Recombinant human erythropoietin increases cerebral cortical width index and neurogenesis following ischemic stroke

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Abstract
The cerebral cortical expansion index refers to the ratio between left and right cortex width and is recognized as an indicator for cortical hyperplasia. Cerebral ischemia was established in CB-17 mice in the present study, and the mice were subsequently treated with recombinant human erythropoietin via subcutaneous injection. Results demonstrated that cerebral cortical width index significantly increased. Immunofluorescence detection showed that the number of nuclear antigen antibody/5-bromodeoxyuridine-positive cells at the infarction edge significantly increased. Correlation analysis revealed a negative correlation between neurological scores and cortical width indices in rats following ischemic stroke. These experimental findings suggested that recombinant human erythropoietin promoted cerebral cortical hyperplasia, increased cortical neurogenesis, and enhanced functional recovery following ischemic stroke.

Key Words: cerebral infarction; erythropoietin; neural regeneration; neurogenesis

Abbreviations: rEPO, recombinant human erythropoietin; MCAO, middle cerebral artery occlusion; BrdU, 5'-bromo-2'-deoxyuridine-5'-monophosphate; NeuN, neuronal nuclei

INTRODUCTION
Stroke is increasingly recognized as the leading cause of mortality and long-term disability. Thrombolysis in combination with recombinant tissue plasminogen activator, the only licensed drug for acute stroke, is applied to a restricted number of stroke patients due to multiple contraindications [1-2], and its efficacy has been limited by toxicity and reperfusion injury [3]. Therefore, there is an urgent need for novel therapies for acute ischemic stroke patients who are not qualified for recombinant tissue plasminogen activator treatment. Recent studies have focused on the role of a local protective mechanism that underlies an anti-inflammatory response and anti-apoptotic actions during the acute phase of ischemic stroke [4-5]. Angiogenesis and neurogenesis have been shown to contribute to neuroprotection and functional recovery after stroke [6-8], and a promising neuroprotective therapy can improve histological functions, as well as neurological functions. To date, erythropoietin and its analogues have been widely tested in animal models and clinical trials, showing the protective effects against cerebral ischemic impairment [9-13].

Erythropoietin prevents neuronal apoptosis induced by a variety of stimuli [14]. Erythropoietin affords significant neuroprotection against ketamine-induced injury in neurons via the PI3K/Akt-mediated signaling pathway [15] and protects cortical neurons from glutamic acid toxicity, which could be related to signal transduction of nuclear factor-kappa B [16]. Erythropoietin also promotes proliferation and angiogenesis in vitro and in the subventricular zone [17-19]. Erythropoietin significantly decreases expansion of the subventricular zone unilaterally and improves functional outcomes following experimental neonatal stroke [20]. However, little is known about the effects of erythropoietin on morphological changes due to ischemic cerebral cortex, as well as the relationship between these changes and neurological functional improvement. The present study evaluated the effects of recombinant human erythropoietin (rEPO) on neurological functional improvement, neurogenesis, and cerebral cortical expansion following intraperitoneal injection into a permanent middle cerebral artery occlusion (MCAO) CB-17 mouse model. The relationship between neurological functional improvement and cerebral cortical expansion was also evaluated.
RESULTS

Quantitative analysis of experimental animals
A total of 20 CB-17 male mice were selected to establish MCAO models. No mice died during the study, and model establishment was considered successful. Twenty surviving mice were randomly assigned to two groups: rhEPO (treated with rhEPO; \( n = 10 \)) and model (treated with phosphate-buffered saline; \( n = 10 \)). All 20 mice were included in the final analysis, with no loss.

rhEPO-induced cerebral cortex expansion following ischemic stroke
To determine cerebral cortical expansion, the cortical width index was utilized as a quantitative hallmark. Quantitative analysis revealed that the cortical width index significantly increased in the rhEPO treatment group compared with the model group at 35 days post-stroke (\( P < 0.05 \); Figure 1).

rhEPO-increased neurogenesis at the infarction edge
Immunofluorescence stainings were performed with 5′-bromo-2′-deoxyuridine-5′-monophosphate (BrdU; synthetic nucleoside thymidine analogue used for detection of proliferating cells) and neuronal nuclei (NeuN; neuronal-specific nuclear protein) monoclonal antibody to determine neurogenesis at the infarction edge at 35 days post-stroke. Quantitative analysis revealed that the number of NeuN/BrdU-positive cells significantly increased in the rhEPO group compared with the model group (6.8 ± 2.3 vs. 2.5 ± 1.5, \( n = 10 \), \( P < 0.05 \); Figure 2).

Figure 2  Neurogenesis at infarct edge following phosphate-buffered saline and recombinant human erythropoietin (rhEPO) treatment (immunofluorescence, laser scanning confocal microscopy).

(A–C) rhEPO group; (D–F) model group.
Green fluorescence indicates 5′-bromo-2′-deoxyuridine-5′-monophosphate (BrdU)-positive cells (FITC), red fluorescence (Texas Red) indicates neuronal nuclei (NeuN)-positive cells; yellow fluorescence is an indicator of NeuN/BrdU double-labeling.

A greater number of NeuN/BrdU-positive cells is visible in the rhEPO group compared with model group at 35 days post-stroke. Arrow represents neurogenesis (NeuN/BrdU-positive cells). Scale bar: 50 μm.

rhEPO-accelerated neurological function recovery following ischemic stroke
Neurological function recovery was assessed using a modified neurological functional rating scale. Compared with the model group, mice treated with rhEPO exhibited accelerated functional recovery at 35 days post-stroke (neurological function score: 1.3 ± 0.3 vs. 2.4 ± 0.2, \( n = 10 \), \( P < 0.05 \)). The neurological function score of ischemic mice negatively correlated with cortical width index (\( n = 10 \) per group, \( r = –0.23, P < 0.05 \)).

Figure 1  Recombinant human erythropoietin (rhEPO)-induced cerebral cortical expansion in mice after ischemic stroke. Using a digital camera system, whole brain images were captured. Scale bar: 2 mm.

(A) Model group.
(B) rhEPO treatment at 35 days post-stroke.
(C) Cortical width index measured using NIH image software; a: maximum width from midpoint to edge of infarcted hemisphere; b: maximum width from midpoint to edge of non-infarcted hemisphere;
(D) Cortical width index after treating with phosphate-buffered saline and rhEPO (mean ± SD, \( n = 10 \), two-sample t-test). *\( P < 0.05 \), vs. model group.
RESULTS

The results show a significant correlation between cortical width index and neurologic function score. The correlation coefficient is $r = -0.23$, with $P < 0.05$.

**Figure 3** Correlation between cortical width index and neurologic function in mice.

The neurologic function score negatively correlates with the cortical width index ($1.3 \pm 0.3$ vs. $2.4 \pm 0.2$, mean±SD, $n = 10$; regression analysis, $P < 0.05$, $r = -0.23$).

DISCUSSION

The MCAO animal model correlates with many human clinical characteristics and is recognized as a standard animal model for focal cerebral infarction studies. The present study introduced a highly reproducible model of permanent cerebral ischemia in CB-17 mice. Results showed that rhEPO significantly accelerated neurological function recovery and promoted neurogenesis at the infarct edge, as well as induced cortical expansion, at 35 days post-ischemic stroke. In addition, neurological function improvement directly correlated with cortical expansion.

Compared with the model group, rhEPO treatment resulted in significant neurological functional improvement. These experimental results were consistent with previous studies.[20-21] EPO treatment has been shown to significantly decrease infarct size, thereby maintaining cognitive function after ischemic stroke.[22]

The subventricular zone in the rodent forebrain contains a population of neuronal progenitor cells throughout life.[23] Various types of injury have been shown to stimulate progenitor proliferation in the subventricular zone. Following MCAO, increased size and proliferation of progenitor cells in the subventricular zone have been shown in the adult and in a neonatal rodent stroke model.[24] Erythropoietin and its receptor play important roles in subventricular zone proliferation.[25] In contrast to results from other previous studies, the present study did not quantify subventricular zone progenitors. However, results demonstrated that rhEPO significantly increased the number of BrdU/NeuN-positive cells at the ischemic boundary. Expression of the EPO receptor has been described in a permanent MCAO animal model.[26]

Erythropoietin is an important physiological determinant for mobilizing immature hematopoietic stem cells, including endothelial progenitor cells, which results in a significantly increased numbers of circulating endothelial progenitor cells,[27-29] which subsequently have the capacity to participate in neovascularization of ischemic tissues and accelerate angiogenesis by incorporating into the neovascularature at the ischemic site, thereby limiting tissue damage.[30-31] Disruption of the blood-brain barrier after ischemic stroke might facilitate hematopoietic stem cell entry into ischemic tissue. Therefore, erythropoietin administration is thought to increase mobilization of circulating hematopoietic stem cells to the damaged area, subsequently stimulating cell division. Erythropoietin is also a survival factor for endothelial cells and indirectly acts on endothelial cells via activation of vascular endothelial growth factor receptor system.[32] Vascular endothelial growth factor is the most important specific regulator of endothelial cell growth and differentiation and a major angiogenesis factor. Following administration of rhEPO, there are a greater number of hematopoietic stem cells and newly formed vessels that promote neurogenesis.

Neovascularization is essential for neuronal regeneration after stroke, and therapeutic neovascularization is a potential effective means for enhancing functional recovery. Results from the present study indicated that rhEPO promoted neurogenesis at the infarction border, as well as significantly increased the number of cells labeled with BrdU and NeuN antibodies in the ischemic border. The increased neurogenesis might be due to proliferation of immature hematopoietic stem cells mobilized by rhEPO or migration from the striatum to the cortex via the hippocampus.

To the best of our knowledge, the present study identified, for the first time, that neurological function improvement directly correlated with cortical expansion after cerebral infarction. Cortical expansion in MCAO mice treated with rhEPO suggested ongoing neurogenesis. Erythropoietin is a potent inhibitor of ischemia-induced apoptosis,[33] and provides neuroprotection by maintaining Bcl-2 and Bcl-x expression or inactivating caspases.[34] Therefore, the beneficial effects of rhEPO on neurological functional improvement comprise multiple mechanisms.

Some limitations exist in the present study. First, the effect of rhEPO on angiogenesis was not analyzed, nor the growth of new blood vessels from the existing vasculature, to explore probable mechanisms underlying neurogenesis. Second, the numbers of circulating hematopoietic stem cells were not quantified. Third, the effect of rhEPO on erythropoiesis was not determined and could be detrimental following stroke.

In conclusion, these results showed that rhEPO provided protection against cerebral infarction in a mouse MCAO model. Increased neural regeneration has been shown to contribute to long-term repair of the ischemic brain.[35] Early use of rhEPO promotes neurobehavioral development in preterm infants.[36] Results from the present study suggested that rhEPO provided neuroprotection and therapeutic effects, and might be helpful for treatment of patients following...
ischemic stroke.

**MATERIALS AND METHODS**

**Design**
A randomized, controlled, animal experiment.

**Time and setting**
This experiment was performed at the Laboratory of Cerebral Circulation and Metabolism, the Second Affiliated Hospital of Soochow University, China from January to October 2009.

**Materials**
A total of 20 male, CB-17 mice, aged 8 weeks and weighing 10–12 g, were purchased from Animal Department of Soochow University, China (license No. SYKK (Su) 2009-0048). All mice were allowed access to food and tap water *ad libitum*. Powdered chow and sterilized water were also provided during the first 7 days after stroke induction. All experiments were conducted according to the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China[37].

**Methods**

**Establishment of cerebral ischemic infarction model**
Permanent focal cerebral infarction was established by ligation and disconnection of the distal portion of the left middle cerebral artery, as previously described[38-39]. Briefly, general anesthesia was induced and maintained by inhalation of 3% and 1.5% halothane (7025 Rodent Ventilator, Ugo Basile, Italy) through a face mask, and a skin incision was made between the left eyeball and left ear hole. The left salivary gland and veins were carefully removed to visualize the zygomata. The left zygomata was dissected under a surgical microscope, and the jaw joint was detached to allow visualization of the middle cerebral artery through the cranial bone. A hole (2 mm diameter) was made in the bone using a dental drill. Subsequently, the dura mater was carefully removed to avoid damage to the brain surface. The middle cerebral artery was then isolated, electrocauterized, and disconnected distally to the olfactory tract (distal M1 portion of middle cerebral artery). The middle cerebral artery was coagulated from the distal to the proximal portion. After confirming no re-vascularization, the skin incision was closed with sutures. Body temperature was maintained at 36.5–37.0°C through use of a heat lamp around the ischemic cortex edge using a high-power microscope (Olympus). For immunofluorescence, green (FITC for BrdU), red (Texas Red for NeuN), and yellow (double-stained with BrdU and NeuN) was visualized by laser beam excitation. Areas of interest were scanned with a 40 × oil-immersion objective lens. For quantitative analysis, the number of cells double-stained with BrdU and NeuN was quantified around the ischemic cortex edge using a high-power lens field under microscopy (Olympus).

**Statistical analysis**
Data were analyzed using SPSS 11.5 software (SPSS, Chicago, IL, USA), and the results were statistically evaluated in each group. Measurement data were expressed as mean ± SD, and the difference between two groups was evaluated using the two-sample t-test. Regression analysis was performed between cortical width index and neurological functional score. Statistical significance was set at $P < 0.05$.

**Author contributions:** Zhongmin Wen conceived and designed the study, analyzed data, and drafted the manuscript. Peij Wang authorized the manuscript and guided the study.

**Conflicts of interest:** None declared.

**Ethical approval:** This study received permission from the Animal Ethics Committee of Soochow University in China.
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