE-positive cells in the striatum of the EA; **C**) NSE-positive cells in the striatum of the EA+BMSC group; **D**) NSE-negative control group. **A-D**, 20  $\mu$ m.

which are released by BMSC or other parenchymal cells such as astrocytes stimulated by BMSC, can mediate neuroprotective effects in acute cerebral infarction. Our data showed that BMSC can down-regulate the expression of NSE.

Studies<sup>57-62</sup> show that EA may alleviate neurological dysfunction by suppressing neuronal apoptosis<sup>57</sup> and accelerating angiogenesis<sup>58</sup> in the rat stroke model. EA has been shown to exert neuroprotective effects by inhibiting inflammatory cytokines (caspase-3<sup>59</sup> and c-Fos<sup>60</sup>), reducing glutamate release<sup>61</sup> and increasing dopamine secretion<sup>62</sup>. In the EA+BMSC treatment group, rats with ischemic stroke showed a significant decrease in the expression of NSE in the stratum, compared to the MCAO group or BMSC treatment-alone group. These results may reflect the severity of ischemic stress and provide evidence that EA combined with BMSC is more effective in relieving neuronal damage and improving the release of cytokines in the cerebral infarction region.

In addition to their neuroprotective effects, BMSC can be induced to differentiate into functional neurons belonging to MSC-derived neuronal cell types that contribute to the recruitment and replacement of damaged neural tissue<sup>63-65</sup>. However, BMSC are multipotential cells that differentiate into neural progenitor cells, chondrocytes, or adipocytes in the host brain<sup>66,67</sup>. Therefore, stimulating specific BMSC differentiation into neural and astroglial lineages but not chondrocytes or adipocytes may be challenging in the clinical setting. Several protocols have used growth factors<sup>68</sup>, neurotrophic factor<sup>69</sup> and lentivirus-mediated transfection<sup>70</sup> to induce stem cells into becoming neuronal cells. Continuous EA has been proposed<sup>71</sup> to promote endogenous MSC mobilization into the peripheral blood. Previous reports<sup>72</sup> also showed that MSC transplantation combined with EA treatment increased MSC differentiation into neuronal cells and promoted remyelination. However, in our study, while the expression of nestin mRNA was significantly increased in



the EA+BMSC group, we did not detect nestin protein expression in the cells, indicating that both BMSC alone and BMSC combined with EA did not induce the differentiation of BMSC into neurons one week after the transplantation. We may speculate, that the substantial neuron regeneration requires longer period of highly efficient and specific therapeutic environment that can only be achieved in *in vitro* cell cultures.

### Limitations

While this study objectively assessed the degree of neuronal damage, as indicated by the intracranial NSE expression, and found that acupuncture combined with BMSC could effectively repair neuron injury, it still has several limitations. Our results showed that EA and BMSC were ineffective alone. We may speculate that a synergistic effect can be achieved by combining EA and BMSC therapy with neuroprotective agents. Further studies to test this hypothesis are needed. There was only a slight repair effect in the BMSCs group. It is necessary to take into account and evaluate the potential damage that may be associated with the procedure of the intracerebral BMSC injection. The efficiency of combined EA+BMSC treatment in long-term neuronal remodeling needs to be further confirmed.

### Conclusions

In summary, transplantation of BMSCs combined with EA treatment is more conducive to treat damage to local neurons in ischemic foci. Improvement in the ischemic microenvironment surrounding MSCs affects the seeding, expansion, survival, renewal, growth, and differentiation of BMSCs in the lesion area. However, further longitudinal studies are needed to determine the efficacy of EA in promoting BMSCs differentiation, and whether the proliferation and differentiation of BMSCs into neurons is augmented by EA in the long term.

### Authors' Contributions

Z.S. and Y.Q.C. conceived and designed the study, B.Y.R. and Q.W. collected data and performed data analysis, Z.S. and Y.Q.C. wrote the draft of this manuscript. W.S. and L.N.K. edited the manuscript.

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### Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Conflict of Interest

The authors declare that no competing financial interests.

### Informed Consent

Not applicable.

### Ethics Approval

Relevant protocols were submitted to the Hainan Medical College Ethics Committee (No. HYLL-2022-134, Date: 2022-03-24). All the experiments were performed in accordance with the Guidelines for Animal Research of Hainan Medical University.

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### References

- 1) Hossmann KA. Viability thresholds and the penumbra of focal ischemia. *Ann Neurol* 1994; 36: 557-565.
- 2) Moseley GL. Widespread brain activity during an abdominal task markedly reduced after pain physiology education: fMRI evaluation of a single patient with chronic low back pain. *Aust J Physiother* 2005; 51: 49-52.
- 3) Savitz SI, Fisher M. Future of neuroprotection for acute stroke: in the aftermath of the SAINT trials. *Ann Neurol* 2007; 61: 396-402.
- 4) Koh SH, Kim KS, Choi MR, Jung KH, Park KS, Chai YG, Roh W, Hwang SJ, Ko HJ, Huh YM, Kim HT, Kim SH. Implantation of human umbilical cord-derived mesenchymal stem cells as a neuroprotective therapy for ischemic stroke in rats. *Brain Res* 2008; 1229: 233-248.
- 5) Hayase M, Kitada M, Wakao S, Itokazu Y, Nozaki K, Hashimoto N, Takagi Y, Dezawa M. Committed neural progenitor cells derived from genetically modified bone marrow stromal cells ameliorate deficits in a rat model of stroke. *J Cereb Blood Flow Metab* 2009; 29: 1409-1420.
- 6) Kim SS, Yoo SW, Park TS, Ahn SC, Jeong HS, Kim JW, Chang DY, Cho KG, Kim SU, Huh Y, Lee JE, Lee SY, Lee YD, Suh-Kim H. Neural induction with neurogenin1 increases the therapeutic

- effects of mesenchymal stem cells in the ischemic brain. *Stem Cells* 2008; 26: 2217-2228.
- 7) Zhao LR, Duan WM, Reyes M, Keene CD, Verfaille CM, Low WC. Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. *Exp Neurol* 2002; 174: 11-20.
  - 8) Kuroda S. Bone marrow stromal cell transplantation for ischemic stroke -- its multi-functional feature. *Acta Neurobiol Exp (Wars)* 2013; 73: 57-65.
  - 9) Li G, Yu F, Lei T, Gao H, Li P, Sun Y, Huang H, Mu Q. Bone marrow mesenchymal stem cell therapy in ischemic stroke: mechanisms of action and treatment optimization strategies. *Neural Regen Res* 2016; 11: 1015-1024.
  - 10) Lepski G, Jannes CE, Strauss B, Marie SKN, Nikkhah G. Survival and neuronal differentiation of mesenchymal stem cells transplanted into the rodent brain are dependent upon microenvironment. *Tissue Eng Part A* 2010; 16: 2769-2782.
  - 11) Zhang XG, Shan C, Zhu JZ, Bao XY, Tong Q, Wu XF, Tang XC, Xue T, Liu J, Zheng GQ, Wang Y. Additive Neuroprotective Effect of Borneol with Mesenchymal Stem Cells on Ischemic Stroke in Mice. *Front Physiol* 2017; 8: 1133.
  - 12) Bao C, Wang Y, Min H, Zhang M, Du X, Han R, Liu X. Combination of ginsenoside Rg1 and bone marrow mesenchymal stem cell transplantation in the treatment of cerebral ischemia reperfusion injury in rats. *Cell Physiol Biochem* 2015; 37: 901-910.
  - 13) Cui X, Chopp M, Zacharek A, Roberts C, Lu M, Savant-Bhonsale S, Chen J. Chemokine, vascular and therapeutic effects of combination Simvastatin and BMSC treatment of stroke. *Neurobiol Dis* 2009; 36: 35-41.
  - 14) Zhan J, Pan R, Zhou M, Tan F, Huang Z, Dong J, Qen Z. Electroacupuncture as an adjunctive therapy for motor dysfunction in acute stroke survivors: a systematic review and meta-analyses. *BMJ Open* 2018; 8: e017153.
  - 15) Franco-Santana LE, Torres-Castillo S, González-Trujano ME, González-Ramírez M. Stimulation of the Po-shen and Shen-hun scalp-acupuncture bands modifies levels of inhibitory and excitatory amino acids in the immature rat brain. *Neurochem Int* 2013; 63: 275-282.
  - 16) Tao J, Zheng Y, Liu W, Yang S, Huang J, Xue X, Shang G, Wang X, Lin R, Chen L. Electro-acupuncture at LI11 and ST36 acupoints exerts neuroprotective effects via reactive astrocyte proliferation after ischemia and reperfusion injury in rats. *Brain Res Bull* 2016; 120: 14-24.
  - 17) Kim YR, Kim HN, Ahn SM, Choi YH, Shin HK, Choi BT. Electroacupuncture promotes post-stroke functional recovery via enhancing endogenous neurogenesis in mouse focal cerebral ischemia. *PLoS One* 2014; 9: e90000.
  - 18) Ding Y, Yan Q, Ruan JW, Zhang YQ, Li WJ, Zhang YJ, Li Y, Dong H, Zeng YS. Electro-acupuncture promotes survival, differentiation of the bone marrow mesenchymal stem cells as well as functional recovery in the spinal cord-transected rats. *BMC Neurosci* 2009; 10: 35.
  - 19) Schmechel D, Marangos PJ, Brightman M. Neuron-specific enolase is a molecular marker for peripheral and central neuroendocrine cells. *Nature* 1978; 276: 834-836.
  - 20) Wunderlich MT, Lins H, Skalej M, Wallech C-W, Goertler M. Neuron-specific enolase and tau protein as neurobiochemical markers of neuronal damage are related to early clinical course and long-term outcome in acute ischemic stroke. *Clin Neurol Neurosurg* 2006; 108: 558-563.
  - 21) Schaarschmidt H, Prange HW, Reiber H. Neuron-specific enolase concentrations in blood as a prognostic parameter in cerebrovascular diseases. *Stroke* 1994; 25: 558-565.
  - 22) Samancı Y, Samancı B, Şahin E, Altıokka-Uzun G, Küçükali C, Tüzün E, Baykan B. Neuron-specific enolase levels as a marker for possible neuronal damage in idiopathic intracranial hypertension. *Acta Neurol Belg* 2017; 117: 707-711.
  - 23) Sienkiewicz-Jarosz H, Gałęcka-Wolska M, Bidziński A, Turzyńska D, Sobolewska A, Lipska B, Płaźnik A, Ryglewicz D. Predictive value of selected biochemical markers of brain damage for functional outcome in ischaemic stroke patients. *Neurol Neurochir Pol* 2009; 43: 126-133.
  - 24) Butterworth RJ, Wassif WS, Sherwood RA, Gerges A, Poyser KH, Garthwaite J, Peters TJ, Bath PM. Serum neuron-specific enolase, carnosinase, and their ratio in acute stroke. An enzymatic test for predicting outcome? *Stroke* 1996; 27: 2064-2068.
  - 25) Oh SH, Lee JG, Na SJ, Park JH, Choi YC, Kim WJ. Prediction of early clinical severity and extent of neuronal damage in anterior-circulation infarction using the initial serum neuron-specific enolase level. *Arch Neurol* 2003; 60: 37-41.
  - 26) Wunderlich MT, Ebert AD, Kratz T, Goertler M, Jost S, Herrmann M. Early neurobehavioral outcome after stroke is related to release of neurobiochemical markers of brain damage. *Stroke* 1999; 30: 1190-1195.
  - 27) Barone FC, Clark RK, Price WJ, White RF, Feuerstein GZ, Storer BL, Ohlstein EH. Neuron-specific enolase increases in cerebral and systemic circulation following focal ischemia. *Brain Res* 1993; 623: 77-82.
  - 28) Zhu SZ, Gu Y, Wu Z, Hu YF, Pan SY. Hypothermia followed by rapid rewarming exacerbates ischemia-induced brain injury and augments inflammatory response in rats. *Biochem Biophys Res Commun* 2016; 474: 175-181.
  - 29) Hatfield RH, McKernan RM. CSF neuron-specific enolase as a quantitative marker of neuronal damage in a rat stroke model. *Brain Res* 1992; 577: 249-252.
  - 30) Nik Ramli NN, Omar N, Husin A, Ismail Z, Siran R. Preconditioning effect of (S)-3,5-dihydroxyphenylglycine on ischemic injury in middle

- cerebral artery occluded Sprague-Dawley rats. *Neurosci Lett* 2015; 588: 137-141.
- 31) Kittaka M, Giannotta SL, Zelman V, Correale JD, DeGiorgio CM, Weiss MH, Zlokovic BV. Attenuation of brain injury and reduction of neuron-specific enolase by nicardipine in systemic circulation following focal ischemia and reperfusion in a rat model. *J Neurosurg* 1997; 87: 731-737.
  - 32) Gilyarov AV. Nestin in central nervous system cells. *Neurosci Behav Physiol* 2008; 38: 165-169.
  - 33) Lendahl U, Zimmerman LB, McKay RD. CNS stem cells express a new class of intermediate filament protein. *Cell* 1990; 60: 585-595.
  - 34) Wiese C, Rolletschek A, Kania G, Blyszczuk P, Tarasov KV, Tarasova Y, Wersto RP, Boheler KR, Wobus AM. Nestin expression--a property of multi-lineage progenitor cells? *Cell Mol Life Sci* 2004; 61: 2510-2522.
  - 35) Zhao WJ, Fan YP, Ou GY, Qiao XY. LASS2 impairs proliferation of glioma stem cells and migration and invasion of glioma cells mainly via inhibition of EMT and apoptosis promotion. *J Cancer* 2022; 13: 2281-2292.
  - 36) Huang J, Deng R, Li W, Jiang M, Xiang AP, Zhang X. Nestin+ Mesenchymal Precursors Generate Distinct Spleen Stromal Cell Subsets and Have Immunomodulatory Function. *Int J Mol Sci* 2022; 23: 11819.
  - 37) Li H, Wang Y, Feng D, Liu Y, Xu M, Gao A, Tian F, Zhang L, Cui Y, Wang Z, Chen G. Alterations in the time course of expression of the Nox family in the brain in a rat experimental cerebral ischemia and reperfusion model: effects of melatonin. *J Pineal Res* 2014; 57: 110-119.
  - 38) Chen J, Li Y, Wang L, Zhang Z, Lu D, Lu M, Chopp M. Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. *Stroke* 2001; 32: 1005-1011.
  - 39) The Chick Brain in Stereotaxic Coordinates and Alternate Stains by Luis Puelles, Margaret Martinez-de-la-Torre, Salvador Martinez – Ebook, Scribd. Available at: <https://www.scribd.com/book/394666895/The-Chick-Brain-in-Stereotaxic-Coordinates-and-Alternate-Stains-Featuring-Neuromeric-Divisions-and-Mammalian-Homologies> (accessed 7 December 2022).
  - 40) Jin XL, Li PF, Zhang CB, Wu JP, Feng XL, Zhang Y, Shen MH. Electroacupuncture alleviates cerebral ischemia and reperfusion injury via modulation of the ERK1/2 signaling pathway. *Neural Regen Res* 2016; 11: 1090-1098.
  - 41) Abe K, Yamashita T, Takizawa S, Kuroda S, Kinouchi H, Kawahara N. Stem cell therapy for cerebral ischemia: from basic science to clinical applications. *J Cereb Blood Flow Metab* 2012; 32: 1317-1331.
  - 42) Gervois P, Wolfs E, Ratajczak J, Dillen Y, Vanganswinkel T, Hilkens P, Bronckaers A, Lambrechts I, Struys T. Stem Cell-Based Therapies for Ischemic Stroke: Preclinical Results and the Potential of Imaging-Assisted Evaluation of Donor Cell Fate and Mechanisms of Brain Regeneration. *Med Res Rev* 2016; 36: 1080-1126.
  - 43) Wakabayashi K, Nagai A, Sheikh AM, Shiota Y, Narantuya D, Watanabe T, Masuda J, Kobayashi S, Kim SU, Yamaguchi S. Transplantation of human mesenchymal stem cells promotes functional improvement and increased expression of neurotrophic factors in a rat focal cerebral ischemia model. *J Neurosci Res* 2010; 88: 1017-1025.
  - 44) Munoz JR, Stoutenger BR, Robinson AP, Spees JL, Prockop DJ. Human stem/progenitor cells from bone marrow promote neurogenesis of endogenous neural stem cells in the hippocampus of mice. *Proc Natl Acad Sci U S A* 2005; 102: 18171-18176.
  - 45) Hu G, Cheng Y, Zuo Y, Chang Y, Suo P, Jia Y, Lu A, Wang Y, Jiao S, Zhang L, Sun Y, Yan C, Xu L, Zhang X, Liu K, Wang Y, Zhang L, Huang X. Comparisons of Long-Term Survival and Safety of Haploidentical Hematopoietic Stem Cell Transplantation After CAR-T Cell Therapy or Chemotherapy in Pediatric Patients With First Relapse of B-Cell Acute Lymphoblastic Leukemia Based on MRD-Guided Treatment. *Front Immunol* 2022; 13: 915590.
  - 46) Deng YB, Ye WB, Hu ZZ, Yan Y, Wang Y, Takon BF, Zhou GQ, Zhou YF. Intravenously administered BMSCs reduce neuronal apoptosis and promote neuronal proliferation through the release of VEGF after stroke in rats. *Neurol Res* 2010; 32: 148-156.
  - 47) Du S, Guan J, Mao G, Liu Y, Ma S, Bao X, Gao J, Feng M, Li G, Ma W, Yang Y, Zhao RC, Wang R. Intra-arterial delivery of human bone marrow mesenchymal stem cells is a safe and effective way to treat cerebral ischemia in rats. *Cell Transplant* 2014; 23: S73-S82.
  - 48) Ohta M, Suzuki Y, Noda T, Ejiri Y, Dezawa M, Kataok K, Chou H, Ishikawa N, Matsumoto N, Iwashita Y, Mizuta E, Kuno S, Ide C. Bone marrow stromal cells infused into the cerebrospinal fluid promote functional recovery of the injured rat spinal cord with reduced cavity formation. *Exp Neurol* 2004; 187: 266-278.
  - 49) Kawabori M, Kuroda S, Ito M, Shichinohe H, Houkin K, Kuge Y, Tamaki N. Timing and cell dose determine therapeutic effects of bone marrow stromal cell transplantation in rat model of cerebral infarct. *Neuropathology* 2013; 33: 140-148.
  - 50) Vaquero J, Zurita M, Oya S, Santos M. Cell therapy using bone marrow stromal cells in chronic paraplegic rats: systemic or local administration? *Neurosci Lett* 2006; 398: 129-134.
  - 51) Chen J, Zhang ZG, Li Y, Wang L, Xu YX, Gautam SC, Lu M, Zhu Z, Chopp M. Intravenous administration of human bone marrow stromal cells induces angiogenesis in the ischemic boundary zone after stroke in rats. *Circ Res* 2003; 92: 692-699.
  - 52) Uccelli A, Benvenuto F, Laroni A, Guinti D. Neuroprotective features of mesenchymal stem cells. *Best Pract Res Clin Haematol* 2011; 24: 59-64.

- 53) Scheibe F, Klein O, Klose J, Priller J. Mesenchymal stromal cells rescue cortical neurons from apoptotic cell death in an in vitro model of cerebral ischemia. *Cell Mol Neurobiol* 2012; 32: 567-576.
- 54) Brenneman M, Sharma S, Harting M, Strong R, Cox CS, Aronowski J, Grotta JC, Savitz SI. Autologous bone marrow mononuclear cells enhance recovery after acute ischemic stroke in young and middle-aged rats. *J Cereb Blood Flow Metab* 2010; 30: 140-149.
- 55) Crigler L, Robey RC, Asawachaicharn A, Gaupp D, Phinnery DG. Human mesenchymal stem cell subpopulations express a variety of neuro-regulatory molecules and promote neuronal cell survival and neuritogenesis. *Exp Neurol* 2006; 198: 54-64.
- 56) Wilkins A, Kemp K, Ginty M, Hares K, Mallam E, Scolding N. Human bone marrow-derived mesenchymal stem cells secrete brain-derived neurotrophic factor which promotes neuronal survival in vitro. *Stem Cell Res* 2009; 3: 63-70.
- 57) Cho NH, Lee JD, Cheong BS, Choi DY, Chang HK, Lee TH, Shin MC, Shin MS, Lee J, Kim CJ. Acupuncture suppresses intrastriatal hemorrhage-induced apoptotic neuronal cell death in rats. *Neurosci Lett* 2004; 362: 141-145.
- 58) Luo JK, Zhou HJ, Wu J, Tang T, Liang QH. Electroacupuncture at Zusanli (ST36) accelerates intracerebral hemorrhage-induced angiogenesis in rats. *Chin J Integr Med* 2013; 19: 367-373.
- 59) Choi DC, Lee JY, Moon YJ, Kim SW, Oh TH, Yune TY. Acupuncture-mediated inhibition of inflammation facilitates significant functional recovery after spinal cord injury. *Neurobiol Dis* 2010; 39: 272-282.
- 60) Jiang K-W, Zhao Z-Y, Shui Q-X, Xia ZZ. Electro-acupuncture preconditioning abrogates the elevation of c-Fos and c-Jun expression in neonatal hypoxic-ischemic rat brains induced by glibenclamide, an ATP-sensitive potassium channel blocker. *Brain Res* 2004; 998: 13-19.
- 61) Pang J, Itano T, Sumitani K, Negi T, Miyamoto O. Electroacupuncture attenuates both glutamate release and hyperemia after transient ischemia in gerbils. *Am J Chin Med* 2003; 31: 295-303.
- 62) Chuang CM, Hsieh CL, Li TC, Lin JG. Acupuncture stimulation at Baihui acupoint reduced cerebral infarct and increased dopamine levels in chronic cerebral hypoperfusion and ischemia-reperfusion injured sprague-dawley rats. *Am J Chin Med* 2007; 35: 779-791.
- 63) Tornero D, Tsupikov O, Granmo M, Rodriguez C, Grønning-Hansen M, Thelin J, Smozhanik E, Laterza C, Wattananit S, Ge R, Tatarishvili J, Grealish S, Brüstle O, Skibo G, Parmar M, Schouenborg J, Lindvall O, Kokaia Z. Synaptic inputs from stroke-injured brain to grafted human stem cell-derived neurons activated by sensory stimuli. *Brain* 2017; 140: 692-706.
- 64) Wislet-Gendebien S, Hans G, Leprince P, Rigo JM, Moonen G, Rogister B. Plasticity of cultured mesenchymal stem cells: switch from nestin-positive to excitable neuron-like phenotype. *Stem Cells* 2005; 23: 392-402.
- 65) Woodbury D, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res* 2000; 61: 364-370.
- 66) Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. *Nat Rev Immunol* 2008; 8: 726-736.
- 67) Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporita S, Janssen W, Patel N, Cooper DR, Sanberg PR. Adult bone marrow stromal cells differentiate into neural cells in vitro. *Exp Neurol* 2000; 164: 247-256.
- 68) Wang F, Chang G, Geng X. NGF and TERT co-transfected BMSCs improve the restoration of cognitive impairment in vascular dementia rats. *PLoS One* 2014; 9: e98774.
- 69) Zhao T, Yan W, Xu K, Qi Y, Dai X, Shi Z. Combined treatment with platelet-rich plasma and brain-derived neurotrophic factor-overexpressing bone marrow stromal cells supports axonal remyelination in a rat spinal cord hemi-section model. *Cytotherapy* 2013; 15: 792-804.
- 70) Song JL, Zheng W, Chen W, Qian Y, Ouyang YM, Fan CY. Lentivirus-mediated microRNA-124 gene-modified bone marrow mesenchymal stem cell transplantation promotes the repair of spinal cord injury in rats. *Exp Mol Med* 2017; 49: e332.
- 71) Liu L, Yu Q, Hu K, Wang B, Zhang Y, Xu Y, Fu S, Yu X, Huang H. Electro-Acupuncture Promotes Endogenous Multipotential Mesenchymal Stem Cell Mobilization into the Peripheral Blood. *Cell Physiol Biochem* 2016; 38: 1605-1617.
- 72) Liu Z, He B, Zhang RY, Zhang K, Ding Y, Ruan JW, Ling EA, Wu JL, Zeng YS. Electroacupuncture Promotes the Differentiation of Transplanted Bone Marrow Mesenchymal Stem Cells Pre-induced with Neurotrophin-3 and Retinoic Acid Into Oligodendrocyte-Like Cells in Demyelinated Spinal Cord of Rats. *Cell Transplant* 2015; 24: 1265-1281.