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Neuroprotective Effect of Erythropoietin on Hypoxic-Ischemic Brain Injury in Neonatal Rats

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Key Words
Erythropoietin · Apoptosis · Neonatal rat · Hypoxia · Ischemia · Infarct volume · Brain

Abstract
Erythropoietin (Epo) prevents ischemia and hypoxia-induced neuronal death in vitro. Recent studies have shown that this cytokine also has in vivo neuroprotective effects in cerebral and spinal ischemia in adult rodents. In this study, we aimed to investigate the effect of systemically administered recombinant human Epo on infarct volume and apoptotic neuronal death in a newborn rat hypoxic-ischemic brain injury model. Our results showed that a single dose of intraperitoneal Epo treatment (1,000 U/kg) significantly decreased the mean infarct volume as compared to the control group. In contrast to the Epo-treated group, histopathological examination by positive terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling of the affected brain in control animals revealed widespread neuronal injury associated with numerous apoptotic cells. Morphometric analysis to determine the extent of damage quantitatively ascertained that the mean infarct volume was significantly lower in the Epo-treated group ($p < 0.003$). These results suggest the beneficial neuroprotective effect of Epo in this model of neonatal hypoxic-ischemic brain injury. To our knowledge, this is the first study that demonstrates a protective effect of Epo against hypoxia-ischemia in the developing brain.

Introduction

Perinatal asphyxia is an important cause of neonatal mortality and subsequent sequelae such as cerebral palsy, mental retardation, learning disability, and epilepsy [1, 2]. Although there is an increased understanding of some of the mechanisms that may underlie neonatal hypoxic-ischemic brain injury, there is currently no clinically utilized treatment for this common disorder. It is clear that destructive processes such as glutamate and nitric oxide (NO) neurotoxicity, free radical formation, intracellular calcium accumulation and immune/inflammatory activation continue to damage the brain for many hours after oxygenation and circulation have been restored [1–5]. Pharmacological agents may provide neuroprotection in

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this condition with any of these processes. One candidate agent that may have an impact on this condition is erythropoietin (Epo).

Epo is a hematopoietic cytokine, which has recently been shown to be expressed in the nervous system [6–8]. The expression of Epo and its receptor in the nervous system is modulated by hypoxia and metabolic insult [6, 9, 10]. Recent in vitro studies suggest that Epo has neuroprotective effects against various insults such as glutamate-induced excitotoxicity, serum deprivation, hypoxia, and growth factor deprivation [10–14]. It has also been shown that Epo possesses neuroprotective action in animal models of global and focal cerebral ischemia and spinal ischemia models in adult rodents [9, 10, 15–18]. Although the exact mechanisms of the neuroprotective action of Epo is not completely known, promotion of cell survival signaling cascades [10, 19], upregulation of the expression of antiapoptotic proteins [20], modulation of intracellular calcium metabolism [13], attenuation of NO production [16, 21], inhibition of glutamate release [12], antiapoptotic [10, 16, 18], antioxidative [22, 23] and anti-inflammatory [17] actions have been suggested as possible mechanisms. These properties suggest that Epo may show beneficial effects against hypoxic-ischemic insults in the neonatal brain.

The purpose of the present study is to investigate the possible ameliorating effect of Epo in the neonatal rat model of hypoxic-ischemic brain injury.

Material and Methods

Animals

This study was performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the Dokuz Eylul University School of Medicine. Wistar rats with dated pregnancies were maintained at the same center and housed in individual cages with free access to water and laboratory chow. Twenty-two offsprings delivered vaginally were reared with their dams until the time of initial experimentation at 7 days of postnatal age (day of birth is day 1). A modification of Levine preparation was used as a model for perinatal hypoxic-ischemic brain injury [24].

Surgical Procedure and Treatment

Twenty-two pups underwent permanent unilateral carotid ligation. The midline of the neck was incised at the longitudinal plane under halothane anesthesia. The left carotid artery was permanently ligated with 5-0 surgical silk. Total time of surgery never exceeded 7 min. Animals were excluded from the study if there was bleeding during ligation or respiratory arrest resulting from anesthesia. Following a 2-hour recovery and feeding period, the animals were exposed to a 2.5-hour period of hypoxia (92% N_2, 8% O_2) by placing them in airtight containers partially submerged in a 37°C water bath to maintain a constant thermal environment. After retrieval from the hypoxia chambers, 11 pups received an intraperitoneal injection of recombinant human Epo at a dose of 1,000 units/kg, whereas the remaining 11 pups received only saline as a control group. After this procedure the pups were returned to their dam for 72 h until sacrifice.

Histopathological Evaluation

After 72 h all animals were perfused by 10% formalin under ether anesthesia. Brain tissues were removed and fixed in 10% formalin in phosphate buffer for 24 h. After detaching the hindbrain, the forebrain was cut coronally with a brain matrix into four equally spaced slices each with a 2-mm interval. The first and the last slices were 1 mm distant from each tips of the forebrain. The slices were processed and embedded in paraffin blocks. The blocks were cut into 5-μm sections at multiple levels and stained with hematoxylin and eosin.

Areas of infarction were delineated at eight preselected coronal levels with the known distance (1 mm) between each cross-sectional level. The infarct areas were determined at each cross-sectional level by one morphometrist with no prior knowledge of the experimental data, using a computer-assisted image analyzer system consisting of a microscope (Labophot-2, Nikon, Tokyo, Japan) equipped with a high-resolution video camera (VKC220E, Hitachi, Tokyo, Japan). The images were processed by an IBM-compatible personal computer, high-resolution video monitor and image analysis software (BS 200Docu Version 2.0, BAB Imaging Systems, Ankara, Turkey). Briefly, the images were grabbed with the video camera at 1 × magnification, and the areas of infarction were viewed on the monitor and outlined by drawing. The infarct volume and the ipsilateral hemispheric volume were determined by multiplying the measured area by the section interval thickness. The total volume of infarction was calculated by summation of the infarcted volume of each section. In order to avoid artifacts due to edema and tissue processing, the infarcted volume size was presented both as the percentage of the infarct to the ipsilateral hemisphere (%) and in absolute terms (mm^3).

In situ Cell Death Detection

To detect DNA fragmentation in cell nuclei, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) reaction was applied to the paraffin sections by using a kit (Roche, Cat. No. 1 684 817). After deparaffinization, the sections were treated with 20 μg/ml proteinase K for 10 min. After treatment with 0.3% H_2O_2 in methanol for 10 min and 0.1% Triton X-100 in 0.1% sodium citrate, for 2 min on ice, the sections were incubated with TUNEL reaction mixture for 60 min at 37°C. Further incubation with peroxidase-conjugated antibody was performed for 30 min at 37°C. The sections were stained with diaminobenzidine solution for 10 min at room temperature and then counterstained with hematoxylin.

Statistical Method

All data regarding the brain infarct were expressed as mean ± SD. Statistical comparison between Epo-treated group and control group was performed by Mann-Whitney U test using computer software (SPSS 10.0, Chicago, Ill., USA). The probability level (p) of 0.05 or less was chosen to represent statistical significance.

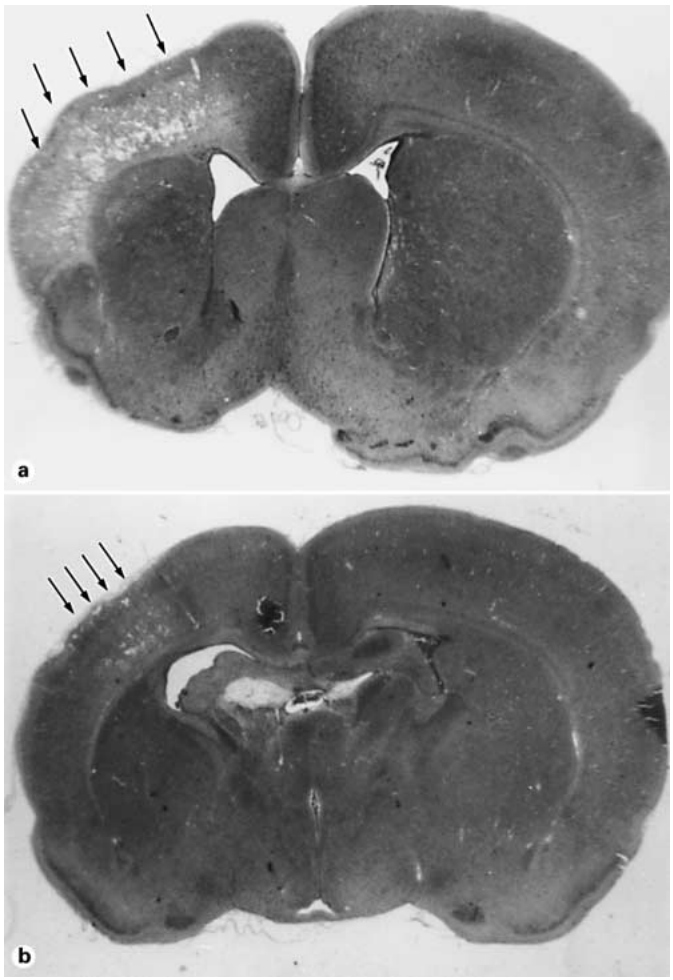

Fig. 1. Brain sections showing hippocampus in the coronal plane. Arrows indicate the infarcted area. a Untreated control group. b The Epo-treated group. Note that the neuroprotective effect is evident in the Epo-treated group with smaller infarcted area. HE stain. Orig. magn. × 4.

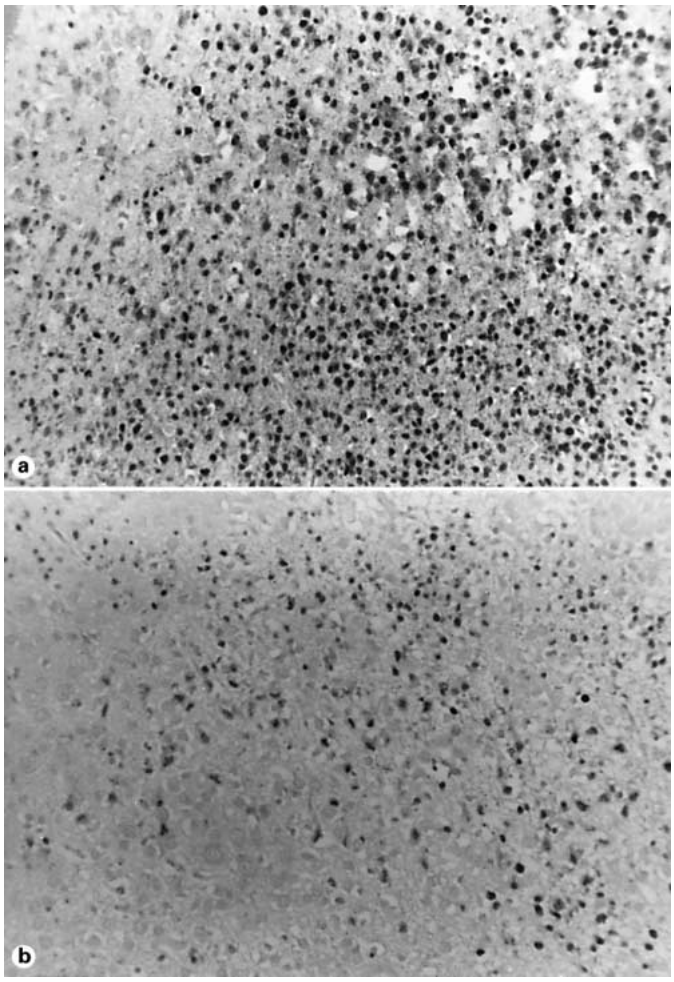

Fig. 2. Apoptotic cells exhibiting DNA fragmentation in the nuclei in the boundary between ischemic and non-ischemic cerebral cortex. a Untreated control group. b Epo-treated group. Note that the anti-apoptotic effect is evident in the Epo-treated group with decreased number of cells. TUNEL staining. × 200.

Results

In the present study, Epo treatment significantly diminished hypoxic-ischemic brain injury assessed by infarction volume measurement at 72 h after the insult. Morphometric analysis to determine the extent of damage quantitatively ascertained that the mean infarct volume and infarct percentage were significantly lower in the Epo-treated group compared to the control group (table 1, fig. 1). TUNEL reaction revealed numerous apoptotic cells in the untreated group, in contrast to the Epo-treated pups, where the apoptotic cell number decrease was evident (fig. 2). For quantitative measurement of the number of cells that underwent apoptosis, we counted 1,000 cells randomly in the infarcted area and calculated the percentage of the apoptotic cells (table 1).

Discussion

The present data indicate that Epo is an effective neuroprotective agent in this particular animal model when administrated consequently after exposure to hypoxic-ischemic insult. This neuroprotection indicates possible human therapeutic implications. Since the treatment of human perinatal asphyxia should be performed imme-

Table 1. Effect of Epo on brain infarct and apoptosis following hypoxic-ischemic injury in neonatal rats

	Epo-treated group (n = 11)	Control group (n = 11)	p
Infarct volume, mm^3 (mean ± SD)	20.7 ± 6.3	32.8 ± 9.3	0.003
Infarct, %	11.2 ± 4.2	16.8 ± 3.7	0.008
Apoptotic cells, %	74.1 ± 17.4	91.4 ± 6.9	0.010

diately after the insult, we investigated the neuroprotective potency of Epo on rat pups with hypoxic-ischemic brain injury.

The exact mechanisms responsible for the in vivo neuroprotective effects of Epo remain to be defined. Numerous mechanisms such as reducing NO overproduction that mediates glutamate neurotoxicity and preventing free radical formation potentially exist whereby Epo protects the immature brain from hypoxic-ischemic damage. Recent studies suggest that excitatory amino acids (EAAs) such as glutamate and aspartate may be important for the development of hypoxic-ischemic brain injury in the newborn [1–5]. Suppression of NO toxicity, known to be involved in the neuropathological mechanisms triggered by EAAs, appears to be involved in the neuroprotective action of Epo in vitro against glutamate neurotoxicity [16]. Free radical injury is also implicated in hypoxic-ischemic brain injury in neonates [1–5]. Epo treatment after starvation restores decreased antioxidant enzymes to normal levels on rat red blood cells suggesting that it might increase the activities of antioxidant enzymes in the central nervous system (CNS) in a similar manner [22]. In vitro strengthening action of Epo on the glial antioxidant defense system supports this hypothesis [23].

Another issue that remains to be clarified is to determine the mode of cell death (apoptosis or necrosis) upon which Epo has its greatest effects in hypoxic-ischemic injury. Apoptosis contributes significantly to cerebral damage in the perinatal period. Infants who die after intrauterine insults have a significant number of cells in the brain with the morphologic characteristics of apoptosis [25]. In the neonatal hypoxic-ischemic model, there is evidence that cerebral ischemia leads to delayed cell death with DNA damage [26]. There is some evidence suggesting that Epo protects neuronal cells that die via apoptosis both in vivo and in vitro. Epo prevents the ischemia-induced delayed neuronal death in the hippocampus in the animal model of global cerebral ischemia [16]. Anti-apoptotic action of Epo has also been confirmed in focal cerebral ischemia and transient spinal ischemia models [10, 18]. Epo decreases apoptotic cell death of neurons under conditions of hypoxia in vitro, suggesting that it might have a similar effect in vivo [27]. In the present study, histopathological evaluation using the TUNEL method, as well as quantitative measurement of apoptotic cells, confirm this hypothesis and suggests that apoptosis is one of the modes of cell death upon which Epo has its neuroprotective effect in hypoxic-ischemic brain injury.

Whether endogenous Epo or its receptor in the CNS play some role in the pathophysiology of hypoxia-ischemia in the developing brain needs to be further assessed. Epo and its receptor are present in the developing human brain as early as 5 weeks post-conception, and each protein shows a specific distribution that changes with development [27, 28]. It has been shown that Epo stimulates neurogenesis and prevents apoptosis in the embryonic brain [29]. Recent studies suggest that Epo is capable of regulating the production of neuronal progenitor cells from neural stem cells [29, 30]. It is therefore conceivable that endogenous Epo normally plays an important protective role following hypoxia-ischemia in the developing brain and that alteration of its actions by increasing its level by exogenous Epo like that in this study would further modify this role.

Hypoxic-ischemic injury to the CNS can have devastating lifelong effects on the developing fetus or in the neonate. To our knowledge, this study is the first in vivo demonstration to report that Epo can protect the developing brain against hypoxia-ischemia. Further behavioral studies are needed to evaluate the long-term beneficial effects of Epo in this model. Since the wide use of Epo in premature newborns demonstrated an excellent safety profile, this agent may be potentially beneficial in treating asphyxial brain damage in the perinatal period.

References

1 Delivoria-Papadopoulos M, Mishra OP: Mechanisms of cerebral injury in perinatal asphyxia and strategies for prevention. J Pediatr 1998; 132:S30–S34.
2 Vannucci RC, Perlman JM: Interventions for perinatal hypoxic-ischemic encephalopathy. Pediatrics 1997;100:1004–1014.
3 Fellman V, Raivio KO: Reperfusion injury as the mechanism of brain damage after perinatal asphyxia. Pediatr Res 1997;41:599–606.
4 Higuchi Y, Hattori H, Kume T, Tsuji M, Akaike A, Furusho K: Increase in nitric oxide in the hypoxic-ischemic neonatal rat brain and suppression by 7-nitroindazole and aminoguanidine. Eur J Pharmacol 1998;342:47–49.
5 Tan S, Zhou F, Nielsen VG, Wang Z, Gladson CL, Parks DA: Sustained hypoxia-ischemia results in reactive nitrogen and oxygen species production and injury in the premature fetal rabbit brain. J Neuropathol Exp Neurol 1998; 57:544–553.
6 Dame C, Juul S, Christensen RD: The biology of erythropoietin in the central nervous system and its neurotrophic and neuroprotective potential. Biol Neonate 2001;79:228–235.
7 Siren AL, Ehrenreich H: Erythropoietin – A novel concept for neuroprotection. Eur Arch Psychiatry Clin Neurosci 2001;251:179–184.
8 Marti HH, Wenger RH, Rivas LA, Straumann U, Digicaylioglu M, Henn V, Yonekawa Y, Bauer C, Gassmann M: Erythropoietin gene expression in human, monkey and murine brain. Eur J Neurosci 1996;8:666–676.
9 Bernaudin M, Marti HH, Roussel S, Divoux D, Nouvelot A, MacKenzie ET, Petit E: A potential role for erythropoietin in focal permanent cerebral ischemia in mice. J Cereb Blood Flow Metab 1999;19:643–651.
10 Siren AL, Fratelli M, Brines M, Goemans C, Casagrande S, Lewczuk P, Keenan S, Gleiter C, Pasquali C, Capobianco A, Mennini T, Heumann R, Cerami A, Ehrenreich H, Ghezzi P: Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. Proc Natl Acad Sci USA 2001;98:4044–4049.
11 Morishita E, Masuda S, Nagao M, Yasuda Y, Sasaki R: Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. Neuroscience 1997;76:105–116.
12 Kawakami M, Sekiguchi M, Sato K, Kozaki S, Takahashi M: Erythropoietin receptor-mediated inhibition of glutamate release confers neuroprotection during chemical ischemia. J Biol Chem 2001;276:39469–39475.
13 Koshimura K, Murakami Y, Sohmiya M, Tanaka J, Kato Y: Effects of erythropoietin on neuronal activity. J Neurochem 1999;72:2565–2572.
14 Sinor AD, Greenberg DA: Erythropoietin protects cultured cortical neurons, but not astroglia, from hypoxia and AMPA toxicity. Neurosci Lett 2000;290:213–215.
15 Sadamoto Y, Igase K, Sakanaka M, Sato K, Otsuka H, Sakaki S, Masuda S, Sasaki R: Erythropoietin prevents place navigation disability and cortical infarction in rats with permanent occlusion of the middle cerebral artery. Biochem Biophys Res Commun 1998;253:26–32.
16 Sakanaka M, Wen TC, Matsuda S, Masuda S, Morishita E, Nagao M, Sasaki R: In vivo evidence that erythropoietin protects neurons from ischemic damage. Proc Natl Acad Sci USA 1998;95:4635–4640.
17 Brines ML, Ghezzi P, Keenan S, Agnello D, de Lanerolle NC, Cerami C, Itri LM, Cerami A: Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. Proc Natl Acad Sci USA 2000;97:10526–10531.
18 Celik M, Gokmen N, Erbayraktar S, Akhisaroglu M, Konakci S, Ulukus C, Genc S, Genc K, Sagiroglu E, Cerami A, Brines M: Erythropoietin prevents motor neuron apoptosis and neurologic disability in experimental spinal cord ischemic injury. Proc Natl Acad Sci USA 2002; 99:2258–2263.
19 Digicaylioglu M, Lipton SA: Erythropoietin-mediated neuroprotection involves cross talk between Jak2 and NF-κB signaling cascades. Nature 2001;412:641–647.
20 Wen TC, Sadamoto Y, Tanaka J, Zhu PX, Nakata K, Ma YJ, Hata R, Sakanaka M: Erythropoietin protects neurons against chemical hypoxia and cerebral ischemic injury by upregulating Bcl-x(L) expression. J Neurosci Res 2002;67:795–803.
21 Calapai G, Marciano MC, Corica F, Allegra A, Parisi A, Frisina N, Caputi AP, Buemi M: Erythropoietin protects against brain ischemic injury by inhibition of nitric oxide formation. Eur J Pharmacol 2000;401:349–356.
22 Chakraborty M, Ghosal J, Biswas T, Datta AG: Effect of erythropoietin on membrane lipid peroxidation, superoxide dismutase, catalase and glutathione peroxidase of rat RBC. Biochem Med Metab Biol 1988;40:8–18.
23 Genc S, Akhisaroglu M, Kuralay F, Genc K: Erythropoietin restores glutathione peroxidase activity in 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine-induced neurotoxicity in C57BL mice and stimulates murine astroglial glutathione peroxidase production in vitro. Neurosci Lett 2002;321:73–76.
24 Vannucci RC, Vanucci SJ: A model of perinatal hypoxic-ischemic brain damage. Ann NY Acad Sci 1997;835:234–249.
25 Edwards AD, Yue X, Cox P, Hope PL, Azzopardi DV, Squier MV, Mehmet H: Apoptosis in the brains of infants suffering intrauterine cerebral injury. Pediatr Res 1997;42:684–689.
26 Pulera MR, Adams LM, Liu H, Santos DG, Nishimura RN, Yang F, Cole GM, Wasterlain CG: Apoptosis in a neonatal rat model of cerebral hypoxia-ischemia. Stroke 1998;29:2622–2630.
27 Juul SE, Anderson DK, Li Y, Christensen RD: Erythropoietin and erythropoietin receptor in the developing human central nervous system. Pediatr Res 1998;43:40–49.
28 Dame C, Bartmann P, Wolber EM, Fahnenstich H, Hofmann D, Fandrey J: Erythropoietin gene expression in different areas of the developing human central nervous system. Dev Brain Res 2000;125:69–74.
29 Yu X, Shacka JJ, Eels JB, Suarez-Quinan C, Przygodski RM, Beleslin-Cokic B, Lin CS, Nikodem V, Hempstead B, Flanders KC, Costantini F, Noguchi CT: Erythropoietin receptor signalling is required for normal brain development. Development 2002;129:505–516.
30 Shingo T, Sorokan ST, Shimazaki T, Weiss S: Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neuronal stem cells. J Neurosci 2001;21:9733–9743.