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Beneficial Effect of Erythropoietin on Sensorimotor Function and White Matter After Hypoxia-Ischemia in Neonatal Mice

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ABSTRACT: There are mixed reports on the neuroprotective properties of erythropoietin (EPO) in animal models of birth asphyxia. We investigated the effect of EPO on short- and long-term outcome after neonatal hypoxic-ischemic (HI) brain injury in mice and compared the effect of two different dose regimens of EPO. Nine-day-old mice were subjected to HI, and EPO was injected i.p. at 0, 24, and 48 h after HI in a dose of either 5 or 20 kU/kg. Paw preference in the cylinder rearing test (CRT) was used as a measure of sensorimotor function. Only in female mice, administration of EPO at 5 kU/kg but not 20 kU/kg improved sensorimotor function, reduced striatum atrophy and hippocampal lesion volume, and enhanced myelin basic protein (MBP) staining as determined at 4 and 9 wk after HI. In addition, at 72 h after HI, more Ki67 cells were found in the subventricular zone and dentate gyrus after EPO 5 kU/kg treatment, indicating an increase in progenitor cell proliferation. In conclusion, EPO improves sensorimotor function after neonatal HI and protects against striatum atrophy, hippocampus injury, and white matter loss. The protective effect of EPO is dose-dependent and only present in females. **(*Pediatr Res* 69: 56–61, 2011)**

Neonatal hypoxic-ischemic (HI) brain injury occurs in 1–4 per 1000 live-born infants and is an important cause of cerebral palsy, epilepsy, and adverse developmental outcome (1,2). Experimental studies in newborn animals with HI showed that antioxidative, anti-inflammatory, and neurotrophic agents are neuroprotective (3,4). However, in clinical studies with newborns with HI encephalopathy, only mild hypothermia showed a modest neuroprotective effect if started within 6 h after birth in asphyxiated term newborns with moderate encephalopathy (5,6).

Erythropoietin (EPO), a glycoprotein primarily recognized as a mediator promoting maturation and proliferation of erythroid progenitor cells (7,8), is an attractive drug for this purpose. EPO has been proven to cross the blood-brain barrier after systemic administration at a high dose and to reduce free radical formation and proinflammatory and apoptotic activity in models of brain damage (8–11). EPO has also been shown to stimulate formation of new neurons because of its actions as a neurotrophic factor (12,13). The effects of EPO are mediated by binding of EPO to its receptor (EPOR), which is present in the brain at relatively high levels in regions known to be sensitive to hypoxia (14,15). Most studies in neonatal animals treated with EPO after HI indeed showed an improved histological outcome (8,16), although our study in rats with neonatal HI brain damage showed no histological improvement (17). However, Spandou *et al.* (18) reported that in a similar rat model, using a shorter duration of hypoxia, EPO induced effective regeneration of brain tissue.

In human studies, no adverse effects of EPO treatment have been reported indicating that EPO is a safe drug for neonates (19). EPO has been increasingly used in preterm infants to treat neonatal anemia or to improve neurodevelopmental outcome, at a low (0.4 kU/kg) or a relatively high dose (3 kU/kg) (19–21). Furthermore, EPO treatment (0.3–0.5 kU/kg) of perinatally asphyxiated term infants resulted in improvement of neonatal behavioral neurologic function (22).

In this study, a mild HI brain injury was induced by unilateral carotid artery ligation followed by 10% O_2 for 45 min, leading to reproducible brain injury (23). The mice were treated with 5 or 20 kU/kg EPO or vehicle at 0, 24, and 48 h after the insult. We investigated the contribution of EPO on short- and long-term sensorimotor function and histological outcome in neonatal mice with a special focus on gender.

METHODS

Animals. Experiments were performed in accordance with international guidelines and approved by the Experimental Animal Committee of the University Medical Center Utrecht. C57Bl/6 J mice were bred at the animal facility of Utrecht University, and surgery was performed at the age of 9 d. Mice were housed at 21–23°C in an automatic 12-h light-dark cycle and weaned at the age of weeks. Food and water were available *ad libitum*. All analyses were performed in a blinded setup.

Experimental model. On postnatal d 9 (P9), 115 mouse pups (58 males and 57 females) underwent HI. The right common carotid artery was isolated and electrocauterized under anesthesia [isoflurane (IsoFlo; Abbott Laboratories Ltd., Berkshire, England): 5% induction, 1.5% maintenance in O_2:N_2O; 1:1]. Pups were allowed to recover for 1.5 h, followed by 45-min hypoxia (humidified 10% O_2, 90% N_2, 35°C). Sham controls underwent anesthesia and skin incision only. Seven mice (four males and three females, 6.1%) died from hypoxia before randomization and treatment, and no mortality occurred after treatment.

EPO treatment. To examine the efficacy of EPO (EPREX, Janssen-Cilag B.V., The Netherlands) treatment in P9 mice after HI, mice were randomly assigned to three groups and treated i.p. with EPO (5 or 20 kU/kg) or vehicle at 0, 24, and 48 h after hypoxia.

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Abbreviations: CA, cornu ammonis; **CRT,** cylinder rearing test; **DG,** dentate gyrus; **EPO,** erythropoietin; **EPOR,** erythropoietin receptor; **HE,** hematoxylin-eosin; **HI,** hypoxia-ischemia; **MAP-2,** microtubule-associated protein 2; **MBP,** myelin basic protein; **P9,** postnatal d 9; **SVZ,** subventricular zone

Animal groups. Short-term study: sham $n = 6$ (three males and three females); vehicle $n = 16$ (eight males and eight females); EPO5 (5 kU/kg) $n = 12$ (eight males and eight females); and EPO 20 (20 kU/kg) $n = 16$ (eight males and eight females). Long-term study: sham $n = 10$ (five males and five females); vehicle $n = 20$ (10 males and 10 females); EPO5 (5 kU/kg) $n = 20$ (10 males and 10 females); and EPO 20 (20 kU/kg) $n = 20$ (10 males and 10 females).

Histology. For the short-term study, mice were terminated by 300 mg/kg pentobarbital natrium (Alfasan International BV, Woerden, Holland) at 72 h after HI and perfused with 4% paraformaldehyde in PBS. Brains were paraffin embedded, and coronal sections (8 μm) were cut between ~0.10 and ~−1.68 mm from bregma. Deparaffinized sections were incubated with mouse anti-microtubule-associated protein 2 (MAP-2; 1:1000, Sigma Chemical Co.-Aldrich, Steinheim, Germany), rabbit-anti-α-cleaved-caspase-3 (1:800, Cell Signaling), rabbit-anti-Ki67 (1:400, Abcam) followed by biotin-labeled secondary antibodies and revealed using Vectastain ABC kit (both Vector-Labs, Burlingame, CA) and diaminobenzamidine (Sigma Chemical Co.-Aldrich).

MAP-2 loss was quantified using photoshop CS 4 (Adobe Systems, Mountain View, CA). Both hemispheres (at ~0.10 and ~−1.12 mm from bregma), striatum alone (at ~0.10 mm from bregma), and hippocampus alone (at ~−1.12 mm from bregma) were outlined on full-section images, and MAP-2 loss was calculated as 100 − [(MAP-2 positive area ipsilateral by contralateral hemisphere) × 100] (24). For each animal, three consecutive sections on the same levels were calculated and averaged as the final ratio.

Cleaved-caspase-3 staining was scored using a semiquantitative approach in the ipsilateral hippocampal area with the following scale: 0 = 90–100% caspase-3$^+$ cells (or no brain tissue), 1 = 50–90% caspase-3$^+$ cells, 2 = 10–50% caspase-3$^+$cells, and 3 = 0–10% caspase-3$^+$cells. Scoring was divided into four regions: cornu ammonis (CA) 1, CA2, CA3, and CA4. For each animal, the cells in five randomized sections between ~−1.12 and ~−1.68 mm from bregma were scored and averaged as the final score.

Progenitor cell proliferation was determined by Ki67 staining. The number of immunopositive cells was counted on the level of ipsilateral subventricular zone (SVZ; between ~0.10 and ~−0.56 mm from bregma) and dentate gyrus (DG; between ~−1.12 and ~−1.68 mm from bregma). For each animal, five randomized sections were counted and averaged as the final cell number.

For the long-term histological study, mice were terminated at 10 wk after HI as described for the short-term study. Brains were mounted in paraffin from which coronal sections (8 μM) were cut between ~0.14 and ~−58 mm from bregma. Brain sections were stained with hematoxylin-eosin (HE). Deparaffinized sections were then incubated with mouse-anti-myelin basic protein (MBP; 1:1600, Sternberger Monoclonals, Lutherville, MD) followed by biotin-labeled secondary antibodies, and staining was revealed using Vectastain ABC kit (Vector-labs, Burliname, CA) and diaminobenzamidine (Sigma Chemical Co.-Aldrich). For HE staining, both hemispheres (at ~0.14 and ~−1.58 mm from bregma), striatum alone (at ~0.14 mm from bregma), and hippocampus alone (at ~−1.58 mm from bregma) were outlined on full-section images, and the ratio of ipsi- and contralateral areas was calculated with photoshop CS 4. For MBP staining, the images were made binary, and the white matters of both hemispheres (at ~0.14 and ~−1.58 mm from bregma) were measured with ImageJ 1.42q software (http://rsb.info.nih.gov/ij/, 1997–2006), and the ratio of MBP-positive area ipsilateral by contralateral hemisphere was calculated. For each animal, three consecutive sections on the same level were calculated and averaged as the final ratio. For all the calculations of the hemisphere, the ventricular areas were excluded.

Cylinder rearing test. The cylinder rearing test (CRT) was used to assess sensorimotor function at 9 d, 4 wk, and 9 wk after HI (25). Animals were individually placed in a Plexiglas transparent cylinder between 0900 and 1000 h (9 d and 4 wk: 7.5 cm ø × 15 cm height; 9 wk: 11 cm ø × 30 cm height) and observed for 3 min in the housing room. Initial forepaw (left/right/both) preference of weight-bearing contacts during full rear was recorded. The relative proportion of right (ipsilateral) forepaw contacts was calculated as follows: paw preference = (right − left)/(right + left + both) × 100 (26).

Statistical analysis. Statistical analysis was performed using SPSS 15.0 and Graphpad Prism 4.02 software. Data are presented as mean ± SEM, and a $p < 0.05$ was accepted as statistically significant. One-way ANOVA with Bonferroni post tests was used to analyze group differences.

RESULTS

MAP-2 loss. Loss of ipsilateral MAP-2 staining as determined at 72 h after HI was used as a marker of early gray matter area loss. In our P9 model of neonatal HI brain damage, the loss of MAP-2 chiefly occurred in ipsilateral striatum and hippocampal area. There is no significant neuroprotective effect of EPO treatment (5 or 20 kU/kg) compared with vehicle treatment on the level of MAP-2 staining at this early time point after treatment (Fig. 1*A* and *B*).

Active-caspase-3 staining. To determine whether EPO treatment had an effect on apoptotic cell death, we scored active caspase-3 staining in the hippocampal area and calculated the scoring ratio of ipsilateral/contralateral areas. There was an obviously lower score after HI (either vehicle or EPO-treated animals) in CA1–CA4 areas of the ipsilateral hippocampus, indicating that apoptotic cell death was ongoing in these regions at 72 h after the insult. The i.p. administration of EPO (5 or 20 kU/kg) at 0, 24, and 48 h after the insult had no significant antiapoptotic effect (Fig. 1*C*).

Progenitor cell proliferation. Ki-67, which is expressed during mitosis, is considered as a reliable marker for proliferating cells (27). In the ipsilateral SVZ and DG, the number of Ki67-positive cells was reduced at 72 h after HI. At this time point, more Ki67-positive cells were observed on both areas in females only after EPO 5 kU/kg treatment compared with the vehicle group ($p < 0.001$), but no effect was found after EPO 20 kU/kg administration (Fig. 1*D* and *E*).

Paw preference (CRT). At 9 d, 4 wk, and 9 wk after HI, we assessed sensorimotor function using the CRT. Sham-treated animals did not show any paw preference during rearing in the CRT. HI caused a preference (~15% at 9 d, ~30% at 4wk, and ~25% at 9 wk) to use the unimpaired forepaw. EPO treatment did not improve performance in the CRT of male or female animals when assessed at 9 d after the insult. However, EPO-treated female but not male animals showed a significant improvement of sensorimotor function at 4 wk after HI. Interestingly, the improvement obtained in females of the EPO 5 kU/kg group was ~10% stronger than that obtained in females of the 20 kU/kg treatment group ($p < 0.001$, $p < 0.01$, respectively). At 9 wk after HI, the EPO 5 kU/kg-treated female mice still showed improvement of sensorimotor function ($p < 0.001$). Surprisingly, we no longer detected sensorimotor improvement in the 20 kU/kg EPO-treated female animals at this late time point (Fig. 2). Sensorimotor function in males was not improved by EPO treatment at any of the time points tested.

Analysis of ipsilateral surface area. The effect of EPO on long-term brain damage was determined at 10 wk. The morphological changes in the brain as visualized after HE staining are shown in Figure 3. The lesion volumes of the ipsilateral hemispheres were similar in male and female mice after HI. In addition, we did not detect a significant effect of EPO treatment (5 and 20 kU/kg) in the ratio of ipsilateral/contralateral hemispheres in males or females (Fig. 3*A* and *B*). However, when measuring the striatum and hippocampus area alone, a ~30% reduction of striatum tissue atrophy and a ~40% reduction of hippocampal tissue loss were indicated in females only after EPO treatment at 5 kU/kg ($p < 0.01$).

In contrast to what was observed for total area loss as determined after HE staining, the data in Figure 3*C* and *D* show that a significant reduction in the HI-induced loss of MBP staining was obtained only in females after treatment with 5 kU/kg EPO compared with vehicle ($p < 0.01$). However, there was no effect in either males or females of 20 kU/kg EPO.

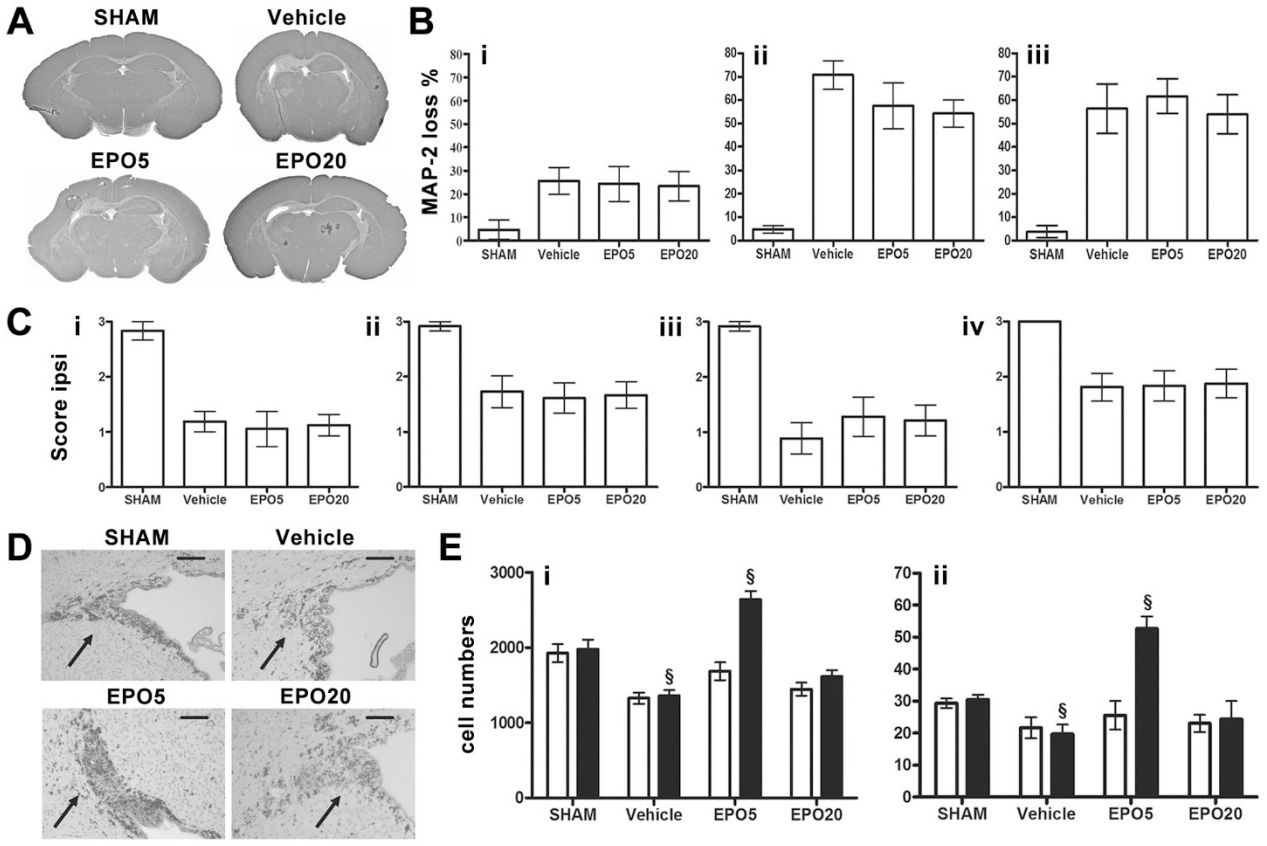

Figure 1. MAP-2 loss, semiquantitative analysis of α-cleaved-caspase-3 activity and Ki67 immunopositive cells counting. P9 mice were exposed to HI and treated with EPO 5 kU/kg, EPO 20 kU/kg, or vehicle. At 72 h after HI brains were collected and analyzed immunohistochemically. *A*, Representative examples of MAP-2 staining of the brain. *B*, Mean MAP-2 loss expressed as ratio of ipsilateral/contralateral MAP-2 positive areas at full hemisphere (*Bi*), striatum alone (*Bii*), and hippocampus alone (*Biii*). *C*, The immuno-positive cell score of ipsilateral hippocampal areas CA1 (*Ci*), CA2 (*Cii*), CA3 (*Ciii*), and CA4 (*Civ*) after α-cleaved-caspase-3 staining was calculated as described in the Methods section. *D*, Representative examples of Ki67 positive cells in the SVZ (*arrows*) as a measure of neural progenitor cell proliferative activities at ×100 magnification. Bars = 600 μm. *E*, Mean numbers of Ki67-positive cells on the SVZ (*Ei*) and DG (*Eii*) for male (□) and female (■) mice. §$p < 0.001$ EPO5 *vs* vehicle; $n = 16$ per group (sham group $n = 6$; *B* and *C*); $n = 8$ per group (sham group $n = 3$; *E*).

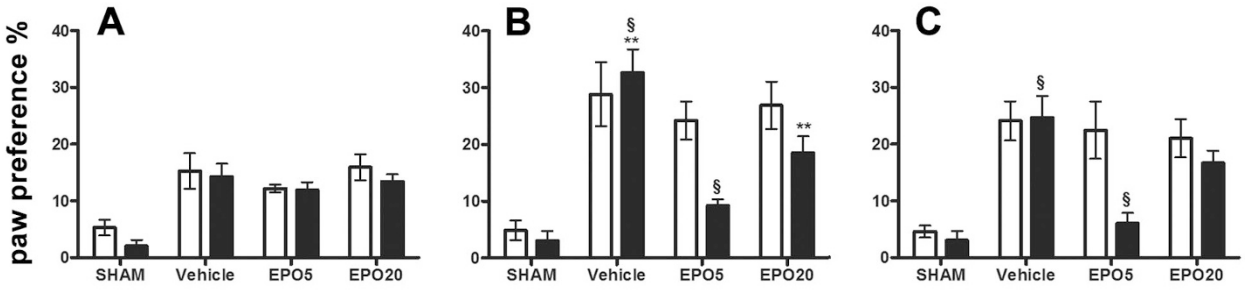

Figure 2. Effect of EPO treatment on preference to use the nonimpaired (ipsilateral) paw in the CRT, male (□) and female (■) P9 mice were exposed to HI and treated with EPO or vehicle as described in the Fig. 1 legend. At 9 d (*A*), 4 wk (*B*), and 9 wk (*C*) after HI, sensorimotor function was assessed in the CRT. **EPO20 *vs* vehicle $p < 0.01$; §EPO5 *vs* vehicle $p < 0.001$; and $n = 10$ per group (sham group $n = 5$).

DISCUSSION

In this study, we found no neuroprotective effect of EPO in our histological parameters when determined 24 h after the last EPO treatment (72 h after HI). In the long term, we observed a positive effect of EPO treatment on sensorimotor function, striatum atrophy, hippocampal lesions, and white matter injury when using 5 kU/kg of EPO. However, the beneficial effect of EPO was only observed in female animals.

Gender effects of posthypoxic neuroprotective interventions have been described after hypothermia and after treatment with the drug 2-iminobiotin (24,28). Moreover, in those studies, only females benefited from these neuroprotective interventions. The results of this study on the gender-specific effects of EPO treatment after neonatal HI are in line with those of the study by Wen *et al.* (29). These authors described gender-specific long-term protective effects of EPO treatment in a neonatal stroke model (29). Although it has been shown convincingly that circulating estradiol can lower the sensitivity for HI injury in females (30), it is not likely that female sex hormones play already a major role in P9 neonatal mice.

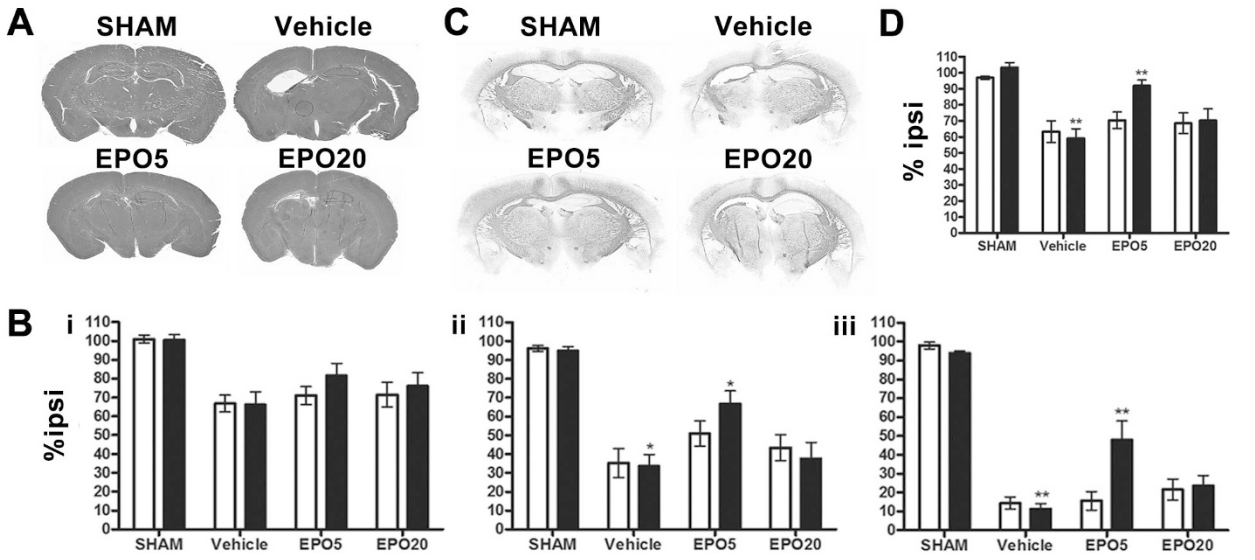

Figure 3. Effect of EPO treatment on brain area loss and white matter loss as determined after HE and MBP staining, respectively, at 10 wk after HI. *A*, Representative examples of brain area loss after HE staining. *B*, The ratio of ipsilateral/contralateral areas [hemispheric area (*Bi*), striatum alone (*Bii*), and hippocampus alone (*Biii*)] after HE staining in male (□) and female (■) mice. *C*, Representative examples of white matter changes of the brain after MBP staining. *D*, The ratio of MBP-positive ipsilateral/contralateral hemispheric areas in male (□) and female (■) mice. **EPO5 *vs* vehicle $p < 0.01$; *EPO5 *vs* vehicle $p < 0.01$; and $n = 10$ per group (sham group $n = 5$).

Indeed, we did not observe any gender difference in the extent of long-term HI brain injury in either sensorimotor function or brain damage in the vehicle group. In a clinical investigation using peripheral blood cells from healthy individuals, the EPOR alleles, EPORA 1 and EPORA 10, were present in a significantly higher frequency in females than in males (31). Although there is no report about the gender difference of EPOR in the human brain, we would like to propose that the higher frequency of EPOR in females may contribute to the gender-dependent neuroprotection of EPO. In any case, our results indicate that gender has to be taken into account even early in life, when designing strategies to protect the neonatal brain against injury.

It has been reported that EPO does not cross the blood-brain barrier in a detectable amounts when given at doses appropriate for erythropoiesis (200–400 U/kg) (32). Although the dose of EPO used in our studies is clearly above the range used for anemia treatment, there is no consensus as to the optimal dose, dose frequency, or dosing interval when using EPO to treat brain damage (33). Pharmacokinetics of EPO have demonstrated that i.p. administration produced a higher plasma concentration than s.c. administration. Meanwhile, i.p. injection also resulted in a more pronounced penetration into the brain than s.c. injection after HI (34). We administered EPO i.p. in our study and used a dose that is well above the dose used for treatment of anemia. Therefore, we expect that in our study EPO could reach the brain in sufficient amounts.

In our study, the beneficial effect of 5 kU/kg EPO on sensorimotor function, striatum atrophy, hippocampal lesions, and white matter damage in females was stronger and lasted longer than the effect of a higher (20 kU/kg) dose. Interestingly, an inverted U-shaped dose-response curve for EPO has also been described in a neonatal rat HI model (11); 5 kU/kg injection resulted in more neuroprotection than 2.5 or 30 kU/kg; three injections had a stronger beneficial effect than one injection, although increasing treatment to seven injections did not further improve the effect of EPO. It is not completely clear which mechanisms underlie the loss of positive effects at high (>20 kU/kg) doses of EPO.

Toxicity of EPO at high dosage has been reported in animal experiments. In a neonatal rat HI model, administration of EPO at 20 kU/kg significantly increased the number of degenerating cerebral neurons (35). However, in our female animals, a mild improvement of sensorimotor function was shown at 4 wk post-HI after EPO treatment with 20 kU/kg, but the improvement did not last into the adult period (9 wk post-HI). Although we could not check the hematocrit values in blood of EPO-treated animals at this age of the animals, the toxicity of the high dose of EPO may partially result from increased hematocrit-associated side effects, such as hypertension and thromboembolism, which may increase the infarction volume of the brain and neuronal cell death (36).

In addition, two types of EPO receptors have been identified in an *in vitro* study: a homodimeric (EpoR/EpoR) receptor and a heterodimeric (CD131/EpoR) receptor (37). The protective action of EPO occurs when EPO binds to the heterodimeric CD131/EpoR, but protective effects of EPO will be lost if all EPO is bound by homodimeric receptors (37). EPO itself is also involved in the degradation of EPOR (38). It is possible that extremely high doses of EPO will promote the degradation of EPOR leading subsequently to a loss of the protective effects of EPO.

The rodent model of neonatal HI injury has been widely used since it was developed in 1981 (39), with some variation in the hypoxic duration (23,40,41). Previous studies have indicated that treatment with EPO was only effective provided a certain amount of brain matrix tissue was left (18). In our study, we used a mild model of HI brain damage by applying only 10% O_2 for 45 min. We specifically used this mild model to increase the potential beneficial effects of EPO. In addition, although the gender-specific neuroprotection of 2-iminobiotin has been found under condition of severe HI (24), we cannot study the gender effects of EPO in severe HI because there are hardly any neuroprotective effects of EPO seen in such a condition (17).

Endogenous regeneration of neuronal tissue after HI insult is obviously not enough. EPO has been reported to have neurogenerative properties (42). There is a 17-mer peptide neurotrophic sequence called epopeptide AB, which exists in the structure of EPO. This peptide has been considered to induce proliferation, differentiation, and survival of neuronal cells (43). During *in vitro* and *in vivo* studies, administration of EPO enhanced differentiation of embryonic neural stem cells into neurons, up-regulated the expression of neurotrophic factors in the SVZ, and increased the generation of neurons in the injured striatum (12,13,44). Our results indicated that (progenitor) cell proliferation in the SVZ and DG was increased only by treatment with EPO at 5 kU/kg after neonatal HI in females. We also observed a beneficial effect of 5 kU/kg EPO treatment on striatum atrophy and hippocampal lesions in HE staining in the long term. This being said, we cannot rule out the possibility that proliferation and differentiation of progenitor cells have contributed to the improved functional outcome in 5 kU/kg-EPO-treated females but not in 20 kU/kg-EPO treatment. Notably, EPO treatment after HI did reduce the loss of MBP staining in females. However, we do not know at present, whether the effect of EPO on MBP staining in the brain is dependent on the protection of oligodendrocyte precursors, increased formation of these cells, or stimulation of maturation oligodendrocytes. However, it may be that EPO improved axonal remodeling in our model in view of the improved sensorimotor function. In conclusion, EPO treatment for neonatal HI is only effective in female mice provided a dose of 5 kU/kg EPO is administrated.

REFERENCES

1. Ferriero DM 2004 Neonatal brain injury. N Engl J Med 351:1985–1995
2. Hagberg B, Hagberg G, Olow I 1993 The changing panorama of cerebral palsy in Sweden. VI. Prevalence and origin during the birth year period 1983–1986. Acta Paediatr 82:387–393
3. van Bel F, Groenendaal F 2008 Long-term pharmacologic neuroprotection after birth asphyxia: where do we stand? Neonatology 94:203–210
4. Gonzalez FF, Ferriero DM 2008 Therapeutics for neonatal brain injury. Pharmacol Ther 120:43–53
5. Edwards AD, Brocklehurst P, Gunn AJ, Halliday H, Juszczak E, Levene M, Strohm B, Thoresen M, Whitelaw A, Azzopardi D 2010 Neurological outcomes at 18 months of age after moderate hypothermia for perinatal hypoxic ischaemic encephalopathy: synthesis and meta-analysis of trial data. BMJ 340:c363
6. Azzopardi D, Edwards AD 2007 Hypothermia. Semin Fetal Neonatal Med 12:303–310
7. Carnot P, Deflandre C 1906 [Haemopoietic activity of the serum in blood regeneration]. C R Acad Sci Paris 143:432–435
8. van der Kooij MA, Groenendaal F, Kavelaars A, Heijnen CJ, van Bel F 2008 Neuroprotective properties and mechanisms of erythropoietin in in vitro and in vivo experimental models for hypoxia/ischemia. Brain Res Rev 59:22–33
9. Ehrenreich H, Degner D, Meller J, Brines M, Béhé M, Hasselblatt M, Woldt H, Falkai P, Knerlich F, Jacob S, von Ahsen N, Maier W, Brück W, Rüther E, Cerami A, Becker W, Sirén AL 2004 Erythropoietin: a candidate compound for neuroprotection in schizophrenia. Mol Psychiatry 9:42–54
10. Sun Y, Calvert JW, Zhang JH 2005 Neonatal hypoxia/ischemia is associated with decreased inflammatory mediators after erythropoietin administration. Stroke 36:1672–1678
11. Kellert BA, McPherson RJ, Juul SE 2007 A comparison of high-dose recombinant erythropoietin treatment regimens in brain-injured neonatal rats. Pediatr Res 61:451–455
12. Shingo T, Sorokan ST, Shimazaki T, Weiss S 2001 Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells. J Neurosci 21:9733–9743
13. Wang L, Zhang Z, Wang Y, Zhang R, Chopp M 2004 Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats. Stroke 35:1732–1737
14. Digicaylioglu M, Bichet S, Marti HH, Wenger RH, Rivas LA, Bauer C, Gassmann M 1995 Localization of specific erythropoietin binding sites in defined areas of the mouse brain. Proc Natl Acad Sci U S A 92:3717–3720
15. Rabie T, Marti HH 2008 Brain protection by erythropoietin: a manifold task. Physiology (Bethesda) 23:263–274
16. Sola A, Wen TC, Hamrick SE, Ferriero DM 2005 Potential for protection and repair following injury to the developing brain: a role for erythropoietin? Pediatr Res 57:110R–117R
17. van der Kooij MA, Groenendaal F, Kavelaars A, Heijnen CJ, van Bel F 2009 Combination of deferoxamine and erythropoietin: therapy for hypoxia-ischemia-induced brain injury in the neonatal rat? Neurosci Lett 451:109–113
18. Spandou E, Papadopoulou Z, Soubasi V, Karkavelas G, Simeonidou C, Pazaiti A, Guiba-Tziampiri O 2005 Erythropoietin prevents long-term sensorimotor deficits and brain injury following neonatal hypoxia-ischemia in rats. Brain Res 1045:22–30
19. Juul SE, McPherson RJ, Bauer LA, Ledbetter KJ, Gleason CA, Mayock DE 2008 A phase I/II trial of high-dose erythropoietin in extremely low birth weight infants: pharmacokinetics and safety. Pediatrics 122:383–391
20. Bierer R, Peceny MC, Hartenberger CH, Ohls RK 2006 Erythropoietin concentrations and neurodevelopmental outcome in preterm infants. Pediatrics 118:e635–e640
21. Fauchère JC, Dame C, Vonthein R, Koller B, Arri S, Wolf M, Bucher HU 2008 An approach to using recombinant erythropoietin for neuroprotection in very preterm infants. Pediatrics 122:375–382
22. Zhu C, Kang W, Xu F, Cheng X, Zhang Z, Jia L, Ji L, Guo X, Xiong H, Simbruner G, Blomgren K, Wang X 2009 Erythropoietin improved neurologic outcomes in newborns with hypoxic-ischemic encephalopathy. Pediatrics 124:e218–e226
23. van der Kooij MA, Ohl F, Arndt SS, Kavelaars A, van Bel F, Heijnen CJ 2010 Mild neonatal hypoxia-ischemia induces long-term motor- and cognitive impairments in mice. Brain Behav Immun 24:850–856
24. Nijboer CH, Groenendaal F, Kavelaars A, Hagberg HH, van Bel F, Heijnen CJ 2007 Gender-specific neuroprotection by 2-iminobiotin after hypoxia-ischemia in the neonatal rat via a nitric oxide independent pathway. J Cereb Blood Flow Metab 27:282–292
25. Schallert T, Fleming SM, Leasure JL, Tillerson JL, Bland ST 2000 CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. Neuropharmacology 39:777–787
26. Chang YS, Mu D, Wendland M, Sheldon RA, Vexler ZS, McQuillen PS, Ferriero DM 2005 Erythropoietin improves functional and histological outcome in neonatal stroke. Pediatr Res 58:106–111
27. Scholzen T, Gerdes J 2000 The Ki-67 protein: from the known and the unknown. J Cell Physiol 182:311–322
28. Bona E, Hagberg H, Løberg EM, Bågenholm R, Thoresen M 1998 Protective effects of moderate hypothermia after neonatal hypoxia-ischemia: short- and long-term outcome. Pediatr Res 43:738–745
29. Wen TC, Rogido M, Peng H, Genetta T, Moore J, Sola A 2006 Gender differences in long-term beneficial effects of erythropoietin given after neonatal stroke in postnatal day-7 rats. Neuroscience 139:803–811
30. Hurn PD, Vannucci SJ, Hagberg H 2005 Adult or perinatal brain injury: does sex matter? Stroke 36:193–195
31. Zeng SM, Yankowitz J, Widness JA, Strauss RG 2001 Etiology of differences in hematocrit between males and females: sequence-based polymorphisms in erythropoietin and its receptor. J Gend Specif Med 4:35–40
32. Juul SE, Stallings SA, Christensen RD 1999 Erythropoietin in the cerebrospinal fluid of neonates who sustained CNS injury. Pediatr Res 46:543–547
33. Bührer C, Felderhoff-Mueser U, Wellmann S 2007 Erythropoietin and ischemic conditioning—why two good things may be bad. Acta Paediatr 96:787–789
34. Statler PA, McPherson RJ, Bauer LA, Kellert BA, Juul SE 2007 Pharmacokinetics of high-dose recombinant erythropoietin in plasma and brain of neonatal rats. Pediatr Res 61:671–675
35. Weber A, Dzietko M, Berns M, Felderhoff-Mueser U, Heinemann U, Maier RF, Obladen M, Ikonomidou C, Bührer C 2005 Neuronal damage after moderate hypoxia and erythropoietin. Neurobiol Dis 20:594–600
36. Wiessner C, Allegrini PR, Ekatodramis D, Jewell UR, Stallmach T, Gassmann M 2001 Increased cerebral infarct volumes in polyglobulic mice overexpressing erythropoietin. J Cereb Blood Flow Metab 21:857–864

37. Brines M, Grasso G, Fiordaliso F, Sfacteria A, Ghezzi P, Fratelli M, Latini R, Xie QW, Smart J, Su-Rick CJ, Pobre E, Diaz D, Gomez D, Hand C, Coleman T, Cerami A 2004 Erythropoietin mediates tissue protection through an erythropoietin and common beta-subunit heteroreceptor. Proc Natl Acad Sci U S A 101:14907–14912
38. Walrafen P, Verdier F, Kadri Z, Chrétien S, Lacombe C, Mayeux P 2005 Both proteasomes and lysosomes degrade the activated erythropoietin receptor. Blood 105:600–608
39. Rice JE III, Vannucci RC, Brierley JB 1981 The influence of immaturity on hypoxic-ischemic brain damage in the rat. Ann Neurol 9:131–141
40. Ditelberg JS, Sheldon RA, Epstein CJ, Ferriero DM 1996 Brain injury after perinatal hypoxia-ischemia is exacerbated in copper/zinc superoxide dismutase transgenic mice. Pediatr Res 39:204–208
41. McAuliffe JJ, Miles L, Vorhees CV 2006 Adult neurological function following neonatal hypoxia-ischemia in a mouse model of the term neonate: water maze performance is dependent on separable cognitive and motor components. Brain Res 1118:208–221
42. Iwai M, Cao G, Yin W, Stetler RA, Liu J, Chen J 2007 Erythropoietin promotes neuronal replacement through revascularization and neurogenesis after neonatal hypoxia/ischemia in rats. Stroke 38:2795–2803
43. Campana WM, Misasi R, O'Brien JS 1998 Identification of a neurotrophic sequence in erythropoietin. Int J Mol Med 1:235–241
44. Gonzalez FF, McQuillen P, Mu D, Chang Y, Wendland M, Vexler Z, Ferriero DM 2007 Erythropoietin enhances long-term neuroprotection and neurogenesis in neonatal stroke. Dev Neurosci 29:321–330