Nanoerythropoietin Is 10-Times More Effective Than Regular Erythropoietin in Neuroprotection in a Neonatal Rat Model of Hypoxia and Ischemia

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Background and Purpose—Erythropoietin (EPO) has been demonstrated to possess significant neuroprotective effects in stroke. We determined if the nano-drug form of human recombinant EPO (PLGA-EPO nanoparticles [PLGA-EPO-NP]) can enhance neuroprotection at lower dosages versus human recombinant EPO (r-EPO).

Methods—Established neonatal rat model of unilateral ischemic stroke was used to compare r-EPO, PLGA-EPO-NP and phosphate-buffered saline, given by daily intraperitoneal injections, followed by infarction volume and Rotarod Performance Test assessment.

Results—PLGA-EPO-NP significantly reduced infarction volumes 72 hours after injury compared with the same concentrations of r-EPO. Functional deficits were significantly reduced by 300 U/kg PLGA-EPO-NP versus controls, with deficit attenuation apparent at significantly lower dosages of PLGA-EPO-NP versus r-EPO.

Conclusions—PLGA-EPO-NP is neuroprotective and beneficial against deficits after brain ischemia, at significantly reduced dosages versus r-EPO. (Stroke. 2012;43:884-887.)

Key Words: erythropoietin • focal ischemia • functional recovery • hypoxia • neonatal

E rythropoietin (EPO), a key endogenous cytokine in hypoxic physiological response, enhances oxygen delivery and attenuates brain injury in vitro and in vivo,1–5 promoting cell survival via the bcl antiapoptotic gene subfamily6 via limited-carrier blood–brain barrier passage, delaying critical timing in clinical stroke.3,7 Nano-medicine enhances brain drug delivery8 up to 50 times,9 bypassing usual blood–brain barrier routes and increasing clinical viability. With successful demonstration of significant neuroprotective function after systemic administration, it was noted that elevated doses of EPO were needed for optimal protection against brain injury for EPO to pass in substantial doses across the blood–brain barrier. EPO, a large glycosylated molecule, is unable to pass directly across the blood–brain barrier, requiring specific carrier transport and/or endocytosis.10 This type of transport is limited and critical time points in clinical management may be lost during the delay of EPO brain entry after systemic administration. Because recombinant EPO (r-EPO) has been previously demonstrated to have long-term beneficial effects on the brain,11 the purpose of this study was to compare the exact same r-EPO in nano forms and non-nano forms for the relative effects on infarction and behavior at different concentrations.

Materials and Methods

Human r-EPO, sharing 80% homology with rodent EPO, without report of immunologic complications,5,12 was epoetin-alfa (Centocor Ortho Biotech). Poly-DL-lactide-coglycolide (PLGA; 3.07 g) and fluorescein in 30 mL of acetonitrile with 0.0408 g 1-ethyl-3-(3-Dimethylaminopropyl)-carbodiimide hydrochloride underwent 24 hours of incubation, lyophilization, precipitate centrifugation, and water washing. PLGA-EPO nanoparticles (PLGA-EPO-NP) nanoprecipitation uses r-EPO double-emulsion solvent evaporation, primarily w/o emulsion of first aqueous phase (EPO 200 μL) with sonication of organic phase 100 mg PLGA in ethyl acetate (5 mL), emulsification with secondary aqueous phase (20 mL polyvinyl acetate; 1.5% weight/volume phosphate-buffered saline) forming secondary water-in-oil-in-water emulsion, and continuous stirring at 1800 rpm. Nanoparticle washing followed frozen dryer evaporation by ethyl acetate and water.

Perinatal Hypoxia-Ischemia Exposure Model

Procedures followed those of Rice-Vannucci12 with approval by the Loma Linda University Institutional Animal Care and Use Committee. Thirteen litters of Sprague-Dawley dams (Harlan Laboratories, Livermore, CA) at 10 days postnatal age (n=156) under isofluorane 1%, 0.7 L/min room air, 300 mL/min O2, and perioperative 38°C warming underwent right common carotid artery 7-0 silk suture ligation (Ethicon) around microscissor transection, all completed at 4 minutes, minimizing anesthesia.13 After 1-hour recovery, mice underwent 75 minutes at 3.5 L/min humidified 8% O2, 92% N, and 36°C, preceded by 75 minutes of 4.0 L/min; 12.8% (20 of 156) of pups died during hypoxia.

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Experimental Groups
r-EPO and PLGA-EPO-NP, in 10 mmol/L phosphate-buffered saline and 0.1% bovine serum albumin were injected intraperitoneally 1 hour after hypoxia and during 2 24-hour intervals, and were randomly assigned across 9 groups (n=8): vehicle; 30 U/kg r-Epo (n=7) or PLGA-EPO-NP; 100 U/kg r-Epo or PLGA-EPO-NP; 300 U/kg r-Epo or PLGA-EPO-NP; 5000 U/kg r-Epo and sham.

Seventy-two hours after injury, 2-mm brain slices in triphenyltetrazolium chloride at 37°C were analyzed by Image J (National Institutes of Health, Bethesda, MD).

For longer-term evaluation of comparative effect of nano forms and original forms of the same r-EPO, Rotarod 21 days after injury (n=8; Figure 2A, B), from 5 rpm (Figure 2A), 300 U/kg r-EPO, and PLGA-EPO-NP outperformed control (P=0.009 and P=0.004), with 300 U/kg PLGA-EPO-NP (41.5 s) approaching 5000 U/kg r-EPO (41.9 s). From 10 rpm (Figure 2B), 300 U/kg PLGA-EPO-NP outperformed 300 U/kg r-EPO (P=0.011), approximating 5000 U/kg r-EPO and sham (P=0.508 and P=0.214).

Brain weight ratios (ipsilateral/contralateral side to injury) 28 days after injury (n=8) showed 300 U/kg PLGA-EPO-NP (Figure 3) approximated 5000 U/kg r-EPO. Three-hundred U/kg PLGA-EPO-NP and 5000 U/kg r-EPO reduced loss (15.1% and 12%) versus control (35.2%; P<0.001).

Results
Infarction Volumes
Thirty U/kg PLGA-EPO-NP (Figure 1A) averaged 20.2% versus vehicle (29.4%) and 30 U/kg r-EPO (29.5%; P=0.092); 100 U/kg PLGA-EPO-NP (12.6%) outperformed 100 U/kg r-EPO and vehicle (P<0.001; Figure 1B); and 300 U/kg PLGA-EPO-NP (7.5%) outperformed control and 300 U/kg r-EPO (P<0.001; Figure 1C), approximating 5000 U/kg r-EPO (6.8%; P=0.574). Rotarod 21 days after injury (n=8; Figure 2A, B), from 5 rpm (Figure 2A), 300 U/kg r-EPO, and PLGA-EPO-NP outperformed control (P=0.009 and P=0.004), with 300 U/kg PLGA-EPO-NP (41.5 s) approaching 5000 U/kg r-EPO (41.9 s). From 10 rpm (Figure 2B), 300 U/kg PLGA-EPO-NP outperformed 300 U/kg r-EPO (P=0.011), approximating 5000 U/kg r-EPO and sham (P=0.508 and P=0.214).

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Discussion
Intravenous 100 000 IU r-EPO proved therapeutic in clinical stroke. Capillary sludging risk prompted studies of EPO,
novel EPO forms, and EPO receptors to develop neuroprotective forms lacking hematopoietic functions. Nanoformulation stabilizes EPO and facilitates blood–brain barrier crossing with controlled release, improving efficacy. PLGA-EPO-NP was effective at 16-times lower dosage (300 U/kg) than 5000 U/kg EPO. PLGA-NP delivery includes siRNA, proteins, antibodies, antibiotics, and cancer treatments. The present study demonstrates that the beneficial effects of EPO are enhanced at lower dosages when loaded to a polymeric NP carrier. EPO has been previously demonstrated to offer neuroprotective value against several types of brain injury, including ischemic stroke, neuronal degeneration, and apoptosis. Presently, dosages of up to 400 IU/kg EPO are administered as clinical treatment for neonatal anemia. Elevated-dose r-EPO has been successfully used in a clinical acute stroke trial with significant long-term recovery benefit seen at 1 month involving intravenous administration of 100 000 U of r-EPO over 3 days.

Figure 2. A and B, Twenty-one days after injury (n=8), 5 m/s starting: 300 U/kg recombinant erythropoietin (r-EPO) and poly-DL-lactide-coglycolide–erythropoietin nanoparticles (PLGA-EPO-NP) outperformed vehicle (P=0.009 and P=0.004); 10 m/s starting: 300 U/kg PLGA-EPO-NP outperformed 300 U/kg r-EPO; 300 U/kg PLGA-EPO-NP (33.4 s) approximated 5000 U/kg r-EPO (36.8 s) and sham (39.9 s). P=0.508 and P=0.214.

Figure 3. Twenty-eight days after injury. Y-axis: hemispheric weight ratios of ipsilateral vs contralateral to injury (n=8); 300 U/kg PLGA-EPO-NP and 5000 U/kg r-EPO (15.1% and 12%) outperformed control (35.2%).

Conclusions
In summary, the benefits we have observed here are the same as those of r-EPO previously published in the literature, except that in this case the same r-EPO has an attached nano-carrier, PLGA-EPO-NP, which allowed for observation of the beneficial effects at much lower dosages of the same r-EPO, with 300 U/kg PLGA-EPO-NP having significant effect comparable to 5000 U/kg without the nano-carrier.

Disclosures
None.

References
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