Neuroprotective Effect of Darbepoetin Alfa, a Novel Recombinant Erythropoietic Protein, in Focal Cerebral Ischemia in Rats

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Background and Purpose—Darbepoetin alfa is a novel erythropoiesis-stimulating protein developed for treating anemia. In animal models, exogenous recombinant human erythropoietin has been reported to be beneficial in treating experimental cerebral ischemia. In this study, we determined whether darbepoetin alfa would protect in a rat model of transient focal cerebral ischemia.

Methods—Rats received 2-hour middle cerebral artery suture-occlusion. The drug (darbepoetin alfa, 10 µg/kg) or vehicle was administered intraperitoneally 2 hours after onset of middle cerebral artery occlusion. Animals were allowed to survive for 3 or 14 days. Behavioral tests were performed sequentially. Infarct volumes and brain swelling were determined.

Results—Darbepoetin alfa-treated rats showed improved neuroscores relative to vehicle-treated animals beginning within 1 hour of treatment and persisting throughout the 14-day survival period. Darbepoetin alfa significantly reduced corrected total (cortical + subcortical) infarct volume (56.3±20.6 and 110.8±6.8 mm³, respectively) and total infarct areas at multiple levels compared with vehicle in the 14-day survival group. Brain swelling was not affected by treatment.

Conclusion—Darbepoetin alfa confers behavioral and histological neuroprotection after focal ischemia in rats. (Stroke. 2005;36:1065-1070.)

Key Words: brain edema ▪ cerebral ischemia ▪ focal ▪ middle cerebral artery occlusion ▪ neuroprotection

E rythropoietin (EPO) is a glycoprotein hormone that is the primary regulator of erythropoiesis.1 It binds to specific receptors on the cell surface of red blood cell precursors in the bone marrow, promoting their proliferation, differentiation, and survival, causing an increase in the circulating red blood cell mass.2 For more than a decade, recombinant human erythropoietin (rHuEpo) has been used to treat anemia associated with chronic renal failure, chemotherapy for cancer patients, and HIV infection, because of its ability to increase hemoglobin concentration, reduce the need for red blood cell transfusions, and improve symptoms associated with these conditions.3 EPO is neuroprotective in a variety of rodent models of hypoxic/ischemic central nervous system disorders when delivered directly into the brain4,5 or systemically.6 By contrast, 1 study showed increased cerebral infarct volumes in polycythemic mice overexpressing erythropoietin,7 suggesting that chronic overexpression of EPO might worsen outcome after stroke either because of the elevated hematocrit or other chronic effects.7

Darbepoetin alfa is a novel erythropoiesis-stimulating agent with additional sialic acid-containing oligosaccharides compared with EPO, and an extended circulating half-life and increased in vivo biological activity.8 Darbepoetin alfa has 2 additional N-glycosylation sites and up to 22 sialic acid moieties, extending its half-life in the serum 3-fold longer than that of rHuEPO.9 Darbepoetin alfa is now being used extensively to treat anemia associated with chronic renal insufficiency and chemotherapy.10,11 Because darbepoetin alfa activates the EPO receptor, we hypothesized that it should also confer neuroprotection in stroke.

Materials and Methods

Animal Preparation
Twenty-nine adult male Sprague–Dawley rats (277 to 339 grams; Charles River Laboratories, Wilmington, Mass) were fasted overnight but allowed free access to water. After atropine sulfate (0.5 mg/kg, intraperitoneally), anesthesia was induced with 3.5% halothane in a mixture of 70% nitrous oxide and 30% oxygen. Rats were orally intubated, immobilized with pancuronium bromide (0.6 mg/kg, intravenous), mechanically ventilated, and underwent insertion of femoral arterial and venous catheters. Rectal and cranial (left temporalis muscle) temperatures were separately monitored and held at normothermic levels. Arterial blood pressure was continuously monitored, and the rats were maintained at normothermic levels. The rats were randomly assigned to the darbepoetin alfa (10 µg/kg) or vehicle (saline) intraperitoneal injection treatment groups. Arterial blood pressure was continuously monitored, and the rats were maintained at normothermic levels.
monitored and blood gases, pH, glucose, and hematocrit were periodically measured as described previously.\footnote{12}

**Middle Cerebral Artery Occlusion**

The right middle cerebral artery (MCA) was occluded for 2 hours by the intraluminal-filament method using a poly-L-lysine–coated suture as previously reported.\footnote{12} The suture was introduced retrogradely into the right external carotid artery and advanced into the internal carotid artery and MCA, a distance of 20 to 22 mm from the carotid bifurcation according to the animal’s weight. The incision was closed and rats were awakened and examined at 1 hour. Rats not having a right upper extremity deficit were excluded from further study (see Behavioral Testing). After 2 hours of MCA occlusion, rats were re-anesthetized and the intraluminal suture was carefully removed. The neck incision was closed with silk sutures, and the animals were allowed to survive for 3 or 14 days with free access to food and water.

**Treatment**

The drug (darbepoetin alfa, 10 $\mu$g/kg) or vehicle (human serum albumin [0.25%], NaCl [140 mmol/L], and sodium phosphate [20 mmol/L], pH 6.0) was administered intraperitoneally at time of reperfusion, ie, 2 hours after onset of MCA occlusion. Four treatment groups were studied: 3-day survival (vehicle, 6; darbepoetin alfa-treated, 8) and 14-day survival (vehicle, 8; darbepoetin alfa-treated, 14). Rats were allocated to treatment groups in a randomized manner.

**Behavioral Testing**

Behavioral tests were performed in all 29 rats. In the 3-day survival groups, repeat testing was performed before MCA occlusion, during occlusion (at 60 minutes), and after treatment at 1 hour and 1, 2, and 3 days. In the 2-week survival groups, additional testing was performed at 7, 10, and 14 days. Examinations were performed by an observer blinded to the experimental condition. The battery, consisting of postural-reflex and forelimb-placing tests, yielded a scale of 0 to 12 (normal score = 0, maximal score = 12) as previously described.\footnote{12} Four rats with convulsions or sustained disturbances of consciousness were excluded from the study; most of these animals proved to have subarachnoid hemorrhage secondary to suture-induced arterial rupture.

**Infarct Assessment**

Animals were allowed to survive for 3 or 14 days. Brains were then perfusion-fixed, paraaffin-embedded, coronally sectioned at 10 $\mu$m, and sections stained with hematoxylin and eosin as previously described.\footnote{13} Sections were digitized at 9 standardized coronal levels (MCID image-analysis system; Imaging Research Corp, St. Catharines, Canada). An investigator blinded to the experimental groups used software developed by us to quantify infarct size and brain swelling.\footnote{13} We performed 3 histopathological analyses of infarction: (1) measurement of cortical and subcortical areas of infarction on a section-by-section basis in individual animals;\footnote{12} (2) traditional total infarct volume estimation by integrating infarct areas from selected section-by-section basis in individual animals;\footnote{12} (3) depiction of infarct frequency distribution by infarct volume estimation by integrating infarct areas from selected section-by-section basis in individual animals;\footnote{12} and (4) depiction of infarct frequency distribution by infarct volume estimation by integrating infarct areas from selected section-by-section basis in individual animals;\footnote{12} as previously described.\footnote{12} Four rats with convulsions or sustained disturbances of consciousness were excluded from the study; most of these animals proved to have subarachnoid hemorrhage secondary to suture-induced arterial rupture.

**Statistical Analysis**

Data are presented as mean values±SEM. Neurobehavioral scores and infarction size data were compared among treatment groups by repeated-measures analysis of variance (ANOVA) followed by Bonferroni tests. Physiological variables were compared by Student $t$ tests. Differences at $P<0.05$ were considered statistically significant.

**Results**

**Physiological Variables**

There were no significant differences with respect to rectal temperature, cranial temperature, arterial blood gases, arterial blood pressure, or arterial blood glucose (Table). Darbepoetin alfa therapy led to moderate increases in hematocrit (by 5% to 16%) compared with vehicle-treated animals during the first 7 days, but this tended to normalize between 7 and 14 days (Table).

**Neurological Assessment**

Neurological score was normal (0) in all animals before MCA occlusion. High-grade contralateral deficits (score, 11±0) were present at 60 minutes of MCA occlusion in all rats (Figure 1); thus, no animals required exclusion based on inadequate ischemia. A significant improvement in neurological score was evident in darbepoetin alfa-treated animals compared with vehicle-treated rats within 1 hour of treatment and was sustained at every observational point throughout the 3-day and 14-day survival periods (Figure 1). By repeated-measures ANOVA, the overall between-group treatment effect was highly significant ($P<0.0001$).

**Infarct Volume and Brain Swelling**

Cortical infarct areas were significantly reduced by treatment with darbepoetin alfa compared with vehicle at 4 coronal levels in the 14-day survival groups, but no intergroup differences were present in animals with 3-day survival (Figure 2A). Total cortical infarct volume was also significantly reduced by darbepoetin alfa compared with vehicle in the 14-day survival series (28.5±14.1 versus 68.0±4.5 mm$^3$, respectively; $P=0.04$), but not in the 3-day series. Striatal (subcortical) infarct areas were by reduced by darbepoetin alfa-treated rats at 1 or 2 coronal levels in both the 3-day and 14-day series (Figure 2B). Total striatal infarct volume was significantly reduced by darbepoetin alfa compared with vehicle in the 3-day survival group (28.9±5.3 versus 46.4±5.3 mm$^3$, respectively; $P=0.04$), and there was a nonsignificant trend at 14 days. Treatment with darbepoetin alfa significantly reduced corrected total (cortical plus subcortical) infarct volume by 49% (56.3±20.6 versus 110.8±6.8 mm$^3$, respectively; $P<0.05$; Figure 3) and reduced total infarct areas compared with vehicle-treated animals at 3 coronal levels in the 14-day but not the 3-day survival series. Brain swelling was not affected by treatment with darbepoetin alfa (3-day survival: vehicle 11±2% and darbepoetin alfa 9±1%; 14-day survival groups: vehicle –7±2% and darbepoetin alfa –2±2%).

Figure 4 displays pixel-based infarct frequency maps. Vehicle-treated animals showed a large cortical and subcortical infarct at multiple coronal levels. By contrast, the darbepoetin alfa-treated animals displayed marked reductions in the frequency of cortical and subcortical infarction in 14-day survival group.

Seven animals died during the experiment in the 3-day survival groups: 5 in the vehicle group (3 on day 1 and 2 on
day 2) and 2 in the darbepoetin alfa-treated group (on days 1 and 2). Eleven animals died in the 14-day survival groups: 9 in vehicle group (2 on days 1, 2, and 5, and 1 on days 6, 7, and 9) and 2 animals died in the darbepoetin alfa-treated group (on day 2). These animals were not included in the histological analysis. Autopsy revealed a large ipsilateral hemispheric infarct and extensive brain edema in all instances.

**Discussion**

The goal of our study was to determine whether the administration of darbepoetin alfa, a novel erythropoiesis-stimulating agent, was efficacious in protecting the brain after transient focal cerebral ischemia. Our results clearly demonstrate that this treatment improves outcome as measured by neurological score and by final pathological estimation of the size of infarction.

The present results show a beneficial effect of darbepoetin alfa in a well-controlled animal model of MCA occlusion. Intraluminal occlusion of the MCA has become increasingly popular as a focal ischemia model because of its relative simplicity and minimally invasive nature. In the present study and in recently published observations, we have used a poly-L-lysine-coated suture and have found that this technique leads to reliable and highly consistent results.

EPO is a glycoprotein that stimulates differentiation and proliferation of erythroid precursor cells, and hypoxic induction of EPO production increases numbers of red blood cells, leading to better oxygen supply to the tissues. In response to the systemic oxygen caused by decreased oxygen concentration after cerebral ischemia, EPO production is stimulated. It has recently been reported that both erythropoietin and its receptor (EPOR) are found in the human cerebral cortex and hippocampus and that in vitro, the cytokine is synthesized by astrocytes and neurons, has neuroprotective activity, and is upregulated after hypoxic stimuli. In animal models, rHuEpo has been reported to be beneficial in treating experimental global and focal cerebral ischemia and reducing nervous system inflammation. Sadamoto et al demonstrated that rHuEpo infused into the cerebral ventricles of stroke-prone spontaneously hypertensive rats with permanent MCA occlusion improved cognitive tests, reduced cortical infarction, and increased numbers of surviving thalamic neurons. In situ hybridization revealed that EPOR mRNA was upregulated at 24 hours in the ischemic penumbra after MCA occlusion. In addition, infusion of rhEPO into the lateral ventricles prevented ischemia-induced learning disability and rescued hippocampal CA1 neurons from global cerebral ischemic injury in gerbils.

<table>
<thead>
<tr>
<th>Physiological Variables</th>
<th>3-Day Survival</th>
<th>14-Day Survival</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>DP-Alfa</td>
</tr>
<tr>
<td></td>
<td>n=7</td>
<td>n=8</td>
</tr>
<tr>
<td>During MCA occlusion</td>
<td></td>
<td></td>
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<tr>
<td>Cranial temperature, °C</td>
<td>36.2±0.09</td>
<td>36.3±0.09</td>
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<tr>
<td>Rectal temperature, °C</td>
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<td>pH</td>
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<td>pO2, mm Hg</td>
<td>114±6</td>
<td>115±6</td>
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<tr>
<td>pCO2, mm Hg</td>
<td>38.1±0.7</td>
<td>39.8±0.7</td>
</tr>
<tr>
<td>MABP, mm Hg</td>
<td>116±7</td>
<td>111±6</td>
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<tr>
<td>Plasma glucose, mg/dL</td>
<td>126±6</td>
<td>138±8</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>43.4±1.3</td>
<td>43.9±0.9</td>
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<tr>
<td>After treatment</td>
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<td>Cranial temperature, °C</td>
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<td>36.8±0.30</td>
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<tr>
<td>Rectal temperature, °C</td>
<td>37.2±0.27</td>
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<tr>
<td>MABP, mm Hg</td>
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<td>115±2</td>
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<tr>
<td>Hematocrit, %</td>
<td>42.0±1.0</td>
<td>45.9±0.5*</td>
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<td>47.9±0.7*</td>
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<td>Day 3</td>
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<td>43.1±1.3</td>
<td>47.8±1.1*</td>
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<tr>
<td>Day 7</td>
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<tr>
<td>Hematocrit, %</td>
<td>45.0±2.0</td>
<td>51.4±1.6*</td>
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<td>Day 14</td>
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<td></td>
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<tr>
<td>Hematocrit, %</td>
<td>43.2±2.5</td>
<td>48.5±1.5*</td>
</tr>
</tbody>
</table>

MABP indicates mean arterial blood pressure; MCA, middle cerebral artery; DP-Alfa, darbepoetin alfa.

*Different from vehicle group (P<0.05, Student t test).
Darbepoetin alfa is a erythropoiesis-stimulating protein that produces a similar physiological response as rHuEpo. It was generated by site-directed mutagenesis of the erythropoietin gene, resulting in an increased number of glycosylation sites and greater carbohydrate content. In a recent report, darbepoetin alfa increased hematocrit levels, hemoglobin content, total red blood cells, reticulocytes, and platelet numbers, and also significantly reduced the degree of inflammation by as much as 80% in a model of experimental allergic encephalomyelitis in rats. Darbepoetin alfa was approved by the Food and Drug Administration for patients with chronic kidney disease in September 2001 and for chemotherapy-induced anemia in July 2002. In clinical trials of patients with renal failure, darbepoetin alfa was shown to have a 3-fold longer terminal half-life than rHuEpo (25.3 hours versus 8.5 hours). The longer half-life allows for less frequent dosing. In particular, in clinical studies this has translated into effectively maintaining the hemoglobin levels within the target range when administered monthly. Because rHuEPO treatment has been reported to improve stroke outcome in a variety of animal models, we wanted to determine whether darbepoetin alfa would also confer protection in a rat model of transient focal cerebral ischemia. There are currently no publications demonstrating neuroprotective effects of darbepoetin alfa.

Stroke in humans is commonly associated with impaired sensorimotor and cognitive function; ≈70% to 80% of all patients experience hemiparesis immediately after the insult. After MCA occlusion, rodents also exhibit a neurological deficit characterized by sensorimotor dysfunction. In the present study, administration of darbepoetin alfa significantly improved the neurological score compared with vehicle throughout the 3-day and 14-day survival periods. There were no adverse behavioral side effects observed with darbepoetin alfa administration. Histology in animals is a sensitive method of detecting neuroprotection. The present study shows that darbepoetin alfa confers histological neuroprotection when administered at 2 hours after the onset of MCA occlusion. Vehicle-treated animals showed a large cortical and subcortical infarct at multiple coronal levels. By contrast, darbepoetin alfa-treated animals showed significantly reduced cortical (by 58%) and total infarct volumes (by 50%) in the 14-day survival group. The 3-day survival group also showed reduction of subcortical...
cal infarction (by 27%). The protective effect of darbepoetin alfa in this study could not be explained by differences in body or brain temperatures, arterial pressure, or arterial blood gases because these variables were carefully controlled and did not differ among groups.

This study focused on whether darbepoetin alfa was neuroprotective in an in vivo model of focal cerebral ischemia in rats, and not on the mechanism(s) of action by which darbepoetin alfa could be neuroprotective. The most profound effect of darbepoetin alfa in this study was found in the 14-day survival series. Several mechanisms by which erythropoietic therapy may confer neuroprotection can be considered. Ehrenreich\(^{20}\) has recently emphasized that rather than modulating disease-specific pathogenic mechanisms, EPO may have more general tissue-protective effects by targeting different neurodegenerative pathways, such as anti-apoptotic, antioxidant, glutamate-inhibitory, anti-inflammatory, neurotrophic, stem cell-modulatory, and angiogenic mechanisms. In the time frame of 14 days after the ischemic stroke in this study, long-term protective effects of EPO may be considered, such as angiogenesis and neurogenesis. It has been reported that EPO, similar to other growth factors such as granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor, can stimulate endothelial proliferation and, in turn, new vessel formation both in vitro and in vivo.\(^{21}\) By driving the formation of new vessels, EPO might protect the ischemic brain by increasing the delivery of oxygen to brain tissue.\(^{22}\) Recently, Wang et al.\(^{23}\) showed that treatment with rHuEpo significantly improved functional recovery, along with increases in density of cerebral microvessels, increased numbers of BrdUrd-positive cells in the ipsilateral subventricular zone, and neuroblasts in the ischemic boundary regions. Direct evidence for the ability of EPO to induce neurogenesis was reported both in vitro and in vivo.\(^{24}\)

In summary, our results demonstrate the neuroprotective efficacy of darbepoetin alfa, a novel erythropoiesis-stimulating agent, in an in vivo model of temporary focal cerebral ischemia as judged by neurological score and infarct size. A pharmacological agent such as darbepoetin alfa thus may have potential utility in treating focal ischemic stroke in the clinical setting. A beneficial outcome in stroke patients treated with EPO has been already reported by Ehrenreich et al.\(^{25}\) In Ehrenreich’s proof-of-concept trial in patients with MCA territory ischemic stroke, EPO (33 333 U/50 mL) or placebo was administered intravenously after enrollment and was repeated 24 and 48 hours later. Neurological follow-up, scoring, and outcome scales at 30 days showed significantly

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**Figure 4.** Infarct frequency maps at 9 coronal levels (bregma +5.2 mm to −7.3 mm) in the 3-day (A, B, C) and in the 14-day survival groups (D, E, F). The color bars in (A), (B), (D), and (E) depict percentages of animals having infarction at each pixel location. C and F, Results of Fisher exact tests performed on a pixel-by-pixel basis to compare the vehicle-treated and darbepoetin alfa-treated groups; color scales denote \((1−p)\), where \(p\) is the significance level of intergroup difference. The lower threshold of the color bars has been set to 0.95 \((1−p)\), so that colored pixels are those with \(P<0.05\). The infarct is large in vehicle-treated animals but markedly reduced in darbepoetin alfa-treated animals in the 14-day series.
better results for the EPO-treated patients compared with placebo.

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