A Comparison of High-Dose Recombinant Erythropoietin Treatment Regimens in Brain-Injured Neonatal Rats

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ABSTRACT: Recombinant human erythropoietin (rEpo) is neuroprotective in neonatal models of hypoxic-ischemic brain injury. However, the optimal rEpo dose, dosing interval, and number of doses for reducing brain injury are still undetermined. We compared the neuroprotective efficacy of several subcutaneous rEpo treatment regimens. Seven-day-old rats underwent unilateral carotid ligation plus 90 min 8% hypoxia. Treatment began immediately after injury. Treatment regimens examined included 1, 3, or 7 daily subcutaneous injections of either 0 (vehicle), 2,500, 5,000, or 30,000 U/kg rEpo. Gross brain injury, neuronal apoptosis (TUNEL), and gliosis (glial fibrillary acidic protein) were assessed at 48 h or 1 wk post injury. Immunoreactive cells and brain injury were quantified for statistical comparison to vehicle controls. rEpo treatment reduced brain injury, apoptosis, and gliosis, in a dose-dependent U-shaped manner at both 48 h and 1 wk. Neither one injection of 2,500, seven injections of 5,000, or three injections of 30,000 U/kg rEpo were protective. Three doses of 5,000 and one dose of 30,000 U/kg rEpo were most protective at both time intervals. rEpo provides dose-dependent neuroprotection. Of the regimens tested, three doses of 5,000 U/kg was optimal because it provided maximal benefit with limited total exposure. (*Pediatr Res* 61: 451–455, 2007)

Neonatal brain injury continues to affect the lives of thousands of infants in the United States and worldwide. In fact, perinatal asphyxia accounts for 23% of deaths globally (1). Hypothermia following hypoxia-ischemia offers promise of improved neurodevelopmental outcomes, however, thus far, the therapeutic benefit has been modest (2). When strict entry criteria are defined, up to 60% of untreated infants exposed to moderate to severe perinatal hypoxia-ischemia die or have significant neurodevelopmental sequelae (3,4). Erythropoietin (Epo), a hematopoietic cytokine known best for its erythropoietic effects, has neuroprotective effects in many animal models of brain injury, including hypoxia-ischemia, trauma, and excitotoxic injury (5–12). Although many neuroprotective strategies appeared promising in animal models, most have failed clinically (often due to toxicity). In contrast, rEpo is a safe treatment for anemia (13) and, more importantly, the neuroprotective effects of rEpo have translated well

from bench to clinical trials for both amyotrophic lateral sclerosis (ALS) (14) and middle cerebral artery stroke (15).

Initial studies provided no evidence that the 37 kD charged glycoprotein rEpo could cross the blood-brain barrier (BBB) (16–18). Therefore, early rEpo neuroprotection experiments used either *in vitro* application (19,20) or direct intracerebral injection (21). More recently, robust neuroprotection was produced after systemic injection of high-dose rEpo (5,000 U/kg i.p.) (5). Although the neuroprotective rEpo dose is clearly above the anemia treatment range, there is no consensus as to the optimal neuroprotective dose, dose number, or interval. Experimental neuroprotection has been reported using systemic doses ranging from 1,000 to 30,000 U/kg rEpo, using single or multiple injections, and after administration at varying intervals (6,7,22,23). Given that high-dose rEpo is now understood to cross the BBB passively (5,24), a systematic comparison of dosing regimens is warranted.

We therefore compared the neuroprotective effects of several dosing regimens of systemic high-dose rEpo given after neonatal hypoxia-ischemia. We hypothesized that rEpo-induced neuroprotection would be dose-dependent. By this comparative approach, we sought to minimize side effects by identifying the optimal regimen that would use the lowest effective dose and the fewest injections. We compared the effects of different rEpo doses, dose intervals, and number of injections, on the extent of brain damage in animals killed 48 h or 1 wk following hypoxic-ischemic injury. These data indicate that, of the treatment regimens tested, three injections of 5,000 U/kg rEpo are preferred.

METHODS

Animals. Seven-day-old (P7) Sprague-Dawley rats were used for all experiments. Rats in litters of no more than 12 were housed at 21–23°C in an automatic 12-h light-dark cycle. Procedures were approved by the Animal Care Committee at the University of Washington.

Hypoxia-ischemia. Animals were maintained in a neutral-thermal environment before surgery, during anesthesia, after surgery, and during hypoxia. Under anesthesia (2.5% isoflurane), a 1-cm vertical neck incision was made, the right common carotid artery isolated and electrocauterized. Incisions were closed with clips and animals recovered for 15 min, and were then returned to the dam for 2 h. Animals were then exposed to hypoxia (humidified 8% O₂, 92% N₂, 90 min, 36°C).

Treatment groups. To examine the efficacy of rEpo (Procrit, Amgen Inc., Thousand Oaks, CA) treatment in P7 rats after hypoxic-ischemic injury, rats were assigned to groups and given subcutaneous (s.c.) rEpo or vehicle

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Abbreviations: Epo, erythropoietin; GFAP, glial fibrillary acidic protein; NeuN, neuron-specific DNA binding protein; rEpo, recombinant human erythropoietin

injections, beginning shortly after hypoxia, using several regimens which were 1, 3, or 7 daily s.c. injections of either 0 (vehicle), 2,500, 5,000 or 30,000 U/kg rEpo, and then rats were killed at 48 h or 1 wk post hypoxia, and gross injury, neuronal apoptosis (TUNEL), and gliosis (GFAP) were evaluated. Random group assignments were made immediately following hypoxia and treatments begun within 5 min. rEpo doses included 0, 2,500, 5,000, or 30,000 U/kg given by 0.1 mL s.c. injection in vehicle (0.1% BSA in 10 mM PBS, pH 7.4). Sex was determined by PCR amplification of a 270-bp region of the rat Y chromatin (PCR: 95°C × 10', 25 cycles [94° × 30 s + 56° × 30 s + 72° × 30 s], 72° × 1 min) using the primers 5' ACT GTT CAA GCA GTC AGC CG 3' and 5' CTC CAT GAA CTT GGG GTC 3'.

Gross brain injury. Animals were euthanized at 48 h or 1 wk after hypoxia with an overdose of Euthasol (0.1 mL i.p., 390 mg pentobarbital, and 50 mg phenytoin sodium/mL, Virbac, Ft. Worth, TX and perfusion fixed with 15 mL of PBS containing 1 U/mL of heparin followed by 15 mL of 10% buffered formalin. Brains were then removed, photographed, and gross injury scores assigned in a blinded manner, using an ordinal scale (0 = normal ipsilateral hemisphere, 1 = mild edema/atrophy with <25% lesion of ipsilateral hemisphere, 2 = moderate atrophy, 25–50% lesion, 3 = severe atrophy with cystic cavitation 50–75% and 4 = severe atrophy with cystic cavitation ≥75%) (25). Brains were later embedded in paraffin, cut into 5 µm sections and mounted.

Immunohistochemistry. Slides with coronal brain sections through the parietal cortex were dewaxed in xylene, sequentially rehydrated in ethanol, boiled in 10 mM citrate buffer, and stained for TUNEL, degenerating neurons and reactive astrocytes with Fluoro-Jade B (FJ B), NeuN, or GFAP. Apoptotic cells in the cortex were identified by immunofluorescent labeling of DNA double-stranded breaks using the Chemicon S7165 ApopTag Red TUNEL kit protocol (Chemicon International, Temecula, CA) and counterstained with Vectashield (Vector Laboratories, Burlingame, CA) DAPI mounting medium. Degenerating neurons and reactive astrocytes were labeled by bathing slides in 0.06% KMnO₄ [17 min room temperature (RT)], followed by FJ B (0.001% Chemicon AG310, 30 min, RT dark). Neurons were stained using a primary anti-NeuN antibody (1:500 Chemicon MAB377, 12 h, 4°C) and a matching secondary (1:200 Chemicon AP124B, 1 h, RT). Gliosis in cortex was identified by labeling reactive astrocytes using a primary GFAP antibody (1:500 z0334 DakoCytomation, 1 h, RT) and a biotinylated secondary (1:200 KPL 15-16-06, 1 h, 4°C). Color was precipitated using Vectastain ABC peroxidase (30 min), Fast DAB (Sigma Chemical Co.-Aldrich D-4168 5 min; Sigma Chemical Co., St. Louis, MO) and slides were counterstained with methyl green or hematoxylin.

Image analysis. Using light (200×) and fluorescent microscopy (400×), digital images of injured parietal cortical regions were collected. Four sections from each brain were evaluated with each label. Cell counts and sample areas were measured by blinded observers using Soft-Imaging Systems AnalySIS software. Fluorescent TUNEL-positive cells were rhodamine-labeled (red 530–550 nm) with the background cells labeled by DAPI (blue 360–370 nm). GFAP-positive cells were DAB-labeled and counted using a light microscope.

Statistical analysis. All replicates were averaged providing a single measure for each subject. Data were compared using ANOVA with post hoc testing, or Mann-Whitney *U* testing (nonparametric) as appropriate based on homogeneity of variance testing (Levene's test) using SPSS 13 statistical analysis software (SPSS Inc., Chicago, IL). Data from rEpo-treated animals killed 48 h after hypoxia were compared with control (vehicle-injected) animals killed 48h after hypoxia, and data from rEpo-treated animals killed 1 wk after hypoxia were compared with control (vehicle-injected) animals killed 1 wk after hypoxia, as described in Figure 2. Power calculations were based on data from previous work and are as follows: To detect a difference of 1.5 with an expected SD of 0.8, an *n* of 8 is required to achieve 80% power with an alpha of 0.05.

RESULTS

Two hundred and five rat pups underwent unilateral hypoxic-ischemia. Fourteen rats (6.8%) died from hypoxia before randomization and treatment, but no mortalities occurred after treatments. Table 1 lists the mean gross injury scores from all rats given vehicle or rEpo and killed at 48 h (*n* = 103) or 7 d (*n* = 88) post injury. All rEpo dosing regimens are combined together for an overall comparison of vehicle to rEpo. rEpo treatment significantly reduced gross injury at both time points, as indicated.

Table 1. Overall effect of rEpo on gross brain injury scores (mean ± SEM, *n*)

Treatment	48 h post injury	7 d post injury
Vehicle	2.0 ± 0.21, 34	2.1 ± 0.25, 33
rEpo*	1.1 ± 0.15, 69†	0.8 ± 0.16, 55†

* All rEpo dosing regimens collapsed together.

ANOVA: 2 × 2 *F*_{3,187} = 11.68, *p* < 0.001, *t* tests; † *p* < 0.001.

Unilateral gross brain injury, TUNEL-labeled apoptosis, and GFAP-labeled gliosis in the parietal cortex were evaluated 48 h and 1 wk after injury. Figure 1 contains serial photomicrographs showing the brain from a severely injured (score of 4) vehicle-treated animal killed 7 d post injury. Injury is evident in the ipsilateral hemisphere with cortical liquefaction and ventricle enlargement (H&E), cortical apoptosis and ventricle enlargement (H&E), cortical apoptosis

Figure 1. Characterization of the unilateral brain injury. These photomicrographs show histological staining of brain from serial coronal sections of a vehicle-treated neonatal rat killed 7 days after hypoxia-ischemia-insult. The stains are indicated in each panel. The top left panel shows an image (5×) of the hematoxylin and eosin-stained section for general orientation. Note the reduced volume and ventriculomegaly present in the injured hemisphere. The lower two left panels (20×) show staining of neurons (NeuN) and astrocytes (GFAP) in regions of the parietal cortex from adjacent sections as indicated. Note the absence of NeuN immunoreactivity in the injured cortex that corresponds to presence of GFAP staining. The right panels (200×) show labeling of apoptosis (TUNEL) and neuronal degeneration (Fluoro Jade B), along with NeuN and GFAP from serial sections. TUNEL-positive cells include both neurons and astrocytes.

Figure 2. Effect of rEpo dose on brain injury, apoptosis and gliosis. This graph illustrates the dose-dependent effects of rEpo treatment on the degree of neonatal brain injury measured in the injured hemisphere of P7 rat pups after an hypoxic-ischemic insult. Unilateral injury was produced by carotid ligation with hypoxia (8% O₂) exposure and groups were then treated with a range of rEpo dosing regimens, and killed at 48 h (left) or 7 d (right) after injury. The dose and number of doses are listed on the x axis. Gross brain injury (top panels) was blindly scored from digital images. Coronal sections of parietal cortex were immunostained for apoptosis (TUNEL, middle panels) and for gliosis (GFAP, bottom panels). Image analysis was performed to count the immunoreactive cells/area. Data shown are means ($\pm$ SEM) and significant Mann-Whitney *U* comparisons to vehicle controls (0) are indicated (**p* < 0.05, ***p* < 0.01, ‡*p* < 0.001). Treatment groups were included in this figure only if *n* $\geq$ 8.

(TUNEL), neuronal death in the striatum and cortex (FJ B), disruption of neuronal columnar layering in cortex (NeuN), and widespread reactive astrogliosis (GFAP).

Figure 2 compares the neuroprotective efficacy of the individual treatment regimens on unilateral gross brain injury, apoptosis, and gliosis in the parietal cortex at 48 h (*n* = 97) and 7 d (*n* = 83) post injury. In this series of experiments, a single injection of 2,500 U/kg rEpo did not provide neuroprotection at 48 h (mean $\pm$ SEM: injury, 2.1 $\pm$ 0.6; TUNEL, 1190 $\pm$ 391; GFAP, 287 $\pm$ 101; *n* = 10). Although three injections of 2,500 U/kg rEpo did not prevent injury at 48 h, this regimen did reduce gross injury and gliosis at 7 d. A single 5,000 U/kg dose of rEpo reduced injury and gliosis at 48 h. The greatest effects were evident for three injections of 5,000 and one injection of 30,000 U/kg rEpo. These regimens substantially reduced gross injury, apoptosis, and gliosis to a similar degree at both time points. In contrast, seven injections of 5,000 U/kg rEpo administered over a week did not decrease brain injury, apoptosis, or gliosis. Similarly, three injections of 30,000 U/kg rEpo were not protective at either time point: at 48 h (mean $\pm$ SEM: injury, 1.7 $\pm$ 0.3; TUNEL, 896 $\pm$ 391; GFAP, 368 $\pm$ 141; *n* = 6), and 1 wk (mean $\pm$ SEM: injury, 0.7 $\pm$ 0.3; TUNEL, 623 $\pm$ 607; GFAP, 144 $\pm$ 147; *n* = 5). These data are not included in Figure 2 due to small group sizes.

A separate analysis to examine whether outcomes differed between males and females was conducted. There were no significant differences in the initial body weights of male and female rat pups at P7 before the experiment (average: 15.1 g male *versus* 14.8 g female). At death, there were no differences in weight gain due to treatment or sex. The growth data (change in weight from P7 until death 48 h or 1 wk post injury) for each treatment group are presented in Table 2 with male and female weights listed separately. There were no effects of sex on injury or response to treatment for any measure. Therefore, data from male and female rats have been combined together.

DISCUSSION

This report profiled the efficacy of several subcutaneous high-dose rEpo treatment protocols to help identify the optimal experimental neuroprotection regimen. We tested doses ranging from 2,500 to 30,000 U/kg, with one to seven doses given once per day over a timeframe ranging from 1 to 7 d after brain injury. We confirm that high-dose rEpo given to P7 rats soon after hypoxic-ischemic injury is neuroprotective. The neuroprotective effects of rEpo are dose dependent and U shaped. At lower doses (2,500 and 5,000 U/kg s.c.), we found three injections to be more effective than one, and increasing

Table 2. Mean rat weight change ($g \pm SEM$, n) by treatment group and sex

rEpo Regimen (doses $\times$ U/kg)	Male	Female
Killed 48 h after injury		
0	2.4 ± 0.5 , 14	2.9 ± 0.5 , 20
$1 \times 2,500$	3.0 ± 0.4 , 5	2.3 ± 1.6 , 5
$3 \times 2,500$	3.1 ± 0.6 , 4	1.9 ± 0.4 , 4
$1 \times 5,000$	1.7 ± 0.9 , 3	3.2 ± 0.6 , 7
$3 \times 5,000$	2.6 ± 0.6 , 9	2.7 ± 0.6 , 8
$1 \times 30,000$	1.4 ± 0.7 , 8	2.5 ± 0.8 , 10
$3 \times 30,000$	0.4 ± 1.3 , 4	3.1 ± 1.1 , 2
Killed 7 d after injury		
0	13.4 ± 0.7 , 16	13 ± 0.9 , 17
$3 \times 2,500$	12.8 ± 0.8 , 7	12.8 ± 1.4 , 3
$3 \times 5,000$	16.7 ± 1.6 , 5	16.1 ± 1 , 15
$1 \times 30,000$	13.7 ± 2.1 , 6	15.8 ± 1.3 , 4
$7 \times 5,000$	12.9 ± 1.3 , 5	11.85 ± 1.0 , 5
$3 \times 30,000$	20.2 ± 1.9 , 2	14.4 ± 3.8 , 3

There were no treatment or sex differences so no post hoc tests were warranted.

length of treatment to 7 d with a large net dose of 35,000 U/kg ($7 \times 5,000$ U/kg/wk) produced no benefit. At the highest dose (30,000 U/kg), one injection had neuroprotective effects at both 48 h and 1 wk, whereas three did not. One limitation of this study is that small numbers of subjects were used in the groups testing multiple injections of 30,000 U/kg at 48h and 1 wk. Our data showing no benefit at the highest rEpo doses is consistent with a recent study published by Weber *et al.* (26), in which extremely high-dose rEpo (40 U/mL) had neurotoxic effects using *in vitro* neuronal culture and brain slice models of mild hypoxia. This was also apparent *in vivo* when neonatal rats with mild brain injury were treated with $2 \times 20,000$ U/kg i.p. Further testing of rEpo in the developing CNS is clearly needed, particularly to test the long-term neurodevelopmental outcomes of such high doses. It is reassuring, however, that more moderate rEpo doses in neonatal rats (2,500 or 5,000 U/kg) show no such toxicity. We have recently tested the long-term effects of high-dose rEpo (0, 2,500, or 5,000 U/kg) in neonatal rats (27). rEpo treatment transiently raised the hematocrit in treated animals and prevented hypoxia-induced delays in geotaxis and growth as well as hypoxia-ischemia-induced learning impairment and substantia nigra neuron loss. No adverse long-term behavioral, structural, or immunohistochemical effects were identified in adult rats that had undergone neonatal rEpo treatment.

Epo receptors are widespread throughout fetal brain (28). Epo receptor binding initiates a signaling cascade that involves multiple antiapoptotic pathways that can account for the rEpo-induced reduction in TUNEL (29). Three of the antiapoptotic pathways associated with Epo are 1) the Janus kinase (JAK)–cGMP-dependent protein kinase (PKG)-mediated activation of protein kinase B (Akt-1), 2) the JAK-signal transducer and activator of transcription 5 (STAT5)-mediated up-regulation of antiapoptotic Bcl-XL gene products, and 3) the JAK-mitogen-activated protein kinase (MAPK)-mediated inhibition of caspases. More generally, in brain, Epo is released by astrocytes (30), triggers dopamine release (14), promotes neurogenesis (31,32), vasculogenesis

(33) and stimulates proliferation of glia (34). Thus, Epo is recognized to have both trophic and antiapoptotic mechanisms of action.

While our systematic approach to testing the efficacy of several rEpo dosing regimens has provided valuable data that can be used to optimize treatment, more complete information about rEpo pharmacokinetics and the nature of hypoxia-ischemia-induced insult is needed before the optimal dosing strategy may be fully defined. For example, the pharmacokinetics of low-dose rEpo used to treat anemia are well documented, but complete kinetic data are still undetermined for high-dose rEpo. It is known that within 2.5 h after intravenous injection of a high dose, approximately 1% of circulating rEpo becomes detectable in cerebrospinal fluid (24). The differences in rEpo pharmacokinetics given by different routes are also as yet undefined. rEpo neuroprotection has been documented with both subcutaneous and intraperitoneal dosing. Furthermore, rEpo clearance from brain has not been characterized. Also, because hypoxia-ischemia-induced brain damage is progressive (35), with ongoing cortical apoptosis and reactive astrogliosis, it will be important to determine whether additional late dosing or adjunctive therapy is effective. Currently, our data suggest that early repeated subcutaneous treatment provides more benefit than prolonged treatment.

Because there is growing interest in identifying whether sex differences in neonatal vulnerability to hypoxia-ischemia exist, we performed a post hoc analysis to compare outcomes between males and females. In this experiment, no sex-specific differences in growth or injury were identified. This observation may be due to the small number of animals in some of the groups, however, even when adequate numbers were available for comparison within a group, no differences were noted. Since this study was not designed to test the gender hypothesis, no conclusions are warranted. To date, the literature regarding the effect of gender on brain injury and repair has been contradictory. For males, androgens improve developing neuron survival in the hypothalamus (36,37) and visual cortex (38). Male neonatal mice injured by unilateral carotid artery ligation also respond preferentially to the neuroprotective effects of dextromethorphan (39). In contrast, hypoxia triggers IGF-1 expression only in developing females, and estrogen reduces brain injury in adult animal models of stroke (40,41). Neonatal males exhibit more brain injury than females after intracerebral injection of a vasoconstrictor (42), but male response to an injury caused by focal cerebral ischemia is similar to females (43). Indeed, whereas male and female rats sustain equivalent injury following focal cerebral ischemia, when evaluated 6 and 12 wk after injury, the female rats showed a better response to rEpo neuroprotection than did the males (43). Thus, the potential sex-specific differences in neonatal vulnerability to brain injury or repair from brain injury remains unanswered.

In summary, we have systematically compared the degree of neuroprotection offered by several subcutaneous high-dose rEpo treatment regimens as a step toward development of an optimal neurotherapeutic regimen. Of the regimens we tested, three injections of 5,000 U/kg s.c. and one injection of 30,000 U/kg s.c. conferred equivalent neuroprotection. Further in-

creases in dose or length of treatment provided no benefit, suggesting a potential upper safety limit of high-dose rEpo administration. Based on these findings we conclude that of the regimens we tested, three doses of 5,000 U/kg s.c. rEpo is optimal because it limits total drug exposure and provides maximal benefit.

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