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Effects of Erythropoietin on Reducing Brain Damage and Improving Functional Outcome after traumatic brain injury in mice

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Abstract

Object—This study was designed to investigate the beneficial effects of recombinant human erythropoietin (rhEPO) treatment of traumatic brain injury (TBI) in mice.

Methods—Adult male C57BL/6 mice were divided into 3 groups: 1) saline group (TBI + saline, n = 13); 2) EPO group (TBI + rhEPO, n = 12); and 3) sham group (sham + rhEPO, n = 8). TBI was induced by controlled cortical impact. Bromodeoxyuridine (100 mg/kg) was injected daily for 10 days, starting 1 day after injury, for labeling proliferating cells. rhEPO was administered intraperitoneally at 6 hours, and at 3 and 7 days post-TBI (5000 U/kg body weight, total dosage = 15,000 U/kg). Neurological function was assessed using the Morris Water Maze and footfault tests. Animals were sacrificed 35 days after injury and brain sections stained for immunohistochemistry.

Results—TBI caused both tissue loss in the cortex and cell loss in the dentate gyrus (DG) and impaired sensorimotor function (footfaults) and spatial learning (Morris Water Maze). TBI alone stimulated cell proliferation and angiogenesis. As compared to saline treatment, rhEPO significantly reduced lesion volume in the cortex and cell loss in the DG after TBI and substantially improved sensorimotor function recovery and spatial learning performance. rhEPO enhanced neurogenesis in the injured cortex and the DG.

Conclusions—rhEPO initiated 6 hours post-TBI provides neuroprotection by decreasing lesion volume and cell loss as well as neurorestoration by enhancing neurogenesis, subsequently improving sensorimotor and spatial learning function. rhEPO is a promising neuroprotective and neurorestorative agent for TBI and warrants further investigation.

Keywords

erythropoietin; mouse; sensorimotor; spatial learning; traumatic brain injury

Introduction

Traumatic brain injury (TBI) is a common cause of mortality and morbidity in the United States, particularly among the young⁶⁰ and may result in permanent functional deficits due to both primary and secondary mechanisms.¹⁷ In addition to primary mechanical damage, TBI is associated with secondary injury that evolves over a period of hours to days after the primary insult, and is the result of biochemical and physiological events that ultimately lead to neuronal cell death. This period of evolution provides a window of opportunity for therapeutic intervention with the potential to improve long-term patient outcome. Although a number of therapeutic trials for TBI have been undertaken, no efficacious treatment has been identified clinically.⁴⁵

Erythropoietin (EPO) is the major hormone that stimulates and regulates erythropoiesis.³² EPO receptors (EPORs) are expressed in neurons, astrocytes, and endothelial cells.⁵ Inhibition of EPO activity by the administration of soluble EPORs worsens the severity of neuronal injury,⁵⁰ suggesting that endogenous EPO is directly involved in an intrinsic neuronal repair pathway. Administration of rhEPO 24 hours before or up to 6 hours after focal ischemic stroke significantly reduced the extent of infarction. rhEPO also attenuated concussive brain injury,⁵ spinal cord injury,^{7,24,25} kainate-induced seizure activity,⁵ and autoimmune encephalomyelitis.^{8,33,50} More recently, it has been reported that rhEPO treatment promotes spatial memory restoration and enhances neurogenesis after TBI in the rat.³⁷ rhEPO also shows neuroprotection in an animal model of stroke.⁶³

A controlled cortical impact (CCI) model of TBI is one of the most widely used models in rats^{34,38,65} and mice.^{54,66} Using a CCI-induced TBI mouse model, we investigated the effects of posttraumatic administration of rhEPO on cortical and hippocampal injury, cell proliferation, neurogenesis, angiogenesis, sensorimotor function and spatial learning recovery.

Materials and Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Henry Ford Health System.

Animal Model

Adult male C57BL/6 mice weighing 24 to 29 g (Charles River Laboratories., Inc., Wilmington, MA) were anesthetized intraperitoneally with 400-mg/kg body weight chloral hydrate. Body temperature was maintained at 37°C by using a circulating water-heating pad. TBI was delivered as previously described^{37,54,65,66} with minor modifications. Each animal was placed in a stereotaxic frame. A 4-mm diameter craniotomy was performed over the left parietal cortex adjacent to the central suture, midway between lambda and bregma. The dura was kept intact over the cortex. Injury was induced by impacting the left cortex (ipsilateral cortex) with a pneumatic piston containing a 2.5-mm-diameter tip at a rate of 4 m/second and 0.8 mm of compression. Velocity was measured with a linear velocity displacement transducer. A sham group of mice underwent the same craniotomy but were not injured. The animals were divided into three groups: 1) EPO group (TBI + rhEPO, n = 12); 2) saline group (TBI + saline, n = 13); and 3) sham group (sham + rhEPO, n = 8). An additional group of rats (n = 4) underwent TBI, were treated with saline and were sacrificed at Day 7.

rhEPO Administration

The dose of rhEPO was selected based on previous studies.^{37,63} rhEPO at a dose of 5000 U/kg body weight (Epoetin alpha, AMGEN, Thousand Oaks, CA) was administered intraperitoneally at 6 hours and at 3 and 7 days (total dosage = 15,000) after TBI or sham.

Mice in the saline-treated group received an equal volume of saline at 6 hours, and at 3 and 7 days after TBI. For labeling proliferating cells, 5-bromo-2'-deoxyuridine (BrdU, 100 mg/kg; Sigma, St. Louis, MO) was injected intraperitoneally into mice daily for 10 days, starting 1 day after TBI. All mice were sacrificed at 35 days after TBI or surgery.

Body Weight

Body weight was recorded before TBI and at 1, 4, 7, 14, 21, 28 and 35 days after TBI or surgery.

Hematocrit

To determine the effects of rhEPO on hematocrit (HCT), a blood sample (50 μ l) was collected via tail vein before injury and weekly after TBI or sham up to 5 weeks. HCT was measured in micro-HCT capillary tubes (Fisher Scientific, Pittsburgh, PA) using standard procedures (Readacrit Centrifuge, Clay Adams, Parsippany, NJ).

Behavioral Tests

Morris Water Maze test—To detect spatial learning impairments, a recent version of the Morris water maze test was used.¹⁴ The procedure was modified from previous versions^{18,43,44,57} and has been found to be useful for chronic spatial memory assessment in rats and mice with brain injury.^{14,37} All animals were tested during the last five days (i.e., from 31–35 days after TBI or surgery) before sacrifice. Data collection was automated by the HVS Image 2020 Plus Tracking System (US HVS Image, San Diego, CA.). For data collection, a white pool (1.2 m in diameter) was subdivided into four equal quadrants formed by imaging lines. At the start of a trial, the mouse was placed randomly at one of four fixed starting points, facing toward the wall (designated North, South, East and West) and allowed to swim for 90 seconds or until they found the platform within 90 seconds. If the animal found the platform, it was allowed to remain on it for 10 seconds. If the animal failed to find the platform within 90 seconds, it was placed on the platform for 10 seconds. Throughout the test period the platform was located in the NE quadrant 1 cm below water in a randomly changing position, including locations against the wall, toward the middle of the pool or off-center, but always within the target quadrant. If the animal was unable to find the platform within 90 seconds, the trial was terminated and a maximum score of 90 seconds was assigned. If the animal reached the platform within 90 seconds, the percentage of time traveled within the NE (correct) quadrant was calculated relative to the total amount of time spent swimming before reaching the platform and employed 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.⁵¹

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 by an investigator blind to the treatment groups. The mice were allowed to walk on a grid (12 cm \times 57 cm with 1.3 cm \times 1.3 cm diameter openings). With each weight-bearing step, a paw might fall or slip between the wires^{2,3} and, if this occurred, it was recorded as a footfault. A total of 50 steps were recorded for each right forelimb and hind limb.

Tissue Preparation and Measurement of Lesion Volume

At 35 days after TBI, mice were anesthetized intraperitoneally with chloral hydrate, and perfused transcardially first with saline solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4. The brains were removed and post-fixed in 4% paraformaldehyde at room temperature for 48 hours. The brain tissue was cut into 7 equally spaced (1 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 hematoxylin and eosin (H&E). To measure lesion volume the 7 brain sections were traced by a microcomputer imaging device (MCID) (Imaging Research, St. Catharine's, Ontario, Canada), as previously described.⁹ The indirect lesion area was calculated (i.e., the intact area of the ipsilateral hemisphere is subtracted from the area of the contralateral hemisphere),⁵⁸ and the lesion volume presented as a volume percentage of the lesion compared with the contralateral hemisphere.

Immunohistochemistry

To examine the effect of rhEPO on cell proliferation and angiogenesis, coronal sections were histochemically stained with mouse anti-BrdU³⁷ and rabbit anti-human von Willebrand factor (vWF),³⁷ respectively. For BrdU detection, 6- μm -thick paraffin-embedded coronal sections were deparaffinized and rehydrated. Antigen retrieval was performed by boiling sections in 10 mM citrate buffer (pH 6.0) for 10 minutes. After washing with PBS, sections were incubated with 0.3 % H₂O₂ in PBS for 10 minutes, blocked with 1 % BSA containing 0.3 % Triton-X 100 at room temperature for 1 hour, and incubated with mouse anti-BrdU (1:200; Dako, Carpinteria, CA) at 4°C overnight. After washing, sections were incubated with biotinylated anti-mouse antibody (1:200; Vector Laboratories, Inc., Burlingame, CA) at room temperature for 30 minutes. After washing, sections were incubated with an avidin-biotin-peroxidase system (ABC kit, Vector Laboratories, Inc., Burlingame, CA). Diaminobenzidine (Sigma, St. Louis, MO) was then used as a sensitive chromogen for light microscopy. Sections were counterstained with hematoxylin.

To identify vascular structure, brain sections were deparaffinized and then incubated with 0.4% Pepsin solution at 37°C for 1 hour. After washing, the sections were blocked with 1% BSA at room temperature for 1 hour, and then incubated with rabbit anti-human vWF (1:200; DakoCytomation, Carpinteria, CA) at 4°C overnight. After washing, sections were incubated with biotinylated anti-rabbit antibody (1:200; Vector Laboratories, Inc., Burlingame, CA) at room temperature for 30 minutes. After washing, sections were incubated with an avidin-biotin-peroxidase system (ABC kit, Vector Laboratories, Inc., Burlingame, CA). Diaminobenzidine (Sigma, St. Louis, MO) was then used as a sensitive chromogen for light microscopy. Sections were counterstained with hematoxylin.

BrdU-positive cells and vWF-stained vascular structures in the DG and the cortex of both contralateral and ipsilateral hemispheres were examined at 20 × magnification and counted. The cells with BrdU (brown stained) that clearly localized to the nucleus (hematoxylin stained) were counted as BrdU-positive cells.

Immunofluorescent Staining

Newly generated neurons were identified by double labeling for BrdU and NeuN. After dehydration, tissue sections were boiled in 10-mM citric acid buffer (pH 6) for 10 minutes. After washing with PBS, sections were incubated in 2.4 N HCl at 37°C for 20 minutes. Sections were incubated with 1% BSA containing 0.3% Triton-X-100 in PBS. Sections were then incubated with mouse anti-NeuN antibody (1:200; Chemicon, Temecula, CA) at 4°C overnight. FITC-conjugated anti-mouse antibody (1:400; Jackson ImmunoResearch, West Grove, PA) was added to sections at room temperature for 2 hours. Sections were then incubated with mouse anti-BrdU antibody (1:200; Dako, Glostrup, Denmark) at 4°C overnight. Sections were then incubated with Cy3-conjugated anti-mouse antibody (1:400; Jackson ImmunoResearch, West Grove, PA) at room temperature for 2 hours. Each of the steps was followed by three 5-minute rinses in PBS. Tissue sections were mounted with Vectashield mounting medium (Vector laboratories, Burlingame, CA). Images were

collected with fluorescent microscopy. NeuN/BrdU-labeled cells in the DG and the cortex were counted at a magnification of 40.

Cell Counting and Quantification

Cell counts were performed by observers blinded to the individual treatment status of the animals. Five sections with 50- μ m intervals from the dorsal DG were analyzed with a microscope (Nikon eclipse 80i) at 400 \times magnification via the MCID system.^{37,39} All counting was performed on a computer monitor to improve visualization and in one focal plane to avoid oversampling.^{69,71} To evaluate whether intraperitoneally administered rhEPO reduces neuronal damage after TBI, all the cells in the DG in each section were counted using the MCID system.³⁹ Counts were averaged and normalized by measuring the linear distance (in mm) of the DG 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, CA3 and CA1 regions of the hippocampus, using the MCID system. 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 in cells/mm² in the lesion boundary zone or in cells/mm in the hippocampus. For analysis of neurogenesis, additional sections used in the above studies were used to evaluate neurogenesis in the DG and the cortex by calculating the density of BrdU-labeled cells and BrdU/NeuN-labeled cells.³⁹ We focused mainly on the ipsilateral DG and its subregions, including the subgranular zone (SGZ), granular cell layer (GCL), and the molecular layer. The number of BrdU-positive cells (red stained) and NeuN/BrdU-labeled cells (yellow after merge) were counted in the DG and the lesion boundary zone. The percentage of NeuN/BrdU-labeled cells over the total number of BrdU-positive cells in the corresponding regions (DG or cortex) was estimated and used as a parameter to evaluate neurogenesis.

Statistical Analyses

All data are presented as the means \pm standard deviations. Data were analyzed by analysis of variance for multiple comparisons of body weight, hematocrit and functional tests (spatial performance and sensorimotor function). For lesion volume, cell counting, cell proliferation and vWF-stained vascular density, a one-way analysis of variance followed by post hoc Student-Newman-Keuls tests were used to compare the difference between the rhEPO-treated, saline-treated and sham groups. A paired t-test was used to compare values between the ipsilateral and contralateral hemispheres. Statistical significance was set at $p < 0.05$.

Results

Body Weight

When compared with preinjury levels, body weight was significantly decreased at 1 and 4 days post TBI in the saline-treated group ($p < 0.05$) and at 1, 4, and 7 days in the rhEPO-treated group ($p < 0.05$). The sham-treated mice also showed a decrease in body weight at 1 and 4 days after surgery ($p < 0.05$, Fig. 1). However, there was no difference between the rhEPO- and saline-treated groups. By 14 days post-surgery, body weight for all three groups had returned to pre-injury levels and continued to increase afterwards.

Hematocrit

The baseline of HCT was similar for all three groups before injury or sham-surgery (Fig. 2). All mice in the TBI and sham groups received rhEPO at a dose of 5000 U/kg body weight injected intraperitoneally at 6 hours and 3 and 7 days after TBI or sham-surgery. The first

two injections (i.e., 6 hours and 3 days) significantly increased HCT when measured at 7 days post TBI ($p < 0.05$ versus pre-injury). The third injection of rhEPO (i.e., 7 days) did not produce further HCT effects. HCT gradually returned to normal by Day 21 post-injury. In the saline-treated animals, HCT was slightly decreased post TBI compared with pre-injury levels but without significance. There was a significant difference between the rhEPO- and saline-treated groups at 7 and 14 days post TBI ($p < 0.05$).

Spatial Learning Test

The water maze protocol in the present study was used to detect spatial learning deficits. To analyze day-by-day differences in the Morris water maze, a repeated measures analysis of variance was performed followed by Student-Newman-Keuls tests for multiple comparisons. As shown in Figure 3, the time spent in the correct quadrant (Northeast) by non-injured mice gradually increased from 42% at Day 31 to 70% at Day 35 after sham surgery. The saline-treated mice with TBI were impaired relative to sham-operated mice ($p < 0.05$) and rhEPO-treated mice with TBI ($p < 0.05$) did not show significant improvement. The rhEPO-treated group exhibited significantly improved spatial performance at 33, 34, and 35 days after TBI as compared with the saline-treated group ($p < 0.05$).

Sensorimotor Function Test

The incidence of forelimb footfaults during baseline (preoperatively) was about 4% to 5% (Fig. 4). Sham surgery alone mildly increased the frequency of footfaults at post-operative Days 1 and 4. TBI significantly increased the occurrence of right forelimb footfaults contralateral to the TBI at 1 to 21 days postinjury as compared with the pre-injury baseline. Treatment with rhEPO significantly reduced the number of contralateral forelimb footfaults at 1 to 21 days after TBI compared to treatment with saline ($p < 0.05$).

Similar results were found for the contralateral hindlimb (Fig. 5). Sham surgery alone significantly increased the number of footfaults at post-operative Day 1 relative to baseline. TBI significantly increased the incidence of contralateral hindlimb footfaults at 1 to 21 days post-injury. Treatment with rhEPO significantly reduced the number of contralateral hindlimb footfaults 7 to 21 days post-injury compared to treatment with saline.

Lesion Volume

Mice were sacrificed at 35 days post TBI for histological measurements. TBI caused about 8.3 % tissue loss in the ipsilateral hemisphere compared with the contralateral hemisphere. Treatment with rhEPO reduced the lesion volume to 4.3% (Fig. 6, $p < 0.001$ vs. the saline-treated group). In the subgroup of mice examined at 7 days after TBI, there was 9.4 ± 2.6 % ($n = 4$) tissue loss relative to the contralateral hemisphere.

Cell Loss in the DG

When examined at 35 days post TBI (Fig. 7), the neuron counts in the ipsilateral DG had decreased to 58% of the number found in the contralateral DG (Fig. 7C, D and G, $p < 0.05$). When examined at 7 days post TBI, the neuron counts per millimeter in the ipsilateral DG was 70% of that in the contralateral DG (419 ± 44 for ipsilateral versus 593 ± 100 for contralateral). In mice sacrificed at day 35, the neuron counts after rhEPO treatment stayed at 77% of the number found in the contralateral DG (Fig. 7E, F and G, $p < 0.05$). A significant difference was observed in the neuron numbers between the EPO- and saline-treated groups (Fig. 7G, $p < 0.05$).

Cell Proliferation

BrdU, an analog of thymidine, is commonly used to detect proliferating cells in living tissues. BrdU can be incorporated into the newly synthesized DNA of replicating cells during the S phase of the cell cycle, substituting for thymidine during DNA replication. The number of BrdU-positive cells found in the ipsilateral DG, CA3 and CA1 areas (Fig. 8G, $p < 0.05$) was significantly increased (3–4 fold) after TBI alone, compared with the number found in the contralateral hemisphere at 35 days after TBI. However, rhEPO treatment did not influence further the number of BrdU-positive cells in these regions after TBI (Fig. 8). rhEPO even slightly decreased BrdU-positive cells in the DG and CA1 area ($p > 0.05$).

TBI alone significantly increased the number of BrdU-positive cells in the ipsilateral cortex (Fig. 9). The rhEPO-treated group showed significantly increased BrdU-positive cells in the ipsilateral cortex after TBI when compared to the saline-treated group (Fig. 9G, $p < 0.05$). BrdU-positive cells were rarely seen in the contralateral cortex of mice or in the cortex and DG of sham animals that had undergone TBI (Fig. 8A, B, 9A, B).

Neurogenesis

Newly generated neurons were identified by double labeling for BrdU (proliferation marker) and NeuN (mature neuronal marker). Approximately 10% of BrdU-positive cells were NeuN/BrdU-labeled cells in the ipsilateral DG and cortex after TBI (Fig. 10). The percentage of NeuN/BrdU-labeled cells was significantly higher in the rhEPO-treated group than in the saline-treated group in the DG and cortex ($p < 0.05$). The percentage of NeuN/BrdU-labeled cells was increased by 47% in the ipsilateral DG and 36% in the ipsilateral cortex treated with rhEPO.

Angiogenesis

vWF-staining has been used to identify vascular structure in the brain after TBI.³⁶ TBI alone significantly increased the density of vessels in the DG (Fig. 11) and the cortex (Fig. 12) of the ipsilateral hemisphere. TBI did not significantly change the density of vessels in the CA3 and CA1 regions of the injured hemisphere compared with the contralateral hemisphere (Fig. 11G). rhEPO treatment did not show significant effects on vascular density in the DG or in the cortex (Figs. 11, 12).

Discussion

The main findings of the present study are: 1) early (6 hours) rhEPO treatment after TBI provided long-term behavioral benefits, as reflected in improvements in spatial learning and sensorimotor functional recovery; 2) the improvements in spatial learning and sensorimotor function appear to result substantially from early rhEPO treatment's neuroprotective effect in reducing tissue and cell loss; and 3) the footfault test and this moving-platform version of the water maze test are sensitive to sensorimotor and spatial performance deficits in mice even one month post TBI.

There are several major differences between the present work and earlier studies. The effects of rhEPO on sensorimotor functions after CCI in rats were not examined in our previous studies.^{37,41} The present study in mice demonstrates that rhEPO not only significantly improves the spatial learning function at Days 33–35 on the WMW test after CCI but also promotes early sensorimotor functional recovery (footfault test). Neurorestorative events include neurogenesis, angiogenesis, and synaptic plasticity, one or all of which may contribute to functional improvement.¹⁰ Our previous work has shown that rhEPO has neurorestorative effects after stroke⁶³ and TBI.³⁷ Our previous studies in rats focused on the neurorestorative effect of rhEPO (cell proliferation or neurogenesis).^{37,41} However, our

present study investigates the neuroprotective effect of rhEPO (reduction in lesion volume and cell loss) in addition to cell proliferation and neurogenesis. Cell proliferation was induced in the DG after TBI in our previous³⁷ and present studies. However, rhEPO treatment in the present study does not further enhance cell proliferation in the DG in mice after CCI, which was seen in rats with rhEPO treatment after CCI.⁴¹ Our data with 6-hour treatment shows that rhEPO reduced cell loss in the DG. This neuroprotective effect of rhEPO was not evaluated in our previous studies. In addition, our present study shows that early rhEPO treatment reduced lesion volume in the ipsilateral cortex after TBI while rhEPO initiated later (one day) after TBI did not reduce lesion volume in rats (data not shown). These data indicate that the therapeutic mechanism of rhEPO treatment initiated early (i.e., 6 hours post TBI) may differ from that of late treatment with rhEPO (1 day). Thus, rhEPO may provide both neuroprotective (reduction in tissue damage and cell loss) and neurorestorative (neurogenesis, angiogenesis or synaptogenesis, i.e., remodeling) therapeutic effects, depending on the administration time after TBI. Third, in another short-term (14-day survival) study with mice after weight-drop-induced TBI, rhEPO injected 1 and 24 hours after TBI improved motor and cognitive functions and reduced inflammation, axonal degeneration and apoptosis,⁶⁷ supporting the view that rhEPO can provide neuroprotective effects. Cognitive function was evaluated based on the differential exploration of familiar and new objects.²¹ A difference in this cognitive function was highly significant between vehicle-treated and rhEPO-treated mice at Day 3 after injury but diminished at Day 8 post injury.⁶⁷ This functional test may not be suitable to evaluate long-term functional recovery. The spatial learning test in our present study revealed a significant difference between vehicle- and rhEPO-treated groups at 33–35 days after TBI. Fourth, the initiation time for the dosing paradigm after weight-drop TBI in mice was 1 hour.⁶⁷ In the present study, the initiation time of 6 hours was used to match as closely as possible the dosing paradigm used in a small but positive clinical study that used rhEPO for treatment of stroke.²⁰ Thus a 6-hour initiation time may be more clinically relevant. To date, to our knowledge, only several published papers^{37,67,41} have attempted to investigate whether rhEPO treatment after TBI improves spatial memory or motor and cognitive function. Other studies evaluated beneficial effects of rhEPO for treatment of TBI, such as lipid peroxidation,⁴⁶ brain edema,⁶¹ lesion volume⁵ and neuron density,¹² without examining functional outcomes.

In our present study, TBI caused a significant cell loss in the ipsilateral DG (especially in the dorsal blade) of 30% at 7 days post-injury and 42% at 35 days post-injury as compared with the contralateral cell number. This suggests that cell death occurred mostly within the first week post TBI. It has been reported that cell death peaks mainly at 24 hours and subsides by 14 days after TBI.⁴⁸ rhEPO treatment reduced cell loss in the DG (i.e., 23% cell loss in the rhEPO-treated group versus 42% cell loss in the saline-treated group at 35 days after TBI, $p < 0.05$), suggesting that rhEPO has a neuroprotective effect. rhEPO has been demonstrated to reduce neuronal injury through inhibition of apoptosis (i.e., promotion of cell survival) in different injury models.^{29,67,72}

In animal models, cell proliferation occurs following brain trauma in the hippocampus,^{13,16,30,37} in the injured cortex,^{13,30,47} in the SVZ^{11,13,47} as well as in the corpus callosum, striatum and thalamus.⁴⁷ In our present study, cell proliferation was observed mainly in the DG and cortex of the ipsilateral hemisphere after TBI. However, 90% of these proliferating cells were not NeuN/BrdU-labeled, indicating that very limited neurogenesis occurs in the injured cortex after TBI. Furthermore, our present study showed that rhEPO treatment significantly enhanced the percentage of NeuN/BrdU-labeled cells in the DG and cortex post injury, suggesting that rhEPO treatment enhanced neurogenesis. This is in agreement with our previous study that rhEPO treatment increases neurogenesis in the DG in rats after TBI.³⁷ Further investigations as to why rhEPO treatment dramatically enhanced neurogenesis are warranted. Neurogenesis normally only occurs in the

subventricular zone (SVZ) and the SGZ of the hippocampus in the rodent adult brain.^{1,27,40} The source of the newborn neurons in the cortex after injury is unclear. However, degeneration⁴⁰ or injury²⁷ induces cortical neurogenesis. Neural precursors were isolated from damaged rat cerebral cortex after TBI.²⁷ A second source is the SVZ. The neural precursor cells migrate toward cortical lesions from the SVZ after TBI.^{47,56} Our recent work demonstrates that newly generated neurons in the SVZ migrate toward the ischemic boundary in rats after stroke.⁷⁰ It has also been reported that rhEPO increased both neurogenesis in the SVZ and migration of neuronal progenitors into the ischemic cortex and striatum.²⁸ Whether rhEPO treatment enhanced migration of SVZ cells into injured cortex after TBI is not known.

Spatial learning deficits in the water maze tasks have been demonstrated in rats^{15,19,31,35,37} and mice^{22,54} in the CCI model of TBI. This was further confirmed in our study. However, these deficits were probably not caused by sensorimotor dysfunction, since the test was performed at 31–35 days after TBI when sensorimotor function had been fully restored even without rhEPO treatment. Swim speeds (in the range between 14 to 17 cm/second, $p > 0.05$) were equal between groups indicating that there was no motor impairment during spatial learning testing that we could detect. This confirmed the findings in rats⁵⁵ and mice^{49,59} that TBI did not affect swim speed. We theorize that these spatial learning deficits may be related to cell loss in the DG because rhEPO significantly reduced cell loss in the DG and concomitantly improved spatial learning performance. However, spatial performance deficits are known to occur after injury to a variety of brain regions.

In this study, TBI impaired sensorimotor function as revealed by the footfault test, which has effectively been used to evaluate sensorimotor deficits in the mouse model of TBI.³ We also found that mice with TBI showed an increased frequency of footfaults for both the contralateral forelimb and the contralateral hindlimb, which persisted for up to 21 days. This sensorimotor impairment may be related to tissue loss in the cortex, striatum or corticostriatal connections after TBI. rhEPO significantly reduced cortical tissue loss and enhanced cell proliferation in the injured cortex, which may contribute to the improved early recovery of behavioral functions.

Practically all brain cells are capable of the production and release of EPO and expression of EPORs.²⁶ Intraperitoneal administration of rhEPO crosses the blood–brain barrier to protect against brain injury.⁵ EPO exerts multifaceted protective effects on brain cells from noxious stimuli such as hypoxia, excess glutamate, serum deprivation or kainic acid exposure *in vitro*²⁶ and in animal models of subarachnoid hemorrhage, intracerebral hemorrhage, traumatic brain injury, spinal cord injury and stroke.^{4–6,23,37,50,53,63} Mechanistically, rhEPO provides neuroprotection mainly by inhibiting apoptosis after brain ischemia and trauma.^{53,62,64,67} Although various anti-inflammatory and anti-apoptotic agents have shown neuroprotective effects in experimental models of TBI,^{52,68} to date, no specific pharmacological agent aimed at blocking the progression of secondary brain injury has been approved for clinical use in TBI patients.

In light of the positive outcome in using EPO to treat stroke in a recent small clinical trial²⁰ and to increase hippocampal response during memory retrieval in humans,⁴² and our present findings that rhEPO improves long-term histological outcome, sensorimotor function and spatial learning recovery, rhEPO is a promising neuroprotective and neurorestorative agent for TBI and warrants further investigation.

Conclusions

TBI induced by CCI in mice caused both tissue loss in the cortex and cell loss in the DG and impaired sensorimotor function (footfaults) and spatial learning (Morris water maze). TBI alone significantly stimulated cell proliferation and angiogenesis in the ipsilateral cortex and DG. rhEPO treatment initiated 6 hours post TBI provides neuroprotection by reducing lesion volume and DG cell loss as well as neurorestoration by enhancing neurogenesis, subsequently improving functional outcome.

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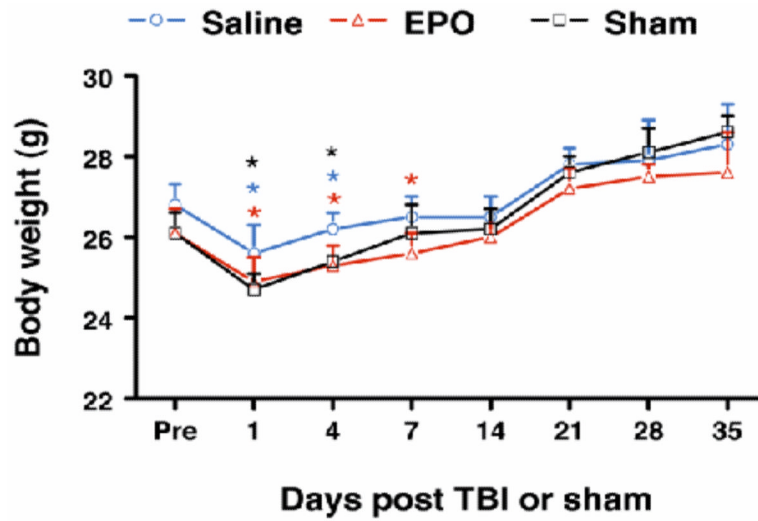


Fig. 1. Changes in body weight before and after TBI. “Pre” represents pre-injury level. Data represent mean \pm SD. * $p < 0.05$ vs. corresponding Pre. N (mice/group) = 12 (EPO); 13 (Saline); 8 (Sham).

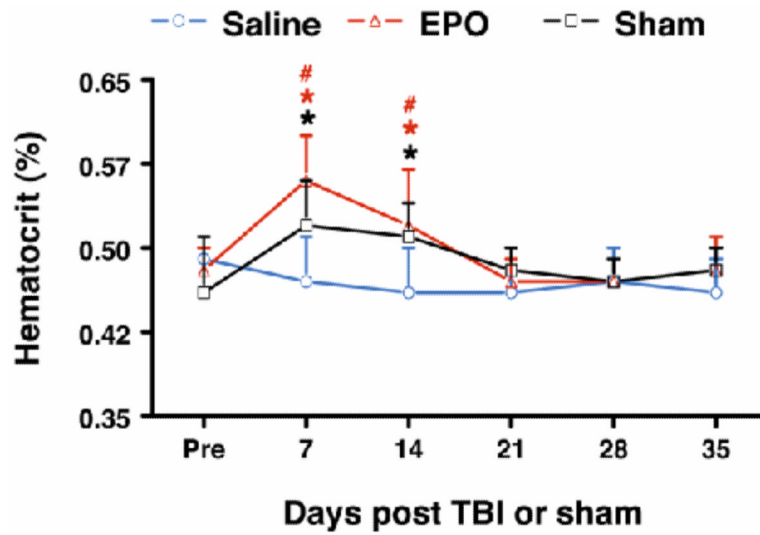


Fig. 2. Changes in hematocrit before and after TBI. “Pre” represents preinjury level. * $p < 0.05$ vs. corresponding Pre. Data represent mean \pm SD. # $p < 0.05$ vs. the saline group at days 7 and 14 after injury. N (mice/group) = 12 (EPO); 13 (Saline); 8 (Sham).

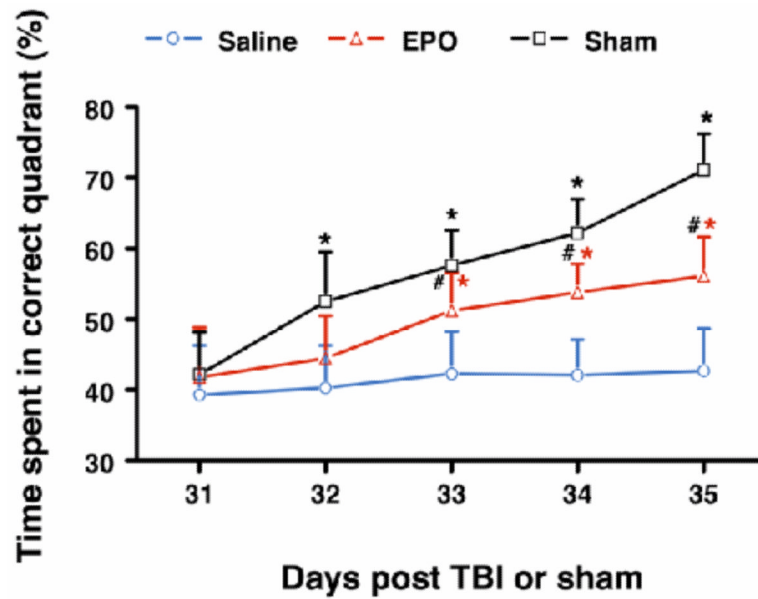


Fig. 3. Effect of rhEPO on spatial learning function 31–35 days after TBI. Delayed treatment with rhEPO improves spatial learning performance measured by a recent version of the water maze test compared with the saline group. Data represent mean \pm SD. * $p < 0.05$ vs. corresponding value at D31. # $p < 0.05$ vs. the saline group at days 33, 34 and 35 after TBI. N (mice/group) = 12 (EPO); 13 (Saline); 8 (Sham).

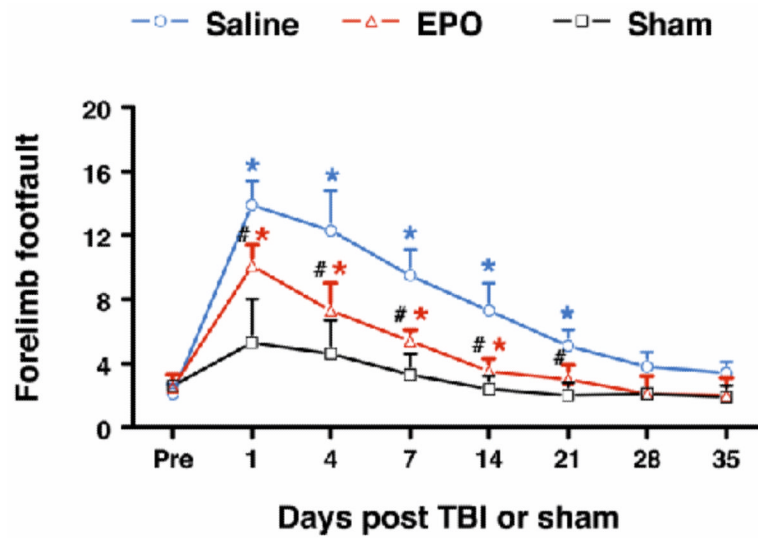


Fig. 4.

Effect of rhEPO on sensorimotor function (forelimb footfault) before and after TBI. “Pre” represents pre-injury level. Delayed treatment with rhEPO improves recovery of sensorimotor performance as compared with the saline group. Data represent mean \pm SD. * $p < 0.05$ vs. corresponding pre. # $p < 0.05$ vs. the saline group at day 1–21 after TBI. N (mice/group) = 12 (EPO); 13 (Saline); 8 (Sham).

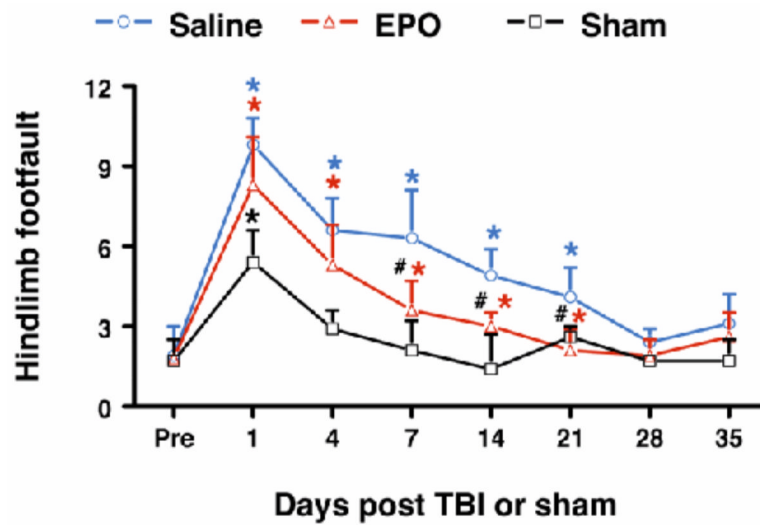


Fig. 5. Effect of rhEPO on sensorimotor function (hindlimb footfault) before and after TBI. “Pre” represents pre-injury level. Delayed treatment with rhEPO improves recovery of sensorimotor performance as compared with the saline group. Data represent mean \pm SD. * $p < 0.05$ vs. corresponding pre. # $p < 0.05$ vs. the saline group at day 7–21 after TBI. N (mice/group) = 12 (EPO); 13 (Saline); 8 (Sham).

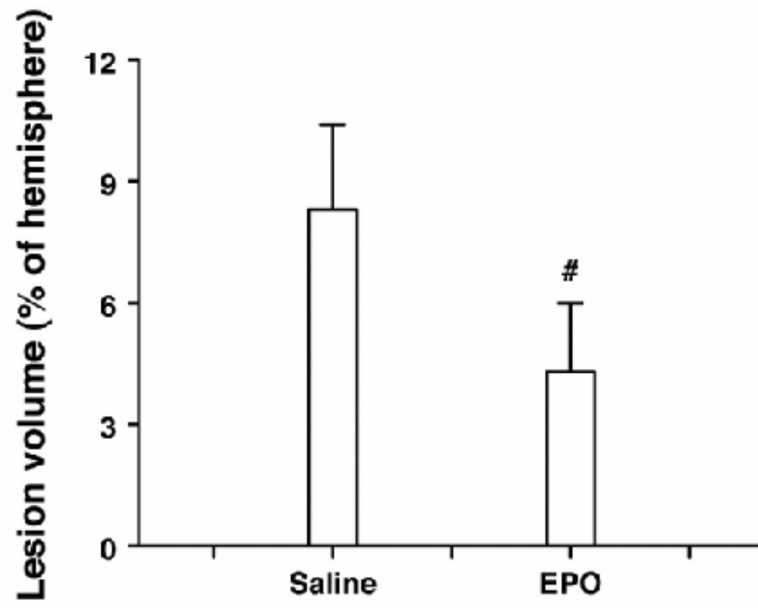


Fig. 6. Lesion volumes at 35 days after TBI. Data represent mean \pm SD. # $p < 0.05$ vs. the saline group. N (mice/group) = 12 (EPO); 13 (Saline).

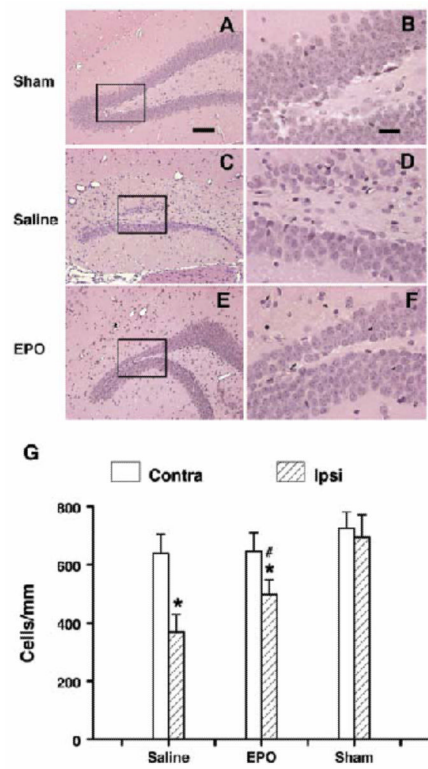


Fig. 7. Effect of rhEPO on cell loss in the DG at 35 days after TBI. H&E staining: A-F. Delayed treatment with rhEPO (E, F) reduces cell loss as compared with the saline group (C, D). Scale bar = 100 μ m (A, C, E); 25 μ m (B, D, F). The cell number in the DG is shown in (G). Data represent mean \pm SD. * $p < 0.05$ vs. corresponding Contralateral. # $p < 0.05$ vs. the saline group. N (mice/group) = 12 (EPO); 13 (Saline); 8 (Sham).

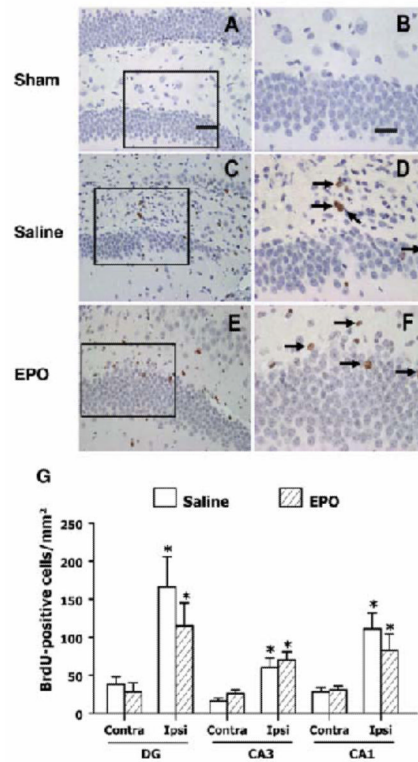


Fig. 8. Effect of rhEPO on BrdU-positive cells in the hippocampus 35 days after TBI. TBI alone significantly increases the number of BrdU-positive cells in the ipsilateral DG, CA3 and CA1. The cells with BrdU (brown stained) that clearly localize to the nucleus (hematoxylin stained) are counted as BrdU-positive cells (arrows in D and F). However, delayed treatment with rhEPO (E, F) does not show significant effects on cell proliferation as compared with the saline group (C, D). Scale bar = 50µm (A, C, E); 25µm (B, D, F). The number of BrdU-positive cells is shown in (G). Data represent mean ± SD. *p < 0.05 vs. corresponding Contralateral. N (mice/group) = 12 (EPO); 13 (Saline); 8 (Sham).

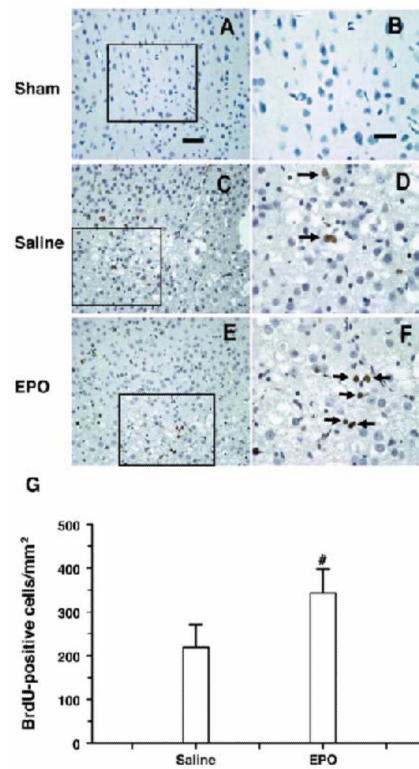
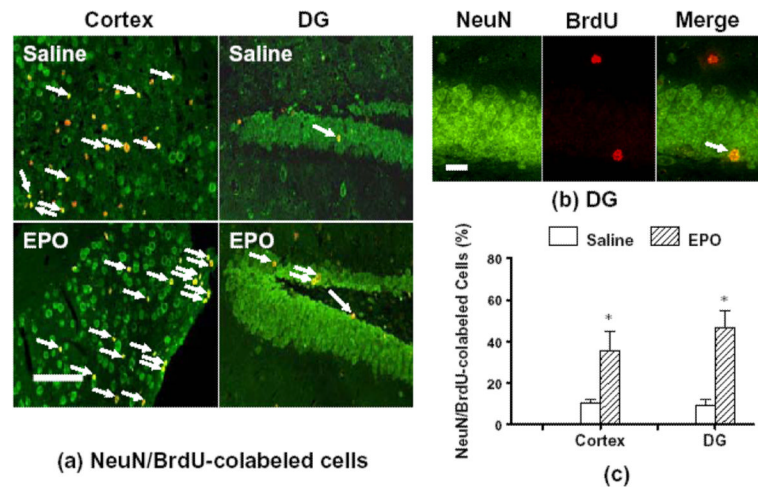


Fig. 9. Effect of rhEPO on BrdU-positive cells in the cortex 35 days after TBI. Delayed treatment with rhEPO (E, F) significantly increases BrdU-positive cells (stained brown, arrows in D and F) in the cortex as compared with the saline group (C, D). Scale bar = 50 μ m (A, C, E); 25 μ m (B, D, F). The number of BrdU-positive cells is shown in (G). Data represent mean \pm SD. # $p < 0.05$ vs. the saline group. N (mice/group) = 12 (EPO); 13 (Saline); 8 (Sham).

**Fig. 10.**

Photographs show the double fluorescent staining for BrdU (red) and NeuN (green) to identify the newly generated cells (red) and neurogenesis (yellow, arrow) in the DG of the ipsilateral, injured hemisphere of the saline-treated and the rhEPO-treated groups at 35 days after TBI (a). (b) NeuN/BrdU-double staining (merge in yellow, arrow): Newborn BrdU-positive cells (red) can differentiate into neurons expressing NeuN (green). (c) The percentage of NeuN/BrdU-labeled cells. Treatment with rhEPO significantly increased the percentage of NeuN/BrdU-labeled cells in the DG and the cortex in TBI mice as compared with the saline group. * $p < 0.05$ vs. saline treatment. Data represent mean \pm SD. N (mice/group) = 12 (EPO); 13 (saline). Scale bar = 50 μ m (a); 25 μ m (b).

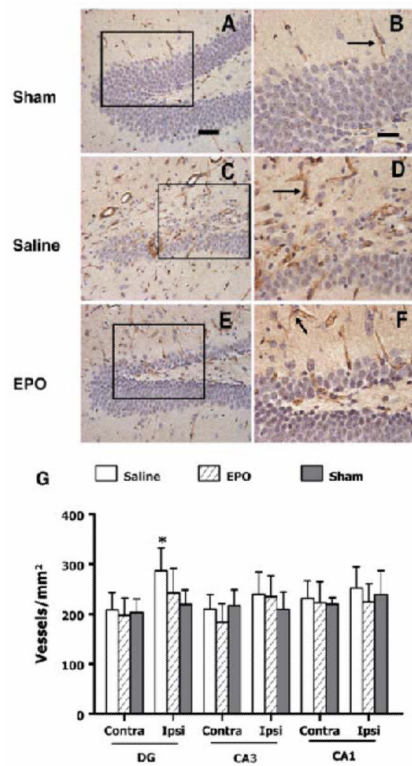


Fig. 11.

Effect of rhEPO on vWF-staining vascular structure in the hippocampus 35 days after TBI. TBI alone (C, D) significantly increases the vascular density (stained brown, arrow as an example) in the DG. rhEPO (E, F) does not have an effect on angiogenesis after TBI. Scale bar = 50 μ m (A, C, E); 25 μ m (B, D, F). The density of vWF-stained vasculature is shown in (G). Data represent mean \pm SD. * $p < 0.05$ vs. Contralateral N (mice/group) = 12 (EPO); 13 (Saline); 8 (Sham). Scale bar = 50 μ m.

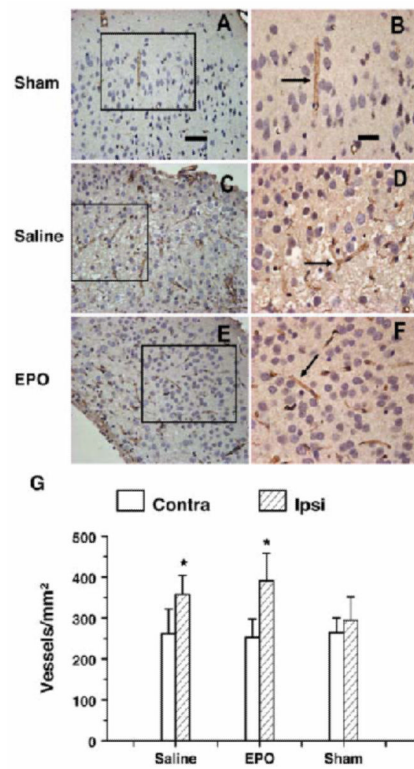


Fig. 12.

Effect of rhEPO on vWF-staining vascular structure in the cortex 35 days after TBI. TBI alone (C, D) significantly increases vascular density (stained brown, arrow as an example) in the cortex. rhEPO (E, F) does not have an effect on angiogenesis after TBI. Scale bar = 50 μ m (A, C, E); 25 μ m (B, D, F). The density of vWF-stained vasculature is shown in (G). Data represent mean \pm SD. * p < 0.05 vs. Contralateral. N (mice/group) = 12 (EPO); 13 (Saline); 8 (Sham).