Neuroprotective effects of erythropoietin pretreatment in a rodent model of transient middle cerebral artery occlusion

Laboratory investigation

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Object. There is an unmet clinical need to develop neuroprotective agents for neurosurgical and endovascular procedures that require transient cerebral artery occlusion. The aim in this study was to explore the effects of a single dose of recombinant human erythropoietin (rhEPO) before middle cerebral artery (MCA) occlusion in a focal cerebral ischemia/reperfusion model.

Methods. Twenty-eight adult male Wistar rats were subjected to right MCA occlusion via the intraluminal thread technique for 60 minutes under continuous cortical perfusion monitoring by laser Doppler flowmetry. Rats were divided into 2 groups: control and treatment. In the treated group, rhEPO (1000 IU/kg intravenously) was administered 10 minutes before the onset of the MCA ischemia. At 24-hour reperfusion, animals were examined for neurological deficits, blood samples were collected, and animals were killed. The following parameters were evaluated: brain infarct volume, ipsilateral hemispheric edema, neuron-specific enolase plasma levels, parenchyma histological features (H & E staining), Fluoro-Jade–positive neurons, p-Akt and total Akt expression by Western blot analysis, and p-Akt–positive nuclei by immunohistochemical investigation.

Results. Infarct volume and Fluoro-Jade staining of degenerating neurons in the infarct area did not vary between groups. The severity of neurological deficit (p < 0.001), amount of brain edema (78% reduction in treatment group, p < 0.001), and neuron-specific enolase plasma levels (p < 0.001) were reduced in the treatment group. Perivascular edema was histologically less marked in the treatment group. No variations in the expression or localization of p-Akt were seen.

Conclusions. Administration of rhEPO before the onset of 60-minute transient MCA ischemia protected the brain from this insult. It is unlikely that rhEPO pretreatment leads to direct neuronal antiapoptotic effects, as supported by the lack of Akt activation, and its benefits are most probably related to an indirect effect on brain edema as a consequence of blood-brain barrier preservation. Although research on EPO derivatives is increasing, rhEPO acts through distinct neuroprotective pathways and its clinical safety profile is well known. Clinically available rhEPO is a potential therapy for prevention of neuronal injury induced by transitory artery occlusion during neurovascular procedures.

Key Words • neuroprotection • rat • recombinant human erythropoietin • transient focal cerebral ischemia • vascular disorders

Abbreviations used in this paper: BBB = blood-brain barrier; BSA = bovine serum albumin; CBF = cerebral blood flow; CCA = common carotid artery; ECA = external carotid artery; EPO, EPOR = erythropoietin, erythropoietin receptor; GSK-3β = glycogen synthase kinase–3β; ICA = internal carotid artery; I/R = ischemia/reperfusion; MCA = middle cerebral artery; NF-κB = nuclear factor–κappa B; NSE = neuron-specific enolase; PBS = phosphate-buffered saline; PI3K = phosphatidylinositol 3–kinase; rhEPO = recombinant human EPO.

Transient vessel occlusion may be unavoidable during neurosurgical or endovascular procedures and might lead to brain damage. Unlike in stroke patients, to which most cerebral ischemia animal studies refer,19,28 in patients undergoing neurovascular procedures the onset of temporary artery occlusion can be planned in advance, allowing the best timing for preventive maneuvers or neuroprotective drug administration to be performed, even before the onset of ischemia.

The risk of brain infarction with temporary artery occlusion in patients undergoing clip ligation for cerebral aneurysm has been reported to be as high as 45%.16 Brain
tolerance to reperfusion injury depends on several features, such as the degree, size, location, and duration of the ischemia; presence of subarachnoid blood; timing of surgery; patient’s age; body temperature; genetic factors; and other comorbidities. Reperfusion injury is attributed to numerous events, including inflammatory response, oxidative stress, loss of blood-brain barrier (BBB) integrity, cerebral edema, and hemorrhagic transformation. Particularly, focal ischemia followed by reperfusion leads to severe damage to the BBB integrity, allowing water and macromolecules to cross into brain tissue as early as 20–45 minutes following permanent middle cerebral artery (MCA) occlusion. The detrimental edema further reduces focal blood flow, and induces cell necrosis and apoptosis. Furthermore, widespread interactions among BBB disruption, edema formation, and lower blood flow become a vicious cycle, which accelerates brain damage.

Erythropoietin (EPO), a 30.4-kD glycoprotein, is a natural hormone originally identified for its role in erythropoiesis and successfully used for anemia treatment in the last 2 decades. The finding that EPO and its receptor (EPOR) are expressed throughout the brain in glial cells, neurons, and endothelial cells suggested that EPO could have hematopoiesis-independent effects on the nervous system. Endogenously produced EPO and/or expression of EPOR gives rise to autocrine and paracrine signaling in different organs, particularly during hypoxia, toxicity, and injury conditions. In the brain, EPO mRNA levels have been shown to remain elevated for more than 24 hours during the duration of the hypoxic stimuli. Accordingly, following ischemic/hypoxic injury, dramatic changes have been reported in the expression of EPO and EPOR within and around infarcts in human brain regions. Erythropoietin has been shown to regulate a variety of cell functions such as ionic balance, neurotransmitter synthesis, and cell survival. Research has also shown that a high dose of systemically administered recombinant human EPO (rhEPO) crosses the BBB, leading to neuroprotective and neurotrophic effects. As a result, several trials are currently ongoing to identify possible benefits in the usage of rhEPO for specific clinical situations requiring neuroprotection.

To our knowledge, we present the first animal study based on an MCA ischemia/reperfusion (I/R) model aiming to investigate the potential benefits of a single dose of 1000 IU/kg intravenous rhEPO as a possible pretreatment against neuronal damage in neurovascular procedures that require transient cerebral artery occlusion. The selected dose and route has been used in several clinical trials with no identified additional safety concerns, and thus could be safely translated to the clinical setting.

**Methods**

A total of 28 adult male Wistar rats weighing between 240 and 340 g and housed under diurnal light conditions with unlimited access to food and water were used (14 per group). Animal care followed the recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for protection of experimental animals). The Institutional Animal Care and Use Committee approved all animal procedures. All efforts were made to minimize animal suffering and to reduce the number of animals used.

**Erythropoietin Administration**

A dose of 1000 IU/kg rhEPO (epoietin β, Hoffmann-La Roche) was administered intravenously in the tail vein 10 minutes before the onset of ischemia. The control group had the same volume of saline administered similarly.

**Middle Cerebral Artery Ischemia-Reperfusion**

Food was withheld from rats 12 hours prior to surgery. Anesthesia was induced by intraperitoneal administration of a ketamine (80 mg/kg) and xylazine (8 mg/kg) mixture, supplemented as needed. Anesthetized rats were placed on a thermostatically controlled heating pad, a rectal probe was inserted, and body temperature was monitored and maintained between 36.5°C and 37.5°C. Transient focal cerebral ischemia was induced by 60-minute right MCA occlusion followed by 24-hour reperfusion, as reported. Briefly, under the operating microscope, the right common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery (ECA) were exposed and isolated from branches through a midline neck incision. The ECA was tied and cut at approximately 5 mm from the bifurcation and a loose 6-0 silk knot was placed around the ECA origin. Afterward, microvascular clips were placed on the CCA and ICA, an ECA stump arteriotomy distal to the loose knot was performed, and a 4-0 nylon silicone monofilament with a rubber-coated tip (Doccol Corp.) was inserted. The suture that had been placed around the ECA stump and intraluminal nylon filament was tightened to prevent bleeding and the clips were removed. The filament was then gently introduced (19–21 mm) into the ICA to the level where the MCA branches out, until the laser Doppler signal decreased to less than 30% of baseline, occluding the right MCA at its origin at the circle of Willis. After a 60-minute period of ischemia the thread was cautiously removed, reestablishing the blood flow in the MCA. The ECA was permanently ligated at the level of bifurcation. Animals were allowed to recover and then were killed with an anesthetic overdose at 24 hours into reperfusion.

**Blood Flow Measurements**

Cortical cerebral blood flow (CBF) was monitored by laser Doppler flowmetry (PeriFlux 4000 System, Probe 407; Perimed Instruments) in the supply territory of the right MCA where transient ischemia was to be induced; CBF was measured before, during occlusion, and within 1 hour of reperfusion. A small burr hole was drilled 2 mm posterior to the bregma and 3.5 mm lateral to the midline, and the micro-Doppler probe was positioned above the dura mater in a holder glued to the bone. Steady-state baseline values were recorded before occlusion, and the
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CBF measured during occlusion and reperfusion was expressed as a percentage of the baseline values. Rats with CBF patterns suggesting subarachnoid hemorrhage, incomplete ischemia (CBF did not decrease to a maximum of 30% of baseline), or incomplete reperfusion (CBF did not recover to more than 80% of baseline within 30 minutes of filament withdrawal) were excluded and replaced.

Neurological Examination

At 24 hours, a 9-point-scale neurological test (0 = normal to 9 = highest handicap) was performed in the rats, as previously described. Four tests were performed by an observer who was blinded to the treatment groups to assess the following: 1) spontaneous activity (moving/exploping = 0, moving without exploration = 1, no displacement = 2); 2) laterality in movement (symmetrical = 0, left drifting when elevated by the tail = 1, spontaneous left drifting = 2, circling to the left without displacement or spinning = 3); 3) resistance to left forepaw stretching (no stretching allowed = 0, stretching allowed = 1, no resistance = 2); and 4) parachute reflex (symmetrical = 0, asymmetrical = 1, contralateral forelimb retracted = 2). Scores for each test were added to obtain the final neurological score.

Infarct Volume and Brain Edema Assessment

The brains were removed, placed in a brain matrix (World Precision Instrument), and sliced in 2-mm-thick coronal sections, beginning 2 mm from the frontal pole and ending rostral to the corticocerebellar junction, resulting in 6 slices per animal (8 rats per group). The sections were stained in 2% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) saline solution for 10 minutes at 37°C in the dark and fixed in 4% paraformaldehyde at 4°C overnight. Sections were scanned and analyzed using ImageJ software (version 1.45). Brain infarction was visualized as areas of unstained (white) tissue, which contrasted from brick-red–stained areas of viable tissue. Summing the infarct area of each coronal slice and multiplying that number by the thickness of the sections allowed the calculation of the total infarct volume. Right and left hemisphere volumes were calculated similarly. The amount of infarction was expressed in absolute terms in cubic millimeters and as a percentage of the infarct volume in the whole forebrain, adjusted for brain edema. An index of brain edema was assessed by calculating the percent increase of size of the ipsilateral (injured) hemisphere compared with the contralateral (uninjured) hemisphere. A single observer blinded to the individual treatment performed the analysis described.

Determination of Neuron-Specific Enolase Plasma Levels

Blood samples were taken by puncture of the left cardiac ventricle prior to killing. Blood was centrifuged at 10,000 rpm for 10 minutes and the isolated serum was frozen and stored until time of assay. Serum neuron-specific enolase (NSE) measurements were performed with an electrochemiluminescence immunoassay by using a sandwich technique with double monoclonal antibodies directed against NSE and an Elecsys 2010 analyzer (antibodies and apparatus both from Roche Diagnostics). Data were normalized to nanograms per milliliter of plasma.

Histological and Immunohistochemical Procedures

Rat brains were removed, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 72 hours at room temperature, dehydrated through a graded ethanol series, and embedded in paraffin (3 per group). Then, H & E staining was performed as previously described and images were acquired using a bright-field microscope (Axioskop, Zeiss).

For Fluoro-Jade staining, 6-μm-thick coronal sections were deparaffinized and rehydrated. Slides were first immersed in 100% alcohol for 3 minutes, followed by 1 minute in 70% alcohol and 1 minute in distilled water. The slides were then transferred to a solution of 0.06% potassium permanganate for 15 minutes on a shaker table and protected from light. After that the slides were rinsed in distilled water for 1 minute and immersed in Fluoro-Jade B (Chemicon) staining solution 0.001% for 30 minutes with moderate agitation. Slides were rinsed for 1 minute in each of 3 distilled water washes and dried at room temperature. The dry slides were cleared by immersion in xylene for at least 1 minute before coverslipping with DPX (Fluka or Sigma-Aldrich). The tissue was then examined using an epifluorescent microscope (Axioskop, Zeiss) with blue (450–490 nm) excitation light. The number of positive Fluoro-Jade B–positive neurons was counted in 4 sections of 0.16 mm² (3 per group) within the region of interest by using ImageJ software version 1.45 and the total was expressed as positive cells/section.

For immunostaining, 6-μm-thick coronal sections were submitted to antigen retrieval in 20 mM citrate buffer with 1.5% H₂O₂ for 15 minutes at room temperature in the dark, incubated for 10 minutes in Tris/EDTA buffer at 84°C, and blocked for 1 hour at room temperature in 1% bovine serum albumin (BSA) in PBS. Primary antibody, rabbit anti–p-Akt (1:100, Cell Signaling Technology) was used in 0.5% BSA in PBS overnight at 4°C. After washing in PBS, sections were incubated for 1 hour at room temperature with anti–rabbit antibodies coupled to AlexaFluor 568 (#A11077, 1:1000; Invitrogen) in 0.5% BSA in PBS, incubated for 20 minutes in DAPI, and mounted with Shandon Immu-Mount Aqueous Nonfluorescing Mounting Medium (Thermo Scientific). Tissue sections were visualized with an epifluorescent microscope (Axioskop, Zeiss), and the total number of nuclei (those with DAPI staining) and those positive for p-Akt were counted to present the results as percentage of p-Akt–positive nuclei (2 sections within the region of interest; 3 per group).

Western Blot Analysis

For Western blot analysis (3 per group), cells from frozen tissue samples were lysed in RIPA (radioimmunoprecipitation assay) buffer containing Tris 50 mM (pH 8.0), 5 mM EDTA (pH 8.0), 150 mM NaCl, 1% NP-40, 10% glycerol, and 0.1% sodium dodecyl sulfate, and sonicated for 20 seconds. The lysate was centrifuged at 14,000 g for 10 minutes at 4°C and the supernatants were collected and stored at ~80°C. Protein concentrations
were determined using Nanodrop ND-1000. Cell extracts containing equal amounts of protein (100–150 μg) were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were blocked with 5% nonfat milk, incubated with the primary antibodies overnight at 4°C (anti–rabbit p-Akt [1:1000, #12178; Cell Signaling], and anti–rabbit Akt [1:1000, #4691; Cell Signaling]), and then with a horseradish peroxidase-labeled secondary antibody for 1 hour at room temperature. After extensive washes, immunoreactive bands were detected by Lumiglo (Cell Signaling) and visualized by autoradiography with Hyperfilm ECL. Phosphorylation levels of Akt were analyzed by the ratio of p-Akt to total Akt levels and expressed as “-fold” change.

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism software, version 6.0. Parametric data were analyzed using the Student t-test for single comparisons between groups, and nonparametric data (neurological scores) were subjected to the 2-tailed Mann-Whitney test. Data are presented as the mean ± SEM for “n” observations, where “n” represents the number of animals studied. For histological scoring and Western blot analysis, each data point represents analyses of brain sections obtained in 3 individual rats. A p value of ≤ 0.05 was the threshold for a statistically significant difference or association.

**Results**

All animals lost between 10% and 18% of body weight during the 24-hour recovery period, with no significant differences between groups. Normothermia was maintained in all animals. There were no significant differences between groups with respect to CBF pattern and glycemia values during the procedure.

Results showed no difference between groups in the infarct volume. The total brain infarct volume was 265.46 ± 13.88 mm³ for the control group and 223.33 ± 12.97 mm³ for the treatment group. Expressed as a percentage,infarct volume was 28.02% ± 1.73% in the control group and 25.71% ± 1.41% in the rhEPO group (Fig. 1). Also, the number of positive Fluoro-Jade degenerating neurons in the area of interest was similar between groups (Fig. 2). Brain swelling index was significantly decreased in the treatment group when compared with the control group (2.11% ± 0.46% and 9.74% ± 1.39%, respectively; p < 0.001) (Fig. 3). Accordingly, histological analysis of the brain parenchyma (with H & E staining) revealed neuropil spongiosis and perivascular edema, which was less marked in the rhEPO-treated group (Fig. 4). The treatment group had significantly lower NSE plasma levels compared with the control group (1.17 ± 0.07 ng/ml compared with 1.88 ± 0.07 ng/ml; p < 0.001) (Fig. 5). This was a relevant parameter for assessing the prognosis of cerebral hypoxia-ischemia, and presented significantly reduced neurological deficits (p < 0.001) (Fig. 6). Although it is believed that EPO activates Akt, no differences were found between groups for the p-Akt/Akt ratio in Western blot analysis (Fig. 7) and for percentage of p-Akt–positive nuclei in immunohistochemical studies (data not shown).

**Discussion**

Our study reveals that rhEPO exerts protective effects in focal cerebral I/R. This is in line with the tissue-protective effects of rhEPO described in several animal models of I/R injury, as demonstrated by our research group. Recombinant human EPO has been consistent
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ly tested at high doses (> 5000 IU/kg) in rodent models of permanent cerebral artery occlusion, and has been shown to be more effective when administered within 6 hours after stroke onset.28 We investigated the effects of a single lower dose of rhEPO as a pretreatment (1000 IU/kg) in an I/R rodent model. The dose used has been safely tested in humans.9,13,29 Our results suggest that intravenous administration of a single 1000-IU/kg dose of rhEPO just prior to a transitory occlusion of a cerebral artery may decrease secondary brain insult, resulting in a better neurological outcome.

Regarding infarct volume outcome, we found no differences between groups at 24-hour reperfusion, although the improved neurological status and significantly lower NSE plasma levels observed at that point could indicate that this outcome may change over time between groups. Because NSE is an enzyme that is rapidly released by injured neurons, lower NSE plasma levels in the treatment group may suggest a smaller penumbra area and a better clinical outcome. Plasma levels reach their maximum at 24 hours, and the best correlation between infarct size and plasma levels occurs at Day 3.14 Plasma levels of this kinase have been found to be increased even before the onset of clinical symptoms, and are strongly correlated with the clinical score and histological damage when clinical symptoms are present.14 The NSE plasma levels probably reflect neuronal cell death, suggesting that NSE is a reliable marker when used to follow neuronal damage.1

There is evidence that EPO treatment activates the antiapoptotic molecule Akt.31,32 On phosphorylation, the serine-threonine kinase Akt promotes cell survival by inactivating several targets, including cell-death antagonist BCL-2, glycogen synthase kinase–3β (GSK-3β), and caspase-9, or else it activates prosurvival molecules,33 suggesting that the phosphatidylinositol 3–kinase (PI3K)/ Fig. 3. Bar graph depicting the percentage of hemispheric edema in both control and rhEPO-treated groups. Data represent the mean ± SEM (8 per group). Values were significant, representing a 78% reduction of ipsilateral hemispheric edema in the treatment group compared with control group. ***p < 0.001.

Fig. 4. Representative photomicrographs of brain sections obtained in control and rhEPO-treated rats. A: Whole-brain-section images with delineated infarct areas. B: Cerebrovascular changes in the infarct area: neuropil spongiosis (arrows) and perivascular edema (black-and-white arrowhead) in control animals or its absence (black arrowhead) in rhEPO-treated ones. Contralateral hemispheres had no relevant changes. H & E. Bar = 200 μm.
Akt/GSK-3β pathway is involved in the neuroprotective effect of rhEPO. Phosphorylation of Akt is coupled to its nuclear translocation where it phosphorylates nuclear targets, suppressing the transcription of death genes. Unexpectedly, our results did not show an increase in the expression of p-Akt/Akt (Western blot) or a p-Akt nuclear translocation (immunohistochemistry). The lack of PI3K/Akt/GSK-3β pathway activation could be explained by the use of lower rhEPO doses in our study when compared with previous ones. In fact, only up to 1% of systemically administered rhEPO crosses the BBB in primates, and poor penetration into the BBB is expected even when administering up to 10,000 IU/kg intravenously as single doses. This poor crossing ratio suggests that high doses of systemically administered rhEPO are needed for direct neuron-protective purposes.

On the other hand, Xiong et al. have previously demonstrated that EPO significantly provides neuroprotection following traumatic brain injury in EPOR-null mice; that is, even in the absence of EPOR in the neural cells, probably mediated through vascular protection. We postulate that the benefits observed in prophylactic rhEPO administration were not due to a direct neuronal action in the infarct area but were the result of an indirect effect on brain swelling as a consequence of diminished BBB disruption. This is also in line with the histological findings and lack of difference between treatment and control groups in the number of positive Fluoro-Jade cells that label degenerating neurons.

**Fig. 5.** Bar graph showing a 38% reduction of NSE plasma levels at 24 hours of reperfusion in the rhEPO-treated group. Data represent the mean ± SEM (n = 8 for control and 13 for treatment group). ***p < 0.001.

**Fig. 6.** Scatterplot showing the effects of rhEPO at 24 hours on a 9-point neurological scale (14 per group). Open circles indicate values for individual animals. Horizontal bars indicate group median values. The rhEPO significantly reduced neurological deficits. ***p < 0.001, Mann-Whitney test.

**Fig. 7.** Western blot showing expression of p-Akt and total Akt, with a bar graph showing the densitometric analysis of the relative intensity of p-Akt, normalized against total Akt. Data represent the mean ± SEM (3 per group). Results did not show differences between groups.
Cerebral edema is a well-recognized factor for high morbidity and mortality in large-territory ischemic strokes. Brain edema following MCA occlusion has been shown to increase gradually over the first 48 hours before peaking. The reduction in brain edema from the early phase may reduce the space-occupying effect and improve regional CBF in the penumbra phase of stroke. Here we suggest that rhEPO pretreatment is associated with reduced perivascular edema and with improved BBB from increased permeability. These effects are probably due to activation of an EPOR-dependent intracellular pathway of the microvascular endothelium, which may inhibit the transcription factor nuclear factor–kappa B (NF-κB), upregulate expression of tight-junction proteins, and improve abnormalities in the free-radical system.

The reperfusion phase leads to generation of reactive oxygen radicals and lipid peroxidation that are highly noxious to the brain’s capillary endothelial cells and its complex tight junctions, which are mostly responsible for the integrity of the BBB. Erythropoietin has been shown to increase nitric oxide synthesis in endothelial cells and, under oxidative stress conditions, nitric oxide may scavenge reactive oxygen species. Detoxification also prevents membrane lipid peroxidation and consequential additional disruption of the BBB in I/R. The NF-κB transcriptional activation pathway has been considered a central regulator of inflammatory response, critical to the regulation of apoptosis, and related to cell adhesion molecule expression in endothelial cells. Liu et al. suggested that the relation between the downregulation of NF-kB and the reversed expression of the tight junction–associated proteins is involved in the mechanism of protection of the BBB in rats with I/R injury.

We recognize some limitations on our study. We speculate that rhEPO promotes BBB integrity but we did not show direct evidence or quantify BBB permeability changes. Also, it would be interesting to perform time course experiments. These dynamics would be of both experimental and clinical relevance.

Nonerythropoietic tissue-protective EPO variants have been developed, most notably asialoEPO (AEPO), intranasal formulations of low-sialic-acid EPO (NeuroEPO), and carbamylated EPO (CEPO) to dissociate the erythropoietic effect from the tissue-protective effect, and thus lessen side effects. However, rhEPO possesses not only neuron antiapoptotic properties but also reduces BBB leakage, enhances blood flow, and promotes angiogenesis after brain injury. It is likely that these nonneuronal effects are not shared by EPO derivatives as part of EPO’s rescue effects after systemic intravenous delivery in patients. Moreover, the clinical safety profile is still under investigation in clinical trials and further research is needed before EPO variants could be used in patients.

Conclusions

We present the first evidence that rhEPO pretreatment at a dose of 1000 IU/kg, which has its safety profile in humans well described, reduces brain edema and preserves the penumbra functional neuronal pool following in vivo I/R injury. Considering that a substantial proportion of the ischemic lesion could be attributed to mechanical compression induced by brain swelling, the development of effective drugs to attenuate the formation and progression of brain edema is crucial. Because rhEPO has a higher innate capacity to cross the BBB in humans than in rodents, and given the neuroprotective effects observed in the present study, we believe that clinically available rhEPO could be a potential therapy to prevent neuronal injury induced by transient ischemia during neurovascular procedures. Further research is needed to completely understand the underlying mechanisms of rhEPO and BBB interactions. A translational clinical trial could be supported.

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Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: Ratilal, Rocha, Fernandes, Sepodes, Mota-Filipe. Acquisition of data: Ratilal, Arrojo, Rocha, Fernandes, Barateiro, Pinto. Analysis and interpretation of data: Ratilal, Arrojo, Rocha, Sepodes. Drafting the article: Ratilal, Arrojo, Rocha, Sepodes. Critically revising the article: all authors. Approved the final version of the manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Ratilal.

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