Dose-dependent neurorestorative effects of delayed treatment of traumatic brain injury with recombinant human erythropoietin in rats

Laboratory investigation

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Object. Delayed (24 hours postinjury) treatment with erythropoietin (EPO) improves functional recovery following experimental traumatic brain injury (TBI). In this study, the authors tested whether therapeutic effects of delayed EPO treatment for TBI are dose dependent in an attempt to establish an optimal dose paradigm for the delayed EPO treatment.

Methods. Experimental TBI was performed in anesthetized young adult male Wistar rats using a controlled cortical impact device. Sham animals underwent the same surgical procedure without injury. The animals (8 rats/group) received 3 intraperitoneal injections of EPO (0, 1000, 3000, 5000, or 7000 U/kg body weight, at 24, 48, and 72 hours) after TBI. Sensorimotor and cognitive functions were assessed using a modified neurological severity score and foot fault test, and Morris water maze tests, respectively. Animals were killed 35 days after injury, and the brain sections were stained for immunohistochemical analyses.

Results. Compared with the saline treatment, EPO treatment at doses from 1000 to 7000 U/kg did not alter lesion volume but significantly reduced hippocampal neuron loss, enhanced angiogenesis and neurogenesis in the injured cortex and hippocampus, and significantly improved sensorimotor function and spatial learning. The animals receiving the medium dose of 5000 U/kg exhibited a significant improvement in histological and functional outcomes compared with the lower or higher EPO dose groups.

Conclusions. These data demonstrate that delayed (24 hours postinjury) treatment with EPO provides dose-dependent neurorestoration, which may contribute to improved functional recovery after TBI, implying that application of an optimal dose of EPO is likely to increase successful preclinical and clinical trials for treatment of TBI.

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Key Words • angiogenesis • cell proliferation • erythropoietin • neurogenesis • rat • sensorimotor • spatial learning • traumatic brain injury

Traumatic brain injury is a leading cause of mortality and morbidity worldwide, particularly among the young. The most prevalent and debilitating features in survivors of TBI are cognitive deficits and motor dysfunctions. To date, despite very encouraging preclinical results, almost all Phase II/III clinical neuroprotection trials in TBI have failed to improve the outcome of patients who have suffered a TBI. The disappointing clinical trials may be due to heterogeneity of the population of TBI patients and variability in treatment approaches. Another important aspect is that most strategies to date have used drugs in clinical trials targeting a single pathophysiological mechanism that contributes to early necrotic cell death. Targeting multiple injury mechanisms that contribute to the secondary injury cascade may reduce brain injury, facilitate repair, and improve functional recovery.

Erythropoietin and EPOR, essential for erythropoiesis, are also expressed in the neurons, astrocytes, and cerebral endothelial cells. Erythropoietin is a multifunctional agent with tissue protection exerting antiapoptotic, antiinflammatory, antioxidative, angiogenic, and neurotrophic properties. Erythropoietin shows neuroprotection in animal models of stroke, spinal cord injury, concussive brain injury, kainate-induced seizure activity, and autoimmune encephalomyelitis. Erythropoietin has been demonstrated to be safe and beneficial in...
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an earlier small clinical trial of stroke but did not show benefits (instead it had a higher mortality than placebo controls) in a recent large clinical trial of stroke. Combination of tPA with EPO may be one of the important factors for this failed clinical trial because a very large number of EPO-treated stroke patients received tPA treatment in this stroke clinical trial, and combination of EPO with tPA has been demonstrated to cause detrimental effects in animal models of stroke. Low doses of EPO used for clinical trials may also contribute to this failure because much higher EPO doses are required to provide neuroprotection and functional benefits in animal stroke models. In a recent small EPO clinical trial, a single dose of EPO administered within 6 hours after TBI did not show efficacy. In addition, several EPO TBI clinical trials are ongoing (www.clinicaltrials.gov: NCT00987454, NCT00313716, and NCT00375869). However, the optimal EPO dose, dosing interval, and number of doses required for reducing brain injury, promoting neurorestoration, and improving functional recovery have not been fully investigated in preclinical trials after TBI. Therefore, it is imperative to investigate dose-response effect of EPO treatment on functional and histological outcomes after TBI. Our recent work demonstrates that delayed treatment (24 hours postinjury) with EPO provides long-term benefits in rats after TBI and after stroke. In an attempt to establish an optimal therapeutic dose, using a CCI TBI rat model, we investigated the effects of the delayed administration of different doses of EPO on lesion volume, cell proliferation, neurogenesis, angiogenesis, and long-term sensorimotor function and spatial learning recovery after TBI in rats.

Methods

All experimental procedures were approved by the institutional animal care and use committee of Henry Ford Health System.

Traumatic Brain Injury Model

A CCI model of TBI in the rat was used for the present study. Young adult male Wistar rats (313.4 ± 8.9 g) were anesthetized intraperitoneally with chloral hydrate (350 mg/kg body weight). Rectal temperature was maintained at 37°C using a feedback-regulated water-heating pad. A CCI device was used to induce injury. Rats were placed in a stereotactic frame. Two 10-mm-diameter craniotomies were performed adjacent to the central suture, midway between lambda and bregma. The second craniotomy allowed for lateral movement of cortical tissue. The dura mater was kept intact over the cortex. Injury was delivered by impacting the left cortex (ipsilateral cortex) with a pneumatic piston containing a 6-mm-diameter tip at a rate of 4 m/second and 2.5 mm of compression. Velocity was measured using a linear velocity displacement transducer.

Experimental Groups and Treatment

Young adult male Wistar rats were randomly divided into 6 groups (8 rats/group): 1) sham; 2) TBI/saline; 3) TBI + EPO1K; 4) TBI + EPO3K; 5) TBI + EPO5K; and 6) TBI + EPO7K. Traumatic brain injury was induced by CCI over the left parietal cortex. Sham rats underwent surgery without injury. Erythropoietin at doses of 0 (saline), 1000 (EPO1K), 3000 (EPO3K), 5000 (EPO5K), and 7000 (EPO7K) U/kg body weight (Epoetin alpha, AMGEN) was administered intraperitoneally at 24, 48, and 72 hours after TBI. Animals in the saline-treated group received an equal volume of saline at 24, 48, and 72 hours after TBI. For labeling proliferating cells BrdU (100 mg/kg, Sigma) was injected intraperitoneally into rats daily for 10 days, starting 1 day after TBI. All rats were killed 35 days after TBI or surgery.

Hematocrit Level

To determine the effects of EPO on HCT, a blood sample (50 μl) was collected via tail vein before injury, on Day 4, and weekly after TBI or sham treatment up to 5 weeks. Hematocrit was measured in micro-HCT capillary tubes (Fisher Scientific) using standard procedures (Readacrit Centrifuge).

Evaluation of Neurological Outcome

All functional tests were performed by investigators blinded to the treatment status.

Morris Water Maze Test. To detect spatial learning impairments, a recent version of the MWM test was used. The procedure was modified from previous versions and has been found to be useful for chronic spatial memory assessment in rats and mice with brain injury. All animals were tested during the last 5 days (that is, 31–35 days after TBI or surgery) before they were killed. Data collection was automated using the HVS Image 2020 Plus Tracking System (US HVS Image). If the animal reached the platform within 90 seconds, the percentage of time traveled within the northeast (correct) quadrant was calculated relative to the total amount of time spent swimming before reaching the platform and used for statistical analysis. The advantage of this version of the water maze is that each trial takes on the key characteristics of a probe trial because the platform is not in a fixed location within the target quadrant.

Footfault Test. To evaluate sensorimotor function, the footfault test was carried out before TBI and at 1, 4, 7, 14, 21, 28, and 35 days after TBI or surgery. The rats were allowed to walk on a grid. With each weight-bearing step, a paw might fall or slip between the wires and, if this occurred, it was recorded as a footfault. A total of 50 steps were recorded for each right forelimb and hindlimb.

Modified Neurological Severity Score Test. Neurological functional measurement was performed using the mNSS. The test was carried out on all rats preinjury and on Days 1, 4, 7, 14, 21, 28, and 35 after TBI. The mNSS is a composite of the motor (muscle status and abnormal movement), sensory (visual, tactile, and proprioceptive), and reflex tests and has been used in previous studies. This test is suitable for evaluating long-term neurological function after unilateral brain injury.
Tissue Preparation and Measurement of Lesion Volume

On Day 35 after TBI, rats were anesthetized intra-peritoneally with chloral hydrate and were perfused transcardially first with saline solution, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4). Their brains were removed and postfixed in 4% paraformaldehyde for 2 days at room temperature. The brain tissue was cut into 7 equally spaced 2-mm coronal blocks and processed for paraffin sectioning. A series of adjacent 6-μm-thick sections were cut from each block in the coronal plane and stained with H & E. For lesion volume measurement, the 7 brain sections were traced using a microcomputer imaging device (Imaging Research), as previously described.52 The lesion volume was presented as a volume percentage of the lesion compared with the contralateral hemisphere.

Immunohistochemical Analysis

To examine the effect of EPO on cell proliferation and angiogenesis, coronal sections were immunohistochemically stained with mouse anti-BrdU (dilution 1:200, Dako) and rabbit anti-human vWF (dilution 1:200, Dako-Cytomation), respectively. The detailed procedures were described in our previous study.33

Immunofluorescent Staining

Newly generated neurons were identified by double labeling for BrdU and NeuN. After dehydration, coronal sections were boiled in 10 mM citric acid buffer (pH 6) for 10 minutes. Sections were incubated in 2.4 N HCl at 37°C for 20 minutes. Sections were incubated with 1% bovine serum albumin containing 0.3% Triton-X-100 in phosphate-buffered saline, and then incubated with mouse anti-NeuN antibody (dilution 1:200, Chemicon) and rabbit anti-human vWF (dilution 1:200, Dako-Cytomation), respectively. Sections were then incubated with fluorescein isothiocyanate (FITC)–conjugated anti–mouse antibody (1:400, Jackson ImmunoResearch) at room temperature for 2 hours. Sections were then incubated with Cy3-conjugated anti–rat antibody (1:400, Jackson ImmunoResearch) at room temperature for 2 hours. Coronal sections were mounted with Vectashield mounting medium (Vector Laboratories).

Cell Counting and Quantitation

Five sections with 100-μm intervals from the dorsal DG were analyzed using a microscope (Nikon i80) at a magnification of 400 via the microcomputer imaging device system.35 All counting was performed on a computer monitor to improve visualization and in one focal plane to avoid oversampling.64 To evaluate whether intraperitoneally administered EPO reduces neuronal damage after TBI, the number of cells were counted in the hippocampus. Although H & E staining is not neuron-specific, the morphological characteristics of neuronal cells in the DG, CA1, and CA3 regions aid in counting them. Counts were averaged and normalized by measuring the linear distance (in mm) of the DG, CA1, and CA3 for each section. Although it is just an estimate of the cell number, this method permits a meaningful comparison of differences between groups. For cell proliferation, the total number of BrdU-positive cells was counted in the lesion boundary zone and the DG. The cells with BrdU (brown stained) that clearly localized to the nucleus (hematoxylin stained) were counted as BrdU-positive cells. The number of BrdU-positive cells was expressed as number of cells per square millimeter. To evaluate neurogenesis, BrdU/NeuN-colabeled cells were counted in the DG and the cortex.50

Statistical Analysis

All data are presented as means ± SDs. Data on mNSS were first evaluated for normality. The rank data were used for the analysis since data were not normal. Analysis of covariance, PROC MIXED with CONTRAST statement in SAS, was used to test the group difference on mNSS. The analysis began testing the overall group effect, followed by pairwise group comparisons if the overall group effect was detected at the 0.05 level; otherwise the pairwise group comparisons would be considered as exploratory analysis. Data on HCT, body weight, footfault, and spatial learning function were analyzed by ANOVA for repeated measurements. For lesion volume, cell counting, and vWF-stained vascular density, a 1-way ANOVA followed by post hoc Student-Newman-Keuls test was used to compare the difference between the EPO-treated, EPO-hemodilution-treated, saline-treated, and sham groups. Statistical significance was set at p < 0.05.

Results

Body Weight and HCT

There was no significant difference in the body weight among groups before TBI (Fig. 1A). Compared with preinjury level, the body weight significantly decreased at Day 1 (p < 0.001) and Day 4 (p < 0.001), returned to preinjury level 1 week after TBI, and then gained steadily until the rats were killed. Erythropoietin treatment at different doses did not significantly change the body weight. The baseline HCT was similar for all animals before injury (Fig. 1B). Compared with saline treatment, EPO treatment significantly increased HCT up to 2 weeks (p < 0.001), which returned to normal thereafter. There was no significant difference in HCT among EPO-treated groups.

Lesion Volume

Lesion volume measurements were performed at 35 days post-TBI. Delayed (24 hours postinjury) EPO treatment did not affect the lesion volume after TBI (p > 0.05) compared with saline controls. The lesion volume was 17.5% ± 0.8%, 17.1% ± 0.3%, 16.3% ± 0.3%, 16.4% ± 0.6%, and 17.0% ± 0.4% for TBI rats treated with saline, EPO1K, EPO3K, EPO5K, and EPO7K, respectively.

Spatial Learning Test

To analyze day-by-day differences in the MWM, a repeated-measures ANOVA was performed and followed by Student-Newman-Keuls tests for multiple compari-
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As shown in Fig. 2A, the time spent in the correct quadrant (northeast) by sham rats gradually increased from Days 31 to 35 after surgery. The saline-treated rats with TBI were significantly impaired compared with sham-operated rats at Days 32–35 (that is, for Days 32, 33, 34, and 35, p = 0.001, < 0.001, < 0.001, and < 0.001, respectively) after injury. Compared with saline treatment, EPO-treated rats with TBI showed significant improvement at Day 32 (p = 0.010 and = 0.013 for EPO3K and EPO5K, respectively), Day 33 (p = 0.020, = 0.002, and < 0.001 for EPO1K, EPO3K, and EPO5K, respectively), Day 34 (p = 0.022, < 0.001, < 0.001, and = 0.040 for EPO1K, EPO3K, EPO5K, and EPO7K, respectively), and Day 35 (p = 0.013, < 0.001, < 0.001, and < 0.001 for EPO1K, EPO3K, EPO5K, and EPO7K, respectively). However, compared with other EPO groups, the EPO5K group showed a significant improvement in spatial learning (that is, a larger percentage of time spent in the correct quadrant) at Day 34 (p < 0.05) and Day 35 (p < 0.05).

Footfault Test

The incidence of forelimb footfaults during baseline (preoperatively) among all groups was comparable (Fig. 2B). Sham surgery alone mildly but significantly increased the incidence of footfaults at postoperative Day 1 (p = 0.015) and Day 4 (p = 0.003). Traumatic brain injury significantly increased the incidence of right forelimb footfaults contralateral to the TBI at 1–35 days postinjury compared with the preinjury baseline (p < 0.001 for Days 1–35). Erythropoietin treatment at all doses (1000–7000 U/kg) significantly reduced the number of contralateral forelimb footfaults at 4–35 days after TBI compared with treatment with saline (p < 0.05) while EPO5K showed better benefits at Days 7–35 (p < 0.05) compared with other EPO groups.

Modified Neurological Severity Score

Figure 2D shows that there is no significant difference in the mNSS among the saline and EPO-treated groups at Day 1 post-TBI. Traumatic brain injury significantly increased the mNSS (the higher the mNSS, the worse the sensorimotor function) at 1–35 days postinjury compared with the sham groups (p < 0.001 for Days 1–35). However, significantly improved scores (that is, reduced scores) were measured at Days 7–35 after TBI in the EPO-treated groups compared with the saline-treated groups (p < 0.05). The medium dosage (EPO3K and EPO5K) showed significantly better benefits at Day 4 compared with saline treatment (for EPO3K and EPO5K, p = 0.014 and < 0.001, respectively). Moreover, EPO5K provided optimal efficacy in reducing mNSS at Days 7–35 (p < 0.05 vs other EPO groups).

Cell Loss in the Hippocampus

When examined at 35 days post-TBI (Fig. 3), the neuron counts in the CA3, DG, and CA1 of the ipsilateral hippocampus significantly decreased after TBI (Fig. 3B, E, and H; p < 0.001) compared with sham controls (Fig. 3A, D, and G). Compared with saline controls, EPO treatment significantly increased the neuron counts in these regions (Fig. 3C, F, and I; p < 0.05). The EPO5K rats had significantly higher neuron counts in these regions compared with other EPO dose groups (Fig. 3J, p < 0.001).

Angiogenesis

Traumatic brain injury alone significantly increased the density of vessels in the cortex, DG, and CA3 of the ipsilateral hemisphere (Fig. 4B, E, and H; p < 0.001) compared with sham controls (Fig. 4A, D, and G). Erythropoietin treatment significantly increased the vascular density in the cortex, DG, and CA3 (Fig. 4C, F, and I; p < 0.05) compared with saline treatment. A significantly higher
vascular density was observed in the EPO5K group than in the other EPO groups (Fig. 4J, p < 0.001).

**Cell Proliferation**

BrdU labeling and staining is commonly used to detect cell proliferation. The number of BrdU-positive cells found in the ipsilateral cortex and DG (Fig. 5B and E, p < 0.001) was significantly increased at 35 days after TBI compared with sham controls (Fig. 5A and D). However, EPO treatment further increased the number of BrdU-positive cells in the cortex and DG (Fig. 5C and F, p < 0.001) after TBI compared with saline controls. A significantly higher density of BrdU-positive cells in these regions was observed in the EPO5K group compared with other EPO-treated groups (Fig. 5M, p < 0.001).

**Neurogenesis**

Newly generated neurons were identified by double labeling for BrdU (proliferating marker, Fig. 5H and K) and NeuN (mature neuronal marker, Fig. 5G and J). The number of NeuN/BrdU-double stained cells (newborn neurons) was significantly higher in the injured cortex and DG (Fig. 5N, p < 0.001) after TBI compared with sham controls. Erythropoietin treatment significantly increased the number of newborn neurons in the injured cortex and DG (Fig. 5N, p < 0.001) compared with saline controls. In addition, the number of newborn neurons in the injured cortex and DG was significantly higher in the EPO5K group than in the other EPO groups (Fig. 5N, p < 0.001).

**Correlation of Histological Changes With Functional Recovery**

Although delayed EPO therapy did not reduce cortical lesion volume caused by TBI, EPO treatment significantly improved sensorimotor and spatial learning recovery assessed by footfault and mNSS tests as well as MWM tests. Correlation analyses indicate that sensorimotor functional deficits (as measured by forelimb, hindlimb...
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footfault, and mNSS) are highly and inversely correlated to increased vascular density (Fig. 6A), cell proliferation (Fig. 6B), and neurogenesis (Fig. 6C) in the injured cortex examined at Day 35 after TBI (p < 0.05). Similarly, spatial learning (percentage of time spent in the correct quadrant) is highly and positively correlated to increased neuron number (Fig. 6D) and vascular density in the injured DG, CA3, and CA1 (Fig. 6E) as well as to increased cell proliferation and newborn neurons in the DG (Fig. 6F) assessed at Day 35 after TBI (p < 0.05). These data indicate that EPO may improve neurological recovery by reducing neuronal loss and promoting angiogenesis and neurogenesis.

Discussion

Our present study demonstrates a significant dose-dependent long-lasting improvement in sensorimotor and spatial learning after TBI in rats treated with EPO. The main findings are as follows: 1) Delayed (24 hours postinjury) EPO treatment after TBI provides long-term behavioral benefits with a wide range of effective EPO doses (from 1000 to 7000 U/kg intraperitoneally). 2) Although all EPO doses tested show substantial benefits after TBI, the repeated 5000 U/kg paradigm provides an optimal functional outcome. 3) The improvements in spatial learning and sensorimotor function are highly and
significantly correlated to the effect of EPO on reducing hippocampal cell loss and promoting angiogenesis and neurogenesis in the injured cortex and hippocampus.

Erythropoietin given within 6 hours after TBI reduces brain damage and improves functional recovery in rats. Our recent findings demonstrated that delayed (24 hours postinjury) EPO therapy does not reduce cortical lesion volume but improves functional outcomes after TBI, indicating that the therapeutic time window may not be limited to the early hours after TBI. However, the optimal EPO dose, dosing interval, and number of doses for reducing brain injury and promoting neurorestoration after TBI have not been fully investigated. In the present study, EPO doses used for treating TBI were much higher than those used for correcting anemia and therefore significantly increased HCT. Although no significant difference in the HCT level was observed among the EPO-treated groups, there was a significant functional improvement in different EPO dose groups. This suggests that EPO-induced therapeutic effects are independent of increased HCT, which is consistent with our previous finding that normalization of the HCT by normovolemic hemodilution does not affect the histological and functional outcome of EPO therapy for TBI. The present study demonstrated that delayed EPO treatment improved functional recovery, reduced hippocampal neuron loss, and increased angiogenesis and neurogenesis in a dose-dependent manner. The medium dose (5000 U/kg given intraperitoneally at 24, 48, and 72 hours post-TBI) provided the maximal functional recovery. In addition, EPO treatment significantly increased the total number of newborn neurons found in the injured cortex, suggesting that EPO treatment induces SVZ-derived neurogenesis (that is, migration of neuroblasts from the SVZ into the injured cortex where some of the SVZ-derived neuroblasts differentiate into mature neurons, which has been demonstrated after stroke and TBI). Erythropoietin therapy also significantly increased the total number of newborn neurons in the DG in the present study, which agrees with findings from our previous studies. Newborn granule neurons in the DG are capable of establishing anatomical integration into the CA3 region after TBI. Our present data suggest that EPO-promoted newborn neurons may participate in brain repair and functional recovery after TBI.

The adult brain vascular system is stable under nor-
mal conditions but is activated in response to certain pathological conditions including injuries. Adult vascular remodeling includes either angiogenesis by mature endothelial cells (that is, the formation of new capillaries from preexisting vessels) or vasculogenesis by endothelial progenitor cells, or a combination of the two. Endothelial progenitor cells are present in the bone marrow and peripheral blood, and are mobilized to the peripheral blood following TBI. Our present study shows that EPO promotes TBI-induced angiogenesis in a dose-dependent manner. Angiogenesis may provide the critical neurovascular microenvironments for neuronal remodeling. The coupling of angiogenesis and neurogenesis has been demonstrated in rodents after brain injuries. Treatment with EPO contributes to neurovascular remodeling, leading to improved neurobehavioral outcomes following ischemic brain injury. Erythropoietin stimulates vascular repair by facilitating endothelial progenitor cell migration into the brain and neovascularization, and it promotes neurogenesis. Erythropoietin treatment upregulates the EPOR level in vascular endothelial cells, confers neurovascular protection, and enhances angiogenesis after permanent focal cerebral ischemia in adult mice. Our recent study demonstrated that tumor necrosis factor–α primes cerebral endothelial cells for erythropoietin-induced angiogenesis. In addition, our in vitro study showed that EPO enhances VEGF secretion in neural progenitor cells through activation of the phosphatidylinositol-3 kinase/protein kinase B (PI3K/Akt) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) signal-transduction pathways and that neural progenitor cells treated with EPO upregulate VEGFR2 expression in cerebral endothelial cells, which along with VEGF secreted by neural progenitor cells promotes angiogenesis. In addition to its robust angiogenic effects, VEGF is capable of promoting hippocampal neurogenesis in the SVZ and subgranular zone in response to TBI. Erythropoietin and EPOR are weakly expressed in normal adult brains. Erythropoietin expression is transiently upregulated the first 3 days after TBI while upregulated expression of EPOR lasts for at least 1 week. These findings suggest that the transient increase in endogenous EPO expression may not match prolonged increased expression of EPOR and therefore may not provide sufficient neuroprotection after TBI. Prolonged EPOR upregulation provides a platform for exogenous EPO treatment and suggests that multiple doses may be required. In the present study, low (1000 U/kg) or high (7000 U/kg) doses of delayed EPO therapy show substantial benefits, while the medium dose of EPO (5000 U/kg) provides optimal outcomes. Erythropoietin has a limited capacity to cross the blood-brain barrier. The low dose of EPO would lead to a low brain EPO level, which may limit the benefits as demonstrated in models of stroke and TBI in animals. However, the higher dose of EPO did not show better benefits than the medium dose in the present study.

Fig. 6. Correlation of functional outcomes with cell loss, angiogenesis, cell proliferation, and neurogenesis. A–C: Line graphs showing that the functional outcomes (hindlimb, forelimb footfault, and mNSS) are significantly and inversely correlated with the number of vessels (A), BrdU-positive cells (B), and NeuN/BrdU-positive cells (C) in the injured cortex measured at Day 35 after TBI and EPO treatment (p < 0.05). D–F: Line graphs showing that spatial learning performance is significantly and positively correlated with the number of neuron cells (D), vessels (E), and BrdU-positive and NeuN/BrdU-positive cells (F) in the ipsilateral hippocampus measured at Day 35 in rats after TBI and EPO treatment (p < 0.05). Data represent mean ± SD. There were 8 rats per group.

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Some studies have shown that high doses of EPO are associated with a lack of or reduced efficacy compared with lower doses. Systemic administration of higher dose EPO will fully activate its hematopoietic (for example, increased HCT) and other vascular activities, including strong procoagulant and hemodynamic effects. Recent clinical EPO studies confirmed these concerns, with a significantly increased incidence of thrombosis or risk of serious complications (intracerebral hemorrhage and brain edema) and death, especially when combined with tPA after stroke.

Several EPO clinical trials in patients with TBI or aneurysmal SAH are ongoing or have been completed. In a small pilot randomized trial, TBI patients either received a single dose of EPO (40,000 U intravenously [11 patients]) or saline placebo (5 patients) within 6 hours after injury, and the primary outcome measure was serum S100B and neuron-specific enolase (NSE) levels. The EPO treatment did not impact NSE or S100B levels compared with placebo. Outcome failure in this small clinical study does not necessarily indicate a lack of therapeutic potential of EPO, but it suggests that EPO is safe to use and that a higher dose or different dosing regimen may be required to demonstrate efficacy. In addition, several EPO TBI clinical trials are ongoing (www.clinicaltrials.gov: NCT00987454, NCT00313716, and NCT00375869) with doses based on the EPO stroke clinical trials. In a double-blind randomized clinical trial, the beneficial effects of EPO (500 U/kg/day for 3 days) in patients with aneurysmal SAH could not be concluded. However, in a recent Phase II randomized, double-blind, placebo-controlled trial, within 72 hours of aneurysmal SAH, 80 patients were randomized to receive intravenous EPO (30,000 U) or placebo every 48 hours for a total of 90,000 U. This preliminary study showed that EPO reduced delayed cerebral ischemia following aneurysmal SAH via decreasing severity of vasospasm and shortening impaired autoregulation. Among the 71 survivors, the EPO group had fewer deficits measured using the National Institutes of Health Stroke Scale. These inconclusive results encourage further investigation of the potential of EPO as a candidate drug for the treatment of brain injury by improving preclinical experimental design and performing associated clinical trials.

There are several limitations in the present study. First, the brain EPO level was not measured, although previous studies show that a dose-dependent increase was observed in the brain EPO level in rats treated with EPO after stroke and TBI. Second, although we found that EPO treatment at doses from 1000 to 7000 U/kg similarly increases HCT, the effects of these doses on procoagulant activities and hemodynamics as well as PO2, PCO2, and blood pH were not examined, the potential side effects of which may affect the outcome. Third, the optimal dose (5000 U/kg intraperitoneally at 24, 48, and 72 hours postinjury) cannot be simply extended to other therapeutic time windows or paradigms such as intravenous injection, a single-dose treatment, and other therapeutic times of multiple-dose chronic treatment. Fourth, although our data show that the EPO-induced effects on hippocampal cells, cell proliferation, and angiogenesis and neurogenesis may contribute to functional recovery, other beneficial effects of EPO cannot be excluded. For example, our recent study demonstrated that EPO-induced corticospinal axonal sprouting from contralateral cortex is highly correlated to sensorimotor functional recovery after TBI. Further investigation of these aspects is warranted.

**Conclusions**

Delayed (24 hours) repeated EPO therapy for TBI significantly reduces hippocampal cell loss, enhances angiogenesis and neurogenesis, and improves sensorimotor function and spatial learning recovery in a dose-dependent manner. These findings suggest that selecting the optimal EPO dose for treatment of TBI is crucial and should be emphasized in designing preclinical and clinical trials.

**Disclosure**

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