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Epo and other hematopoietic factors

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Abstract

The growth factors erythropoietin and granulocyte-colony stimulating factor have hematopoietic and non-hematopoietic functions. Both are used clinically in their recombinant forms. Both also have interesting tissue-protective effects in other organs, which are unrelated to their hematopoietic functions. They have clinical hematopoietic uses in neonatal populations and in experimental non-hematopoietic research, and clinical potential as neuroprotective or tissue-protective agents.

Keywords

Development; Erythropoietin; G-CSF; Growth factor; Neonatal brain injury; Neurogenesis; Neuroprotection

Introduction

Hematopoietic growth factors are classified into two groups: those responsible for the regulation of myeloid and erythroid growth and differentiation, and those concerned with immunity. There is functional overlap between hematopoietic growth factors, and each factor has a multiplicity of biological actions. In other words, more than one cytokine controls cells in any cell lineage, and most factors affect cells in more than one lineage.[1] Both the hematopoietic and non-hematopoietic functions of these cytokines are characterized by these properties.

Many of the hematopoietic cytokines were discovered by virtue of their growth-promoting effects on hematopoietic cell lines, or because of their specific immune functions. It was initially assumed that their effects were specific to the hematopoietic system. This view has been challenged, as functional receptors are expressed by other cell types with clear nonhematopoietic functions, as reviewed by Schneider et al[2] and Juul.[3] For example, both glia and neurons produce many of the cytokines once thought to be restricted to the hematopoietic system. Furthermore, they express receptors for these peptides, suggesting the capability of both paracrine and autocrine interaction.[4,5] Erythropoeitin (Epo) and granulocyte colony-stimulating factor (G-CSF) are both available in recombinant form and are FDA approved for clinical use. This chapter discusses the clinical application of these cytokines, as well as their possible non-hematopoietic effects. This latter topic is the focus of active research.

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Erythropoietin and erythropoiesis

The principal growth factor that regulates erythropoiesis is Epo. This 30.4-kDa glycoprotein contains 165 amino acids and is extensively glycosylated. Epo maintains the production of red blood cells during fetal, neonatal and adult life by inhibiting apoptosis of erythroid progenitors and by stimulating their proliferation and differentiation into normoblasts. Although other factors act in addition to, or synergistically with, Epo, the critical importance of Epo is emphasized by the observation that both Epo and Epo receptor (EpoR) null mutation mice die on the 13th day of intrauterine development due to absence of secondary erythropoiesis.[6] As Epo does not cross the placenta, Epo concentrations measured in the fetus reflect fetal synthesis.[7] The primary mechanism of Epo clearance is by EpoR binding, internalization and degradation. During development, the EpoR is widespread, involving erythrocyte precursors not only in the marrow, but also in liver stromal cells,[8] smooth muscle cells,[9] myocardiocytes,[10] endothelial cells,[11] enterocytes,[12] renal tubular cells, epithelial cells in the lung, retinal cells,[13] placental tissues,[14] Leydig cells,[15] and cells specific to the central nervous system (CNS).[4,16,17] The role of Epo in these tissues is under investigation.

To maintain the increase in red-cell volume associated with fetal growth, it is estimated that approximately 50×10^9 erythrocytes per day must be produced. Compared to adult Epo concentrations present at the time of acute anemia, measured fetal Epo concentrations seem low in the face of such production requirements. It has therefore been proposed that Epo is either more efficient in the stimulation of the erythropoiesis during fetal development, that it acts as a paracrine factor during hepatic hematopoiesis, and/or that other growth factors synergize with Epo. Likely candidates include hepatic growth factor (HGF), thrombopoietin (TPO), and insulin-like growth factor-1 (IGF-1).[18,19] Fetal Epo production is clearly regulated by requirements for tissue oxygenation, as elevated Epo concentrations (up to 8000 mU/mL) have been reported in several pathologic states, such as fetal hypoxia, anemia, placental insufficiency, and in infants of diabetic mothers.[20,21]

After birth in healthy-term infants, serum Epo concentrations decrease to reach a nadir between the fourth and sixth week after birth. By 10--12 weeks of life they reach adult concentrations (approximately 15 mU/mL). These changes in Epo concentrations are consistent with physiologic anemia seen in healthy term infants. In preterm infants, the fall in Epo is more profound and persists longer, leading to anemia of prematurity.

Clinical application of recombinant erythropoietin to stimulate erythropoiesis

Preterm infants remain among the most highly transfused patient populations despite attempts to limit phlebotomy losses, the implementation of transfusion guidelines, and the use of recombinant human erythropoietin (rEpo). Common contributors to anemia in the hospitalized preterm infant include phlebotomy loss (which can exceed the infant's circulating blood volume), decreased red blood cell (RBC) life span (70 vs 120 days in adults), growth requirements, iron deficiency, and anemia of prematurity. When measured, circulating Epo concentrations in this population are low relative to the degree of anemia.[22] Low circulating Epo concentrations, and the fact that marrow and circulating erythroid progenitors are sensitive to exogenous rEpo form the basis for rEpo treatment in preterm infants.[23]

The use of rEpo to avoid excessive transfusions in preterm infants has been studied in multiple randomized controlled trials. Several recent reviews have evaluated the safety and efficacy of rEpo treatment.[24--26] Treatment with doses of rEpo > 500 U/kg/week, together with iron and beginning after 8 days of life, is safe, well tolerated, and decreases both the number of transfusions and the volume of blood transfused. However, rEpo treatment does not prevent all transfusions, or even significantly decrease donor exposures. This is because clinical practice is so variable: transfusion guidelines differ in stringency, phlebotomy practices vary

by institution, and the timing and dose of both rEpo and iron vary widely. An increased risk of retinopathy of prematurity (ROP) was identified when rEpo with iron is used in the first 8 days of life [relative risk (RR) −1.71; confidence interval (CI) 1.15--2.54].[24] It is not clear whether this is an effect of rEpo or of early iron administration, as rEpo is always used in conjunction with iron.

One reasonable approach to managing anemia in the extremely preterm infant is to combine the use of blood transfusion and rEpo therapy with the goal of one donor exposure per infant maximum. Optisol®-preserved blood can be stored for up to 42 days. One adult unit of blood can be divided into aliquots and assigned to one infant. The infant can be transfused with these aliquots as needed during the first month of life (one donor exposure), then started on rEpo with iron if he or she remains significantly anemic with low reticulocyte counts. Judicious phlebotomy practices should be implemented.

Several unanswered questions in this field remain: Garcia et al demonstrated that for every increase in 500 U/kg/week of Epo, transfusion requirements decreased by approximately 75%.[26] This raises the question 'Have we determined the optimal rEpo concentration to stimulate erythropoiesis in preterm newborns?' Recent studies by Pollak et al have used higher rEpo doses, together with high iron dosing, with promising results.[27,28] The addition of other factors that are important for erythropoiesis (e.g. vitamin B12, folate, vitamin E) can help to optimize erythropoiesis, as preterm infants are at risk for deficiencies in these nutrients.[27,28] Finally, Aranesp (darbEpoietin alfa) -- a biologically modified form of Epo -- is now available. Studies in adults and children indicate that Aranesp can be administered less frequently than rEpo with equivalent erythropoietic effects. Although this preparation has not been well studied in preterm infants, it might be applicable to this population.[29]

Non-hematopoietic effects of erythropoietin

Following the observations of EpoR expression within the CNS, and the capacity for Epo production by astrocytes,[30] a broader concept of Epo as a neuroprotective molecule has emerged.[31] Epo might also function as a paracrine--autocrine tissue-protective hormone in other organs such as heart and kidney.[32]

The tissue-protective properties of rEpo have been more extensively studied in adult models than in developing organisms, and evidence supports rEpo treatment as a protective strategy for both perinatal brain damage and lung injury.[33] As rEpo has a long track record of use in preterm infants and high-dose rEpo, given systemically, can cross the injured and also the intact blood--brain barrier,[34] it has promise as a neuroprotective agent for the immature CNS.

The following paragraphs highlight recent findings on the neuroprotective potential of Epo in experimental animal models, with a particular focus on the immature CNS.

Mechanisms of action of erythropoietin in the immature brain

The fact that Epo has biological functions beside erythropoiesis was discovered in the early the 1990s, when Epo mRNA was detected in the brain in response to hypoxia.[30] Ischemic or other metabolic stressors can induce Epo and EpoR expression in animal models,[35] and also in human disease.[36,37] Whereas constitutive expression of Epo and EpoR in the adult brain is restricted, it is highly prominent during fetal development diminishing shortly after birth.[17,38]

The effects of Epo are receptor mediated. Although the classic EpoR is a homodimer, some of the non-hematopoietic effects of Epo are mediated by a heterodimeric receptor. This heterodimer is composed of an alpha subunit, EpoR, and a common beta subunit (βcR), which

is the signal-transducing subunit shared by the granulocyte-macrophage colony stimulating factor (GM-CSF) and the IL-3 and IL-5 receptors. This βcR has recently been discovered to be responsible for transducing neuroprotective signals. Epo derivatives such as carbamylated Epo (cEpo) and asialated Epo (asialoEpo) bind exclusively to the βcR, have no hematopoietic activity, but produce neuroprotection in experimental models of brain injury.[39,40] Interestingly, EpoR null mutation embryos (−/−) have abnormal heart and brain development with extensive neuronal apoptosis. This phenotype can largely be rescued by the human EpoR transgene, which restores erythropoiesis and reduces apoptosis.[41]

Although the precise mechanisms of Epo action in the CNS have not been fully defined, potential effects include induction of anti-apoptotic signaling pathways, e.g. activation of nuclear factor-κB (NF-κB) by Jak2[42] and Akt.[43] Data on signal-transduction pathways modulated by Epo in developmental brain injury are limited and might well differ from those in adults.[44,45] Table 1 summarizes neuroprotective properties of rEpo in the developing brain. The effects of rEpo include many pathways, including apoptosis,[46--48] inflammation,[49] excitotoxicity,[50] and glutamate toxicity.[51] Late brain recovery includes neurogenesis, angiogenesis and migration of regenerating neurons, which are all processes enhanced by rEpo.[52,53] In neonatal brain injury, rEpo has also led to significant improvement of long-term neurological outcome, possibly through similar mechanisms.[54,55]

Anti-apoptotic effects can be mediated by downregulation of the pro-apoptotic proteins Bax and DP5, and upregulation of anti-apoptotic Bcl-2.[56] Modulation of growth factor signaling cascades also plays a role, as was shown in a recent study using differentiated human neuroblastoma SH-SY5Y cells, which demonstrated that the combined activation of multiple signaling pathways, including STAT5, Akt, and -- potentially -- MAPK were required for the protective action of Epo.[57] In a model of NMDA-antagonist MK-801-induced drug toxicity, rEpo conferred 50% neuroprotection, due to stimulation of brain-derived neurotrophic factor and glial cell-line-derived neurotrophic factor. In addition, phosphorylation levels of extracellular signal-regulated protein kinase-1/2 (ERK1/2) and Akt were preserved, indicating enhancement of neurotrophin-associated signaling pathways.[58]

Epo is directly involved in prevention of oxidative stress with generation of anti-oxidant enzymes, inhibition of nitric oxide production and decrease of lipid peroxidation.[59,60] These properties might be relevant in the therapeutic prevention of injury in developing brains of premature infants, in whom anti-oxidant systems are immature. Inflammation is also an important component in the pathogenesis and progression of both preterm and term brain injury. In adult stroke and neonatal HI models, rEpo treatment results in a substantial decrease of pro-inflammatory cytokines and an associated reduction of apoptotic injury.[49,61]

Endogenous Epo production is stimulated by hypoxia-inducible factor 1α (HIF-1α). Systemic application of the HIF-1α stabilizing agent deferoxamine has been shown to be protective in a neonatal stroke model, suggesting that endogenous Epo is also neuroprotective.[62]

Clinical studies of neonatal neuroprotection with erythropoietin

No randomized, controlled trials designed to assess the long-term effects of high-dose rEpo on neurodevelopment have yet been published. In a study that reported on the long-term neurodevelopmental effects of erythropoietic doses of rEpo, infants weighing ≤ 1250 g at birth were randomized to rEpo or control treatment from day 4 of life until 35 weeks corrected gestational age (n = 87 rEpo, n = 85 placebo/control). There were no differences in neurodevelopmental outcomes at 18--22 months.[63] By contrast, in preterm infants < 1000 g treated with rEpo (400 U/kg three times per week) for similar duration, those with serum Epo concentrations > 500 U/mL had higher Mental Development Index scores than infants with Epo concentrations < 500 mU/mL when tested at 18--22 months corrected age (n = 6 rEpo,

n = 6 placebo/control).[64] This suggests that higher circulating Epo concentrations might be of benefit.

Clinical studies testing the safety, pharmacokinetics, and efficacy of rEpo for neuroprotection are underway in Europe and the United States, focusing primarily on preterm infants. More information is needed regarding the ideal population to target, optimal rEpo dose and duration of therapy. It is also unclear whether rEpo has a role as a prophylactic measure for at-risk extremely preterm infants, or whether it will be a rescue treatment. Similarly, for term infants with perinatal brain injury, more data are needed. It is now possible to treat near-term brain-injured infants with hypothermia.[65] The question is, will additional adjunctive therapies further improve outcomes such that infants with severe HIE also benefit? This is promising research and we anticipate that progress will be made to address these important questions in the next decade.

Potential side effects of high-dose rEpo

rEpo is a potent erythropoietic growth factor. Thus, we can expect that using high doses of rEpo as neuroprotective treatment will have transient hematopoietic effects. These include increasing erythropoiesis and -- possibly -- megakaryocytopoiesis. In the neonatal population in whom anemia is ubiquitous, this is unlikely to be a negative consequence but rather a beneficial side effect. It is not known, however, how this potential increase in hematopoiesis might affect iron balance. This is important because preterm infants are at high risk of iron deficiency, because the bulk of iron transfer occurs in the third trimester. Iron is essential for normal growth and development because it is an important component of proteins and enzymes required for oxygen transport, cell division, neurotransmitter synthesis, myelination, and cellular oxidative metabolism.[66] Iron deficiency can lead to adverse neurodevelopmental consequences, with deficits in executive function and memory.[67] Administration of supplemental iron to preterm infants in the first 2 weeks of life is generally not advised because of the potential oxidative effects in the face of deficient anti-oxidative mechanisms.[68] Thus, with the data we have available, it is not clear whether early rEpo administration for the purposes of neuroprotection should be accompanied by supplemental iron.

rEpo administration in the first week of life has been associated with an increased relative risk of ROP in at-risk infants.[24] It is not known whether this increased risk is due to rEpo administration or to the iron supplementation. ROP occurs in two phases, the first involving a loss of retinal vasculature following birth, the second involving uncontrolled proliferation of retinal vessels. EpoR are present on endothelial cells, and rEpo stimulation increases their angiogenic expression.11 Early high-dose rEpo might have a protective effect on the retina by ameliorating the first stage of ROP. Alternatively, the angiogenic properties of Epo might prevail, resulting in an increase in ROP. This issue must be rigorously studied.

Clinical use of recombinant G-CSF

The primary role of G-CSF is to promote the maturation of neutrophil progenitors and to increase the functional activities of mature neutrophils.[69,70] G-CSF is a glycoprotein consisting of four anti-parallel α helices and with a molecular mass of 19.6 kDa. Administration of recombinant G-CSF (rG-CSF) has long been used to prevent infections in patients with non-myeloid malignancies receiving anti-cancer drugs and suffering febrile neutropenia. In addition, rG-CSF is used to facilitate hematopoietic recovery following bone marrow transplantation and to mobilize peripheral blood progenitor cells in healthy donors.[71] In neonates, rG-CSF is used to treat congenital neutropenia[72] and severe neonatal sepsis associated with neutropenia.[73] Although rG-CSF appears to have no significant adverse side effects, its usefulness in treating and preventing sepsis in preterm infants remains uncertain.

Adequately powered, randomized, controlled clinical trials of neutropenic infants are needed to further evaluate its efficacy as an adjunct to antibiotic therapy.[74,75]

Non-hematopoietic effects of rG-CSF

As with EpoR, G-CSF receptor expression is not limited to the hematopoietic system. G-CSF receptor expression occurs in a variety of cell types including endothelial cells, glia and neurons.[76,77] Both G-CSF and its receptor are expressed on neuronal cells.[2,78] Recent work, primarily in adult models of ischemia, has demonstrated that G-CSF has important neuroprotective properties.[2,79] However, the precise mechanisms of action of C-CSF in the brain remain unclear.

G-CSF displays anti-inflammatory properties by reducing disruption to the blood--brain barrier and decreasing the number of infiltrating neutrophils in the infarct penumbra.[76,77] G-CSF increases neurogenesis[2] and enhances angiogenesis following an ischemic insult.[77] Whether G-CSF can decrease neuronal injury by mobilization of hematopoietic stem cells into damaged brain areas is currently a matter of discussion.[80]

Expanding evidence on the anti-apoptotic effect of G-CSF comes from several in-vitro and in-vivo studies. G-CSF exerts its anti-apoptotic effect by interference with various apoptotic signaling pathways, such as activation of the anti-apoptotic target Bcl-XL and PI3K/phosphoinositide-dependent kinase/Akt pathway controlling neuronal survival.[2]

However, data from adult studies might not be transferable to neonates. In contrast to the neuroprotective effects of G-CSF in adult models, the systemic administration of G-CSF and stem cell factor (SCF) surprisingly worsened excitotoxic brain injury in newborn mice.[81]

Remaining questions and future experimental studies

Features unique to the developing brain must be addressed when considering rEpo, rG-CSF, or any novel agent as a potential therapeutic agent for treatment of newborns. For example, physiologic apoptosis is an important aspect of normal brain development. Treatment with high-dose rEpo decreases apoptotic cell death. Will this have an adverse, unanticipated outcome on long-term neurodevelopment, or will the protective effects of the drug provide a net benefit? The answer to this question will depend on whether rEpo is used prophylactically or to treat known brain injury, and what the side effects are found to be. In addition, the effect of high-dose rEpo on ROP in vulnerable preterm infants must be determined.

It is of some concern that very-high-dose rEpo (40 U/mL) was recently demonstrated to have neurotoxic effects in in-vitro neuronal culture and brain-slice models of mild hypoxia, as well as in an in-vivo neonatal rat model of mild brain injury (20,000 U/kg IP × 2).[82] These findings demonstrate that further testing of rEpo in the developing CNS is warranted. It is reassuring that lower doses (2500 or 5000 U/kg) in neonatal rats show no such toxicity. In a series of experiments to test safety, the long-term effects of high-dose rEpo (0, 2500, or 5000 U/kg) administered in the first week of life in three experimental groups was assessed: (1) normoxic newborn rats; (2) normal newborn rats exposed to 2 hours of hypoxia (8% O_2) daily; or (3) animals that underwent unilateral brain injury.[83] rEpo treatment transiently raised the hematocrit in treated animals. It also prevented hypoxia-induced delays in geotaxis and growth as well as hypoxia--ischemia (HI)-induced learning impairment and loss of neurons in the substantia nigra. No adverse long-term behavioral, structural, or immunohistochemical effects were identified. Limitations of this study include the fact that more complex behavioral testing comparable to a human infant cannot be assessed in rats.

More data are needed as to the optimal timing of treatment, optimal dose, number of doses, and mode of application of exogenously administered rEpo. It is important to keep in mind that the answers to these questions will vary with the mechanism of injury under discussion. Thus, a dose that is optimal for neuroprotection after HI encephalopathy might not be the optimal dose to use for prophylaxis of extremely low-birth-weight preterm-infant white-matter disease. Furthermore, more research is needed on asialoEpo and cEpo as possible alternatives to rEpo. These agents might provide more specific neuroprotection without the hematopoietic potential of rEpo.

It is important to note that one cannot necessarily extrapolate data from adult models of brain injury to neonatal treatment strategies. The expression of neurotransmitters and their receptors changes with development, and treatments that are viable in adults might increase injury in neonates, as was found with G-CSF.[81]

Practice points

Receptors for Epo and G-CSF are not limited to hematopoietic cells.

rEpo plus iron beginning at > 8 days of life is safe, well tolerated, and decreases both the number of transfusions and the volume of blood transfused when used at doses of > 500 U/kg/week.

A reasonable approach to managing anemia in the extremely preterm infant is to combine judicious phlebotomy practices, transfusion guidelines, aliquoted assigned packed red blood cells, and rEpo treatment.

One cannot extrapolate data from adult models of brain injury to neonatal treatment strategies. Treatments that are viable in adults might be ineffective, or might even increase injury, in neonates.

Research directions

Further studies are warranted to understand dosing, acute, and chronic effects of hematopoietic growth factors with tissue protective properties on the immature central nervous system.

More information is needed regarding the ideal population to target.

Carefully planned clinical trials are needed to evaluate a possible prophylactic effect of rEpo on neurodevelopment outcome of preterm infants.

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Table 1

Effects of rEpo following injury to the immature CNS

Experimental model	Species	Method	Effect of rEpo	Reference
Hypoxia, HI	Rat	rEpo s.c. (0--2500 U/kg) daily at P1--P5	Improved neurodevelopmental outcome, prevention of dopamine neuron loss	McPherson et al [83]
Excitotoxicity	Mouse	Single and repetitive injections of rEpo i.p. (5000 U/kg) at P5 0--4 h after the insult	Small therapeutic window with protection up to 1 h, no gender differences	Keller et al 2006 [50]
Neonatal stroke	Rat	rEpo i.p. (10,000 U/kg) 1 h before the insult followed by daily injections until sacrifice	Upregulation of STAT-5 and Bcl-2, reduction of infarct volume	Wei et al 2006 [56]
HI	Rat	rEpo i.p. (300 U/kg) directly after hypoxia	Positive effect on auditory processing	McClure et al 2006 [84]
HI	Rat	rEpo i.p. (1000 U/kg) together with the insult	Reversed upregulation of Bax and DP5 and downregulation of Bcl-2	Kumral et al 2006 [46]
Mild hypoxia	Rat	rEpo i.p. (20,000 IE/kg) together with the beginning of hypoxia	Significant increase of neuronal death rates	Weber et al 2005 [82]
HI	Rat	rEpo s.c. (2500 U/kg) at the end of hypoxia	Protection of dopaminergic neurons, improvement of neurobehavioural outcome, no reduction of infarct volume	Demers et al 2005 [55]
HI	Rat	rEpo i.p. (2500 U/kg) 24 h after the insult	Attenuation of brain injury, increase of IL-1? , decreased infiltration of leukocytes	Sun et al 2005 [49]
HI	Rat	rEpo i.p. (2000 U/kg) after the insult	Reduction of injury, improvement of neurobehavioural outcome at day 42	Spandou et al 2005 [85]
Neonatal stroke	Rat	rEpo i.p. (5000 U/kg) immediately on reperfusion	Reduction of hemispheric volume loss, improvement of functional outcome at day 21	Chang et al 2005 [54]
Neonatal stroke	Rat	rEpo i.p. (1000 U/kg) single dose 15 min after FCI or three injections (100, 1000, or 5000 U/kg) 15 min after and repeated at 1 and 2 days after the insult	Reduction of apoptosis, activation of Janus kinase and STAT-5, upregulation of Bcl-xL , no effect on Epo-R and on NF-κB, most effective dose 1000 U/kg for 3 days	Sola et al 2005 [44]
HI	Rat	rEpo i.p. (300 U/kg) 24 h before the insult and for 2 consecutive days after the insult	Reduction of mortality, reduction of DNA fragmentation, reduction of TUNEL-positive cells, activation of heat shock protein 27	Sun et al 2004 [47]
NMDA-antagonist toxicity	Rat	rEpo i.p. (5000, 10,000, 15,000, 20,000, 30,000) IU/kg together with the insult	Reduction of apoptosis at 5000--20,000 U/kg, restoration of BDNF, GDNF mRNA, prevention of phosphorylated levels of ERK1/2 and Akt	Dzietko et al 2004 [58]
HI	Rat	rEpo i.p.(10,000 U/kg), non-erythropoietic asialo-Epo (80 mg/kg) 4 h before the insult	Reduction of infarct volume, reduction of ERK activation, upregulation of SNAP-25	Wang et al 2004
HI		I.p. Injection of naked plasmid containing Epo cDNA 24 h before the insult	Increase of Epo protein for 14 days, reduction of apoptosis, reduced glial activation	Wang et al 2004 [86]
HI	Rat	rEpo i.p. (1000 U/kg) together with the insult	Increase of glutathione peroxidase and decrease of lipid peroxidation	Kumral et al 2005 [87]
HI	Rat	rEpo i.p. (2000 U/kg)	Prevention of DNA	Spandou et

Experimental model	Species	Method	Effect of rEpo	Reference
		after the insult	fragmentation	al 2004 [48]
HI	Rat	rEpo i.p. (1000 U/kg) together with the insult	Reduction of spatial memory deficits at 22 days	Kumral et al 2004 [88]
HI	Rat	rEpo i.p. (1000 U/kg) together with the insult	Inhibition of nitric oxide production	Kumral et al 2004 [60]
HI	Rat	rEpo i.p. (1000 U/kg) together with the insult	Reduction of infarct volume	Kumral et al 2003 [89]
HI	Rat	rEpo i.c.v. (20 U) after the insult	Reduction of infarct volume	Aydin et al 2003 [90]
HI	Mouse	rEpo i.p.(1,000 U/kg and 5000 U/kg) 1 h before HI	Decreased caspase-3 and NF-?B positive neurons	Matsushita et al 2003 [45]

BDNF, brain-derived neurotrophic factor; ERK, extracellular signal-regulated protein kinase; FCI, xxxxxxxxxxxxx; GDNF, xxxxxxxxxx; HI, hypoxia--ischemia; i.c.v., intracerebroventricular; i.p., intraperitoneally; IE, xxxxxxxxxxxxxx; NF-kB, nuclear factor-κB; P, postnatal day; rEpo, recombinant erythropoietin; s.c., subcutaneously; TUNEL, xxxxxxxxxx; U, unit.