Erythropoietin (EPO), a key endogenous cytokine in hypoxic physiological response, enhances oxygen delivery and attenuates brain injury in vitro and in vivo.1–5 Creating cell survival via the bcl-2 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 in clinical management may be lost during the delay of EPO.

Conclusions—PLGA-EPO-NP is neuroprotective and beneficial against deficits after brain ischemia, at significantly lower dosages versus r-EPO.

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

Materials and Methods

Human r-EPO, sharing 80% homology with rodent EPO, without report of immunologic complications,7–12 was epoetin-alfa (Centocor Ortho Biotech). Poly-DL-lactide-coglycolide (PLGA; 5.07 g) and fluoresceinamine 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), emulsionification 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.

Received August 24, 2011; accepted September 26, 2011.

From the Departments of Physiology and Pharmacology (H.C., F.S., M.B., W.B.R., A.F., J.T., J.H.Z.), Department of Neurosurgery (J.H.Z.), Department of Anesthesiology (J.H.Z.), Loma Linda University, Loma Linda, CA; and Center of Excellence for Infectious Disease (H.D.), Department of Biomedicine Sciences, Texas Tech University Health Sciences Center, El Paso, TX.

Correspondence to John H. Zhang, MD, PhD, Department of Neurosurgery, Loma Linda University, Loma Linda, CA 92324. E-mail Johnzhang3910@yahoo.com

© 2011 American Heart Association, Inc.

Stroke is available at http://stroke.ahajournals.org

DOI: 10.1161/STROKEAHA.111.637090
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 = 1100, 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, Rotarod14 (9 groups of 8 animals) 21 days after injury involved analysis of 3 falling latency trials accelerated from 5- or 10-rpm velocities. Data expressed as mean±standard error of the mean were analyzed using SigmaStat (SyStat), with P<0.05 as statistically significant.

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). 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).

Discussion

Intravenous 100 000 IU r-EPO proved therapeutic in clinical stroke.15 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.

**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**

Nanoerythropoietin Is 10-Times More Effective Than Regular Erythropoietin in Neuroprotection in a Neonatal Rat Model of Hypoxia and Ischemia
Han Chen, Frédéric Spagnoli, Michael Burris, William B. Rolland, Adriel Fajilan, Huanyu Dou, Jiping Tang and John H. Zhang

Stroke. 2012;43:884-887; originally published online December 8, 2011;
doi: 10.1161/STROKEAHA.111.637090
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/43/3/884

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/