

Review article

Erythropoietin in neonatal brain protection: The past, the present and the future

Abdullah Kumral^{a,*}, Funda Tüzün^a, Meryem Gülfer Oner^b, Sermin Genç^b,
Nuray Duman^a, Hasan Özkan^a

^a Department of Pediatrics, Division of Neonatology, School of Medicine, Dokuz Eylül University, Izmir, Turkey

^b Department of Neuroscience, Health Science Institute, Dokuz Eylül University, Izmir, Turkey

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Abstract

Over the last decade, neuroprotective effects of erythropoietin (Epo) and its underlying mechanisms in terms of signal transduction pathways have been defined and there is a growing interest in the potential therapeutic use of Epo for neuroprotection. Several mechanisms by which Epo provides neuroprotection are recognized. In this review, we focused on the neuroprotective mechanisms of Epo and provide a short overview on both experimental and clinical studies, testing Epo as a neuroprotective agent in the neonatal brain injury, and the safety concerns with the clinical use of Epo treatment in neonates.

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1. Introduction

Neonatal brain injury including commonly hypoxic–ischemic encephalopathy (HIE), periventricular leukomalacia (PVL), cerebral-intraventricular hemorrhage and hyperoxic brain damage is an important cause of neonatal mortality and subsequent sequelae such as cerebral palsy, mental retardation, learning disability, and epilepsy [1–3]. Although there are increasing evidence about underlying mechanisms and growing number of studies about treatment strategies, there is currently limited clinically utilized treatment for these common disorders.

Epo is one of the most promising neuroprotective agents which was first identified as a humoral mediator

that is involved in the maturation and proliferation of erythroid progenitor cells [4]. Over the last decade, neuroprotective actions of Epo and its underlying mechanisms in terms of signal transduction pathways have been defined and there is a growing interest in the potential therapeutic use of Epo for neuroprotection [5].

This article overviews the neuroprotective role of Epo on neonatal brain injury. Initially we discussed the existing data on the neuroprotective mechanisms of Epo and then discussed in detail the role of Epo as a neuroprotective agent against neonatal brain injury in animal models and clinical trials. Finally we have highlighted the safety concerns with the clinical use of Epo.

2. Epo as an endogenous product

Epo is a naturally occurring 30.4-kDa glycoprotein that was originally identified for its role in erythropoiesis [6]. The primary production sites of Epo are the fetal liver and the adult kidney [7], where Epo gene

* Corresponding author. Address: Department of Pediatrics, Division of Neonatology, Dokuz Eylül University, Faculty of Medicine, Inciralti, 35340 Izmir, Turkey. Tel.: +90 232 4123645; fax: +90 232 4123649.

E-mail address: abdullah.kumral@deu.edu.tr (A. Kumral).

expression occurs mainly under the control of an oxygen-sensing, hypoxia-inducible factor (HIF) dependent mechanism [8,9].

Epo is produced primarily by astrocytes in the brain, but oligodendrocytes, endothelial cells, neurons and microglia were also found to produce Epo, and the production was upregulated mainly by hypoxia. The homodimeric EpoR has also been demonstrated on neurons, astrocytes, endothelial cells and microglia [10].

3. Neuroprotective mechanisms of Epo

3.1. Epo signaling pathway

Epo has been reported to induce a broad range of cellular responses in the brain directed to protect and repair tissue damage. A fundamental mechanism of Epo-induced neuroprotection is its ability to inhibit apoptosis [11–21]. The other mechanisms of Epo-induced neuroprotection include anti-inflammatory, anti-oxidant, angiogenic, anti-epileptic and neurotrophic effects [10,12,22–33] (Fig. 1). It is not easy to differentiate each mechanism distinctly. To better understand these mechanisms a summary of Epo signaling pathways in neuronal protection has been demonstrated in Fig. 2 [22,23,34–37].

3.2. Anti-apoptotic properties

Modulation of Bcl-2 family genes is one of the most investigated mechanisms in the anti-apoptotic properties

of Epo. Epo consistently increases the expression of the anti-apoptotic gene Bcl-xL, decreases the expression of pro-apoptotic gene Bak and also shifts the Bcl:Bax ratio towards a net anti-apoptotic effect in cultured microglia [38]. Bax, a pro-apoptotic molecule, has been shown to be required for apoptotic neuronal cell death during normal development. Bax also plays a role in the regulation of cell death in the CNS following neonatal hypoxic-ischemia (HI) [39,40]. It has been demonstrated that Epo downregulates Bax gene expression induced by HI and prevents injury-induced bcl-2 gene downregulation. Epo significantly prevents hypoxia–ischemia-induced Bax and DP5 mRNA upregulation in the brain tissue [11].

Nuclear factor-κB (NFκB) functions as an anti-apoptotic factor through its induction of genes that inhibit apoptosis. NFκB has been shown to induce the expression of the inhibitor of apoptosis (IAP) protein family. These proteins specifically inhibit the active forms of caspase-3, caspase-7, and caspase-9. Induction of IAP activity by NFκB also suppresses TNF-alfa initiated apoptosis through the inhibition of caspase-8 activity. Nuclear factor-κB may also prevent apoptosis through the direct activation of Bcl-xL. Loss of NFκB activity negates the neuroprotective effects of Epo suggesting that the activation of NFκB is necessary for the Epo protection in the nervous system [20,41].

Brain-derived neurotrophic factor (BDNF) has been implicated in long-latency action of the Epo. Epo induces both mRNA expression and production of biologically active BDNF in primary hippocampal cells,

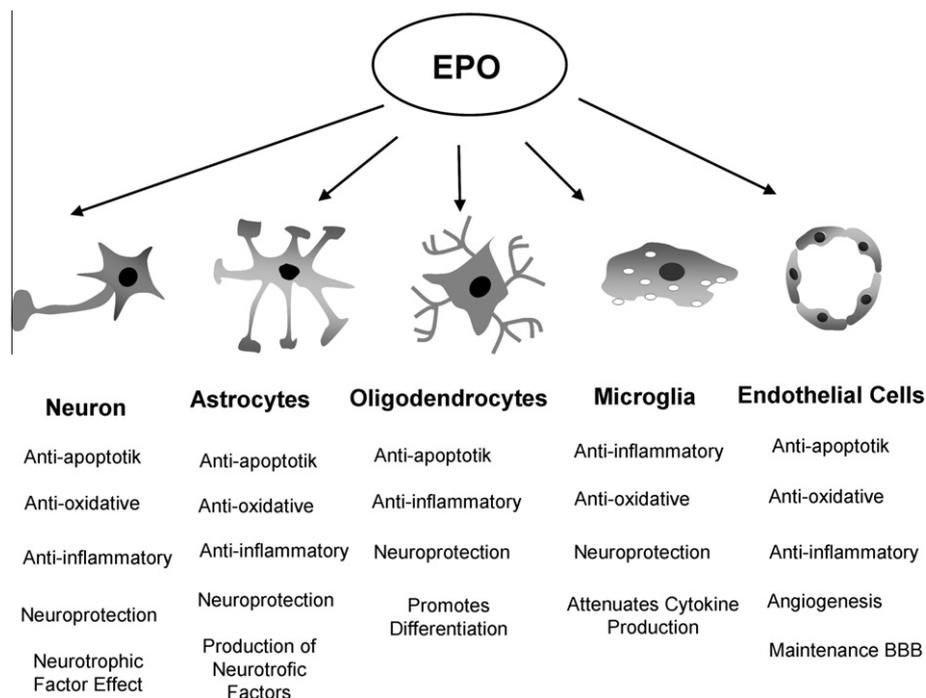


Fig. 1. Protective effect mechanisms of erythropoietin on neuronal and glial cells. BBB: blood–brain barrier.

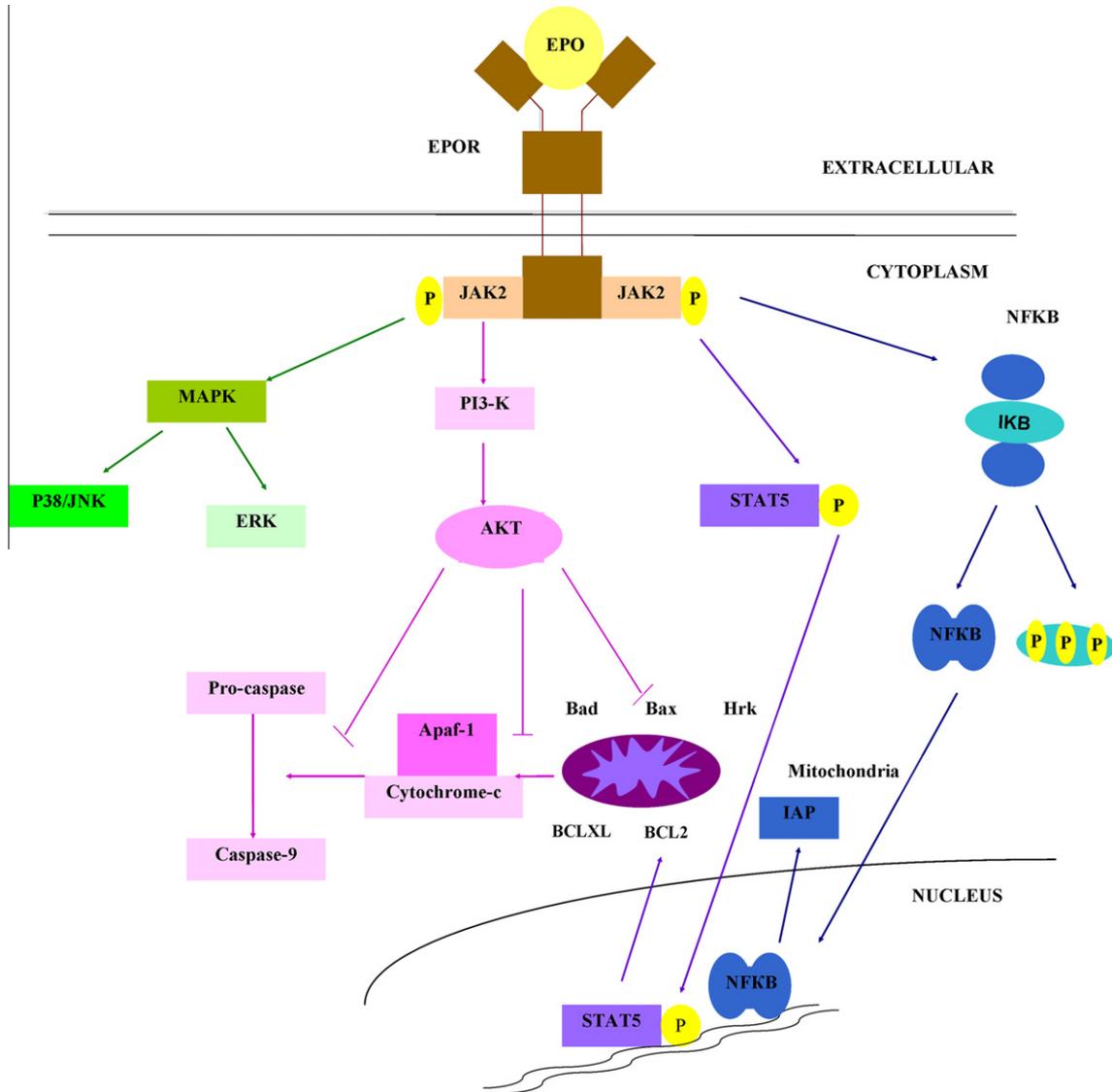


Fig. 2. Summary of EPO signaling in neuronal cell. EPO binds to EPOR dimer and stimulates JAK2 kinase activity which results in phosphorylation of JAK2 and EPOR. Activated JAK2 initiates signal transduction through several downstream molecules such as STAT-5, MAPK, ERK, PI (3) K/AKT and IKB. NFκB dissociates from IKB. NFκB and STAT5 enter into the nucleus, bind to DNA, and transcribe neuroprotective genes such as Bcl-xL and bcl2. Epo: erythropoietin; EpoR: erythropoietin receptor; JAK-2: Janus-tyrosine kinase-2; STAT-5: signal transducer and activator of transcription-5; NFκB: nuclear factor-κB; MAPK: mitogen-activated protein kinase; PI (3) K: phosphatidylinositol-3-kinase; IAP: inhibitor of apoptotic protein.

leading to a long-term activation of its specific receptor TrkB. The reduced neuroprotection and phosphorylation of TrkB triggered by Epo, observed when BDNF is neutralised by a specific antibody, confirm the relevance of both BDNF and TrkB in ruling Epo effect and its role in neuroprotection. Finally, Epo induces BDNF expression also in vivo following i.c.v. administration in mice [42,43].

3.3. Anti-inflammatory properties

Several studies have investigated the ability of Epo to affect inflammatory responses. In a mouse model of

autoimmune encephalomyelitis, Epo treatment upon onset of paresis was reported to significantly improve neurological functional recovery associated with a significant reduction in inflammatory infiltrates and demyelination [24,44]. Epo was found to reduce astrocyte activation and recruitment of leukocytes and microglia in the ischemic brain associated with reduction of levels of inflammatory cytokines including monocyte chemoattractant protein-1 (MCP-1), TNF and IL6 in the ischemic brain in the rat stroke model. Protective effect of Epo against cytotoxicity induced by interferon-gamma (IFN-gamma) and LPS has been shown in primary rat oligodendrocyte cultures [45]. Since Epo

did not reduce cytokine production in response to directly applied lipopolysaccharide (LPS) in vivo and in vitro, the authors suggested that the observed anti-inflammatory effect is due to the inhibition of neuronal apoptosis and not to a direct effect on inflammatory cells [12]. But in a previous study, especially prenatal maternal, Epo treatment has been found to attenuate LPS-induced white matter damage in neonatal rat brain by reducing the expression of inflammatory cytokines and sparing the myeline basic protein. Although the postnatal Epo treatment has been found to prevent LPS-induced brain injury, this effect was partial [46]. The exact mechanisms of the anti-inflammatory effects are unknown. But Epo might reduce leukocyte transmigration through endothelial cells, since Epo enhances the resistance of endothelial cells towards ischemia [47].

3.4. Neurotrophic properties

The reported neurotrophic effects of the Epo include the ability to stimulate axonal regrowth, neurite formation, dendritic sprouting, electrical activity and modulate intracellular calcium and neurotransmitter synthesis and release [16]. Epo was shown to improve functional outcomes by modulating plasticity, synaptic connectivity and activity of memory-related neuronal networks [14,48]. A recent study demonstrated that erythropoietin activates the cAMP response element binding protein (CREB) transcription pathway and increases BDNF expression and production in primary hippocampal neurons, which contributes to erythropoietin mediated neuroprotection [42].

3.5. Angiogenic properties

Besides its direct effects on neurons, Epo induced neuroprotection may be attributed to an improvement in brain perfusion by promoting new vessel growth. The potential roles of Epo and EpoR in vascular function have been indicated in both in vitro and in vivo studies. Epo has been shown to increase microvascular branch formation from rat aortic rings in a standard angiogenic assay. In addition, Epo has been shown to up-regulate expression of several genes involved in vascular function, signal transduction, and energy transfer, in cultured endothelial cells [49–52]. The angiogenic effect of the Epo was also found in the brain, since capillary endothelial cells express two forms of EpoR mRNA and Epo showed a dose-dependent mitogenic activity on brain capillary endothelial cells [53]. This angiogenic effect was confirmed in mice genetically engineered to lack either Epo or its receptor (EpoR) where mutant embryos suffer from severe defects in angiogenesis [32]. In angiogenesis, Epo stimulates proliferation of endothelial precursor cells, production of matrix

metalloproteinase-2, migration of endothelial cells into vascular sites and formation of capillary tubes [54–56].

One concern specific to preterm infants is that the angiogenic effects of Epo might affect the development of retinopathy of prematurity (ROP) [57] and it is further discussed in the relevant section.

3.6. Anti-oxidant properties

Oxygen free radicals are produced at low levels during normal physiological conditions and are scavenged by endogenous anti-oxidant systems that include superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase and small molecule substances such as vitamins C and E [58]. Epo controls a variety of signal transduction pathways during oxidative stress that can involve JAK2, protein kinase B, signal transducer and activator of transcription pathways, mammalian forkhead transcription factors, caspases, and NFκB [29]. Maternal treatment with Epo has been shown to prevent lipid peroxidation in fetal rat brain after ischemia–reperfusion injury [30]. Epo inhibits lipid peroxidation by increasing the activities of cytosolic anti-oxidant enzymes such as SOD and GPX [31,59].

Acute ethanol administration produces lipid peroxidation in the brain as an indicator of oxidative stress. Epo was also shown to have protective effects against ethanol-induced apoptotic neurodegeneration and oxidative stress in the developing mouse brain. Simultaneous administration of Epo along with ethanol attenuated the lipid peroxidation process and restored the levels of anti-oxidants [60].

3.7. Promoting neurogenesis

The developing nervous system has long been known to possess a greater capacity to recover from injury than the adult system. Hypoxic–ischemic injury in the neonatal brain initiates an enduring regenerative response from the subventricular zone [61]. Epo may contribute to the brain repair process after insult as it has a promoting capacity on neurogenesis both in vitro and in vivo [27]. Chemically modified derivatives of Epo such as carbamylated Epo (cEpo) also exert a similar effect on the neural progenitor cells [62]. Repeated doses of Epo treatment immediately after hypoxic-ischemia contribute to neurovascular remodeling by promoting tissue protection, revascularization, and neurogenesis in the neonatal injured brain and improve neurobehavioral outcomes [63]. Delayed administration of Epo also promotes oligodendrogenesis and attenuates white matter injury concurrently with increased neurogenesis. These effects are likely to contribute to the observed improvement in neurological functional outcomes [64].

3.8. Anti-epileptogenic properties

The loss of oxygen and glucose supply to the developing brain leads to excitotoxic neuronal cell damage and death. Such over-excitation of nerve cells can also manifest as seizures. Clinical neonatal seizures in the setting of birth asphyxia are associated with worse neurodevelopmental outcome, independent of the severity of hypoxic–ischemic brain injury [65]. In contrast to this, a recent experimental study has shown that both sub-clinical and clinical seizures are associated with increased severity of hypoxic–ischemic injury in a term model of neonatal hypoxic–ischemic injury [66]. Anyway, these results suggest that anti-epileptogenic medication may contribute to the neuroprotective strategies. Recent experimental studies performed in adult rodents have indicated that Epo and its peptide derivative also exerted anti-epileptogenic effect and decreased seizure-induced neural cell death [23,28,67,68]. Administration of a single dose of Epo directly after an acute hypoxic event at P10 has significantly decreased seizure susceptibility [33]. In a recent clinical pilot study on infants with mild/moderate hypoxic–ischemic encephalopathy, significantly less seizure has been observed in Epo-treated group. Epo also showed significantly improved abnormal electroencephalography findings [69]. Overall, these results suggest that the use of Epo in HIE may also decrease the need for anti-epileptic medication.

4. Experimental models evaluating the neuroprotective role of Epo in the neonatal brain injury

4.1. Protective role of Epo in hypoxic–ischemic brain injury

Increasing evidence suggests that exogenously administered Epo has a protective effect in a variety of different models of brain injury. The neuroprotective effects by Epo and Epo derivatives, shown in *in vitro* [17,70–73] and *in vivo* adult studies [21,67,72,73], were followed by studies involving animal models for neonatal cerebral injury. The most widely studied animal models include the Vannucci–Rice model for neonatal HI and middle cerebral artery occlusion for neonatal stroke [74].

Systemic Epo pretreatment (5 U/g vs vehicle) reduced the infarct volume after hypoxic–ischemic injury model of newborn rats [75]. Single dose systemic Epo treatment given immediately after the hypoxic–ischemic insult in newborn rodents was shown to improve both short- and long-term histological and behavioral changes [76–78]. Delayed administration of exogenous rh-Epo starting 24–48 h after a hypoxic–ischemic insult was also found to be neuroprotective in the neonatal rat models [64,79].

Substantially, timing of assessment of the brain damage is an important factor to evaluate the efficiency of

therapy. In the majority of the studies, assessment of brain damage was performed only a few days (1–7) after the insult. However brain injuries due to a hypoxic–ischemic insult are known to evolve over a period of 6–12 weeks and possibly longer. In few studies, neuroprotective effects of Epo persisted when brain infarcts were assessed at 4, 6 and 10 weeks after MCAO [78,80–83]. Previous studies evaluating the effects of post-insult Epo treatment on functional development (assessed by righting and postural reflexes, grip traction performance, asymmetries of forelimb use, rotation, Morris water maze) revealed that functional outcomes directly correlated with the histological improvement [78,80–82,84,85].

The beneficial effects of Epo seem to be related to timing and dose as well as the type of injury to a great extent. For neuroprotection, the term high-dose rEpo is often used to emphasize that the effective neuroprotective dose range (1000–30,000 U/kg) is well above the range used to treat anemia (500 U/kg) [86]. Pharmacokinetics of high-dose recombinant erythropoietin in the plasma and brain of neonatal rats confirmed that systemic rEpo is only detected in the brain after high doses (5000 U/kg) [87]. Lower multiple Epo doses, such as 1000 U/kg, did not result in significant neuroprotection from early neuronal damage even when combined with deferoxamine, an iron chelator which has been shown to decrease oxidative stress [88]. In a study comparing the neuroprotective effect of dosing regimens, three injections of 5000 U/kg and single injection of 30,000 U/kg were found most protective [89]. Favorably, dosing regimen including multiple injections of the high dose Epo on neonatal hypoxic–ischemic brain injury model in rats was shown to improve short- and long-term physiological and behavioral outcomes with no documented important side effect [90].

Summary of the studies evaluating the neuroprotective effects of Epo in neonatal HIE models is shown in Table 1.

4.2. Protective role of Epo on periventricular leukomalasia

PVL, a common neonatal brain white matter lesion, is frequently associated with cerebral palsy. In addition to the ischemia/reperfusion injury, cytokine-induced brain injury associated with maternal or fetal infection also plays an important role in the pathogenesis of PVL [2]. As mentioned above, several studies have demonstrated the ability of Epo to affect inflammatory responses. In primary rat oligodendrocyte cultures Epo was shown to be protective against cytotoxicity induced by IFN-gamma and LPS [45]. In previous studies post-natal Epo was found to attenuate LPS induced white matter damage, proinflammatory cytokine and chemokine induction in the developing rat brain [46,92].

Table 1
Experimental in vivo studies evaluating the neuroprotective effects of Epo in neonatal HIE models.

Animal	Dose and route	Administration	Assessment	Results–reference
Rat P7*	1000 U/kg (i.p.)	0 h	3 days	37% improvement in neuron count [77]
Mice P7	5000 U/kg (i.p.)	–1 h	7 days	50% improvement in neuron count [75]
Rat P7	20 U/kg (i.p.)	0 h	7 days	53% improvement in neuron count [91]
Rat P7	1000 U/kg (i.p.)	0 h	3 days	Epo decreased NO production [76]
Rat P7	1000 U/kg (i.p.)	0 h	20 weeks	Improved long-term spatial memory deficits and brain injury [80]
Rat P7	2500 U/kg ×3 (i.p.)	0, 1, 2 days	4 weeks	Epo protected mesencephalic dopamine neurons and reduced the degree of behavioral asymmetries at 4 weeks of life [81]
Rat P7	2000 U/kg (i.p.)	0 h	42 days	Epo prevented long-term sensorimotor deficits and attenuated brain injury [78]
Rat P7	5000 U/kg ×3 (i.p.)	24, 48, 72 h	3, 7, 14 and 21 days	Epo attenuated brain injury and prevented both the hypoxia–ischemia-induced increases in IL-1beta mRNA and protein levels [79]
Rat P7	1000 U/kg (i.p.)	0 h	3 days	Epo prevented HI induced Bax and DP5 mRNA upregulation and Bcl-2 downregulation [11]
Rat P7	2500, 5000 or 30,000 U/kg (s.c.)	(1, 3, or 7 dose) 0 day 0, 1, 2 days 0, 1, 2, . . . , 6 days	2 or 7 days	Three doses of 5000 and one dose of 30,000 U/kg rEpo were most protective (79% improvement in neuronal apoptosis) [89]
Rat P7	2500 U/kg ×3 (s.c.)	0, 1, 2 days		Repeated treatment with high-dose rEpo was safe. rEpo prevented hypoxia–ischemia-induced learning impairment and substantia nigra neuron loss [90]
Rat P7	3000 U/kg (i.p.)	0, 24, 48 h	2 weeks	Epo attenuated brain injury, subventricular zone expansion, and sensorimotor deficits [84]
Rat P7	1000 U/kg + deferoxamin 200 U/kg (i.p.)	0, 24, 48 h	72 h	DFO-Epo treatment reduced the number of cleaved caspase 3(+) cells, but did not protect against gray or white matter damage [88]

* P represents postnatal day.

Although the postnatal Epo treatment was shown to prevent LPS-induced brain injury, this effect was partial as compared to prenatal treatment [46]. Epo may also exert its effect on PVL by stimulating oligodendrocyte differentiation. EpoR expression has been detected in O4-positive immature oligodendrocytes, and rEpo treatment or co-culture of these cells with astrocytes enhances oligodendrocyte maturation [93]. Inhibition of this effect by anti-rEpo antibody and/or soluble EpoR suggests that release of Epo by astrocytes may promote oligodendrocyte differentiation. Since the mature oligodendrocyte is less vulnerable to injury, rEpo probably plays a role in reducing white matter injury by stimulating oligodendrocyte maturation.

4.3. Protective effects of Epo on hyperoxic brain injury

Supraphysiological oxygen concentrations are widely used in neonatal intensive care units for resuscitation, pulmonary hypertension and respiratory distress syndrome, and it has been demonstrated that these high concentrations of oxygen exert toxicity to the neonatal brain [3,94]. In recent years, it has been shown that Epo has protective effects on hyperoxic brain damage as well as hypoxic–ischemic brain damage. In a previous study, evaluating the protective effect of Epo against hyperoxic brain injury in the developing rat brain, it was shown that erythropoietin treatment (1000 U/kg/day ×5 dose, i.p.) significantly diminished apoptosis in

the CA1 region and dentate gyrus of hippocampus and parietal cortex in the Epo-treated hyperoxia group [95].

Similarly, systemic treatment with single high dose of Epo (20,000 U/kg i.p.) significantly found to reduce hyperoxia-induced apoptosis and caspase-2, -3, and -8 activity in the developing rat brain [96]. Subsequently the same author group demonstrated that rEpo generates its protective effect against oxygen toxicity by a reduction of diverse oxidative stress parameters and by limiting the stressor-inducible changes in both heme oxygenase 1 (HO-1) and cholinergic functions [97].

5. Clinical trials

Perinatal HIE is associated with high morbidity and mortality rates worldwide. Treatment and care for the sequelae of early brain hypoxic–ischemic injury impose considerable financial and lifelong personal burdens on society and affected families [1]. Hypothermia is rapidly becoming a standard therapy for full-term neonates with moderate-to-severe HIE [98–100]. Drugs added during or after hypothermia that can improve neuroprotection by extending the therapeutic window or providing long-lasting additive or synergistic protection are needed. Unfortunately no proven therapy has been established for preterm brain injury till now [101,102].

Neuroprotective therapeutic strategies except Epo include the use of oxygen free radical inhibitors and

scavengers (i.e. SOD, catalase, N-acetylcysteine, xanthines, deferoxamine, lazaroids); glutamate receptor antagonists (i.e. magnesium sulfate); growth factors (i.e. neurotrophins, IGF-1) and blockage of apoptotic pathways (i.e. minocycline). Although these strategies have been evaluated experimentally, none of them have been replicated in a systematic manner in the human neonate. It is important to consider that drugs administered during the neonatal period may be toxic to the immature brain [102].

Because of the multi-directional mechanisms of action and the safety profile, Epo is the most promising drug for neonatal brain protection. Clinical studies are ongoing to test the safety and efficacy of higher doses of Epo in newborns.

There exist only few studies evaluating the neurodevelopmental outcomes of the premature infants given Epo treatment for stimulation of erythropoiesis [103–107]. Newton et al. reported that Epo, given in clinical trials to reduce transfusions in low birth weight infants under 1250 g, did not significantly influence the neurological or cognitive outcomes at 2.5–8 years [103]. Ohls et al. showed that extremely low birth weight infants (ELBW) who were treated with Epo (400 U/kg body weight 3 times weekly given intravenously or subcutaneously from 96 h after birth until the 35th post-menstrual week) did not benefit in neurodevelopmental outcome at 18–24 months of age [105]. In a posthoc analysis, however, the same group reported that infants with elevated Epo concentrations (>500 mU/mL, $n = 6$) had higher Mental Development Index (MDI) scores than those with lower Epo concentrations (<500 mU/mL, $n = 6$). But these studies had several limitations in terms of sample size and study design to yield a clear comment [104].

A retrospective cohort study with a data set for a group ($n = 366$) of infants of <1500 g and ≤ 30 weeks of gestation revealed a dose–response relationship between Epo treatment and improved MDI scores [106].

In a recently published observational study the neurodevelopmental and school outcome of the ELBW infants receiving Epo treatment for stimulation of erythropoiesis in the first weeks of life ($n = 89$) was compared to that of untreated children ($n = 57$) at the age of 10–13 years. The Epo group scored significantly better than the untreated children in the overall developmental assessment (55% vs 39% normally developed) as well as in the psychological examination using the Hamburg-Wechsler Intelligence Test for Children-III (HAWIK-III). While children with IVH treated with Epo scored significantly better than the untreated children, treated and untreated children without IVH did not differ in their outcome [107].

Two single-center phase I/II prospective trials examining the safety and efficacy of high-dose Epo for preterm infants have been published. The phase I/II trial

by Juul et al. tested the safety and determined the pharmacokinetics of high-dose Epo in ELBW infants. All of the participants were <24 h old and ≤ 28 weeks of gestation. Thirty infants treated with high-dose rEpo were compared to 30 concurrent control subjects. Epo was given 3 i.v. doses of 500, 1000, or 2500 U/kg at 24-h intervals beginning on postnatal 1st day. Both 1000 and 2500 U/kg Epo produced peak serum Epo concentrations that were comparable to neuroprotective concentrations that previously were seen in experimental animals. There were trends towards less IVH ($p = 0.07$) and less severe IVH or PVL ($p = 0.06$) with Epo treatment. No excess adverse events occurred in the Epo-treated infants compared to control infants [108].

In the study by Fauchere et al., newborns born at 24–32 weeks of gestation and less than 1500 g were given Epo [3000 U/kg $\times 3$ i.v. doses, $n = 30$] or placebo ($n = 15$). There were no relevant differences regarding short-term outcomes such as IVH, ROP and PVL. Importantly, any relevant increase in typical adverse effects of Epo, in particular ROP, was not observed in the treatment group [109].

The first trial of Epo therapy for neuroprotection in term infants with moderate-to-severe HIE revealed that repeated, low dose Epo (300 or 500 U/kg every other day for 2 weeks) was safe and resulted in improved neurological outcome for patients with moderate HIE at 18 months of age [110]. Consistent with trials of hypothermia for HIE, Epo was only effective for infants with moderate injury and did not improve outcome for severely affected infants. Clinical studies are ongoing to test the safety and efficacy of Epo [111].

6. Safety concerns with the clinical use of erythropoietin in neonates

Complications that are seen in adults (e.g. hypertension, clotting, seizures, polycythemia and death) have not been identified in infants. Preterm infants have a long history of Epo treatment, with few reported side effects [57]. The safety and efficacy of Epo as an erythropoietic treatment for the prevention or treatment of anemia of prematurity have recently been reviewed [111]. In prospective randomized trials, treatment regimens ranged from 70 to 5000 U/kg/week (35 to 750 U/kg/dose), with duration of therapy ranging from 2 weeks to several months [86,112]. None of these studies are reported as increased risk for stroke, thrombotic events, hemorrhage or death. At the outset, erythropoietic Epo dosing for neonates was extrapolated from adults. But that dosing was found to be too low for neonates who have higher volume of distribution and more rapid clearance than adults [111]. Subsequent trials in preterm infants established the safety, pharmacokinetics and efficacy of higher doses [113–115].

Two single-center phase I/II prospective trials examining the safety and efficacy of high-dose Epo for the preterm infants have been recently published [108,109]. In very immature preterm infants with a gestational age <26 weeks that received early high-dose recombinant Epo, the rates of severe intraventricular hemorrhage were rather increased, although not statistically significant [109]. The phase I/II study by Juul et al. did not report any Epo-related complications [108]. A phase III randomized controlled study is ongoing in Switzerland [111].

Another safety concern which is unique to the preterm population remains whether Epo might increase the risk or severity of ROP. Early repeated high-dose of Epo did not exacerbate (or reduce) ROP in a neonatal rat model [116]. The effects of Epo on ROP might depend on timing because late Epo exposure exacerbated, but early Epo exposure reduced, experimental murine ROP. While early Epo administration decreases ROP by normalizing blood vessel growth after exposure to hyperoxia, late Epo signaling increases ROP pathology by virtue of its angiogenic effects [117].

Because of the reported presence of EpoRs on tumor cells, it was questioned if Epo had the potential for promoting tumor growth through stimulation of EpoRs. It is doubtful whether EpoRs on tumor cells are functional and there is no evidence that Epo can stimulate EpoRs on tumor cells *in vivo* [118,119]. Preclinical and clinical data published to date do not provide compelling support for a role for the Epo in tumor angiogenesis [120]. Preterm infants have a long history of Epo treatment, with few reported side effects [121]. Nonetheless, clinical trials should be proceeded cautiously particularly if the higher doses will be used for neuroprotection and these patients need to be followed closely in terms of the long-term side effects.

Since Epo is a potent erythropoietic growth factor, one can expect that using high doses of Epo as neuroprotective treatment will have transient hematopoietic effects such as increasing erythropoiesis. In the neonatal population in whom anemia is ubiquitous, this is unlikely to be a negative consequence but rather a beneficial side effect. However, the discoveries of EpoR expression in neural tissue and the neuroprotective effect of Epo raised the interest in tissue-specific Epo stimulation with the potential of activation of Epo neuroprotection without increasing erythropoiesis [122,123]. Such activity would be useful in chronic Epo therapeutic intervention for conditions such as ischemic and traumatic events in the brain.

Several modified forms of Epo have been proposed as neuroprotective agents that would limit the extent of injury but would not affect hematopoietic tissue [122,124]. Enzymatic removal of sialic acid residues from Epo shortens its plasma half-life *in vivo* so that it does not stimulate erythropoiesis, but it is able to

provide the neuroprotection in animal model of neonatal hypoxic–ischemic brain injury [125]. Another modification is Epo with carbamylation of lysines that does not appear to activate the EpoR homodimer and lacks erythropoietic activity [124]. Carbamylated Epo exhibits protection in animal models of cerebral infarct and spinal cord injury [126]. The activity of these modified Epo molecules raises the possibility of an alternate EpoR involved in nonhematopoietic protective effects of Epo [123]. Further analyses are necessary to validate the activity of these Epo analogs and the alternate forms of the EpoR and to determine their molecular mode of action.

Clinical studies are ongoing to test the safety and efficacy of Epo for the treatment of different neurological diseases in patient populations that span from newborns to adults [111]. The recent multicenter Epo stroke trial in which more than 60% of patients who received the thrombolytic treatment reported increased risk of serious complications such as mortality, intra-cerebral hemorrhage, brain edema, and thromboembolic events in adult stroke patients receiving Epo after tissue-plasminogen activator (tPA)-induced thrombolysis [127]. Clinical trials in neonatal stroke are still ongoing and it will be interesting to compare the results and to know the differences between the responses of adult and developing brain to exogenous Epo administration in stroke. Although neither term nor preterm infants have exhibited complications after Epo, it is important to proceed cautiously with clinical trials because risks may vary among specific populations, ages and disease states [111]. Conclusively, all safety concerns should be evaluated in preclinical and clinical studies with Epo and its derivatives and analogs.

7. Conclusion

The protective effects of Epo have been demonstrated in *in vitro* studies and in experimental animal models for neonatal cerebral injury. Recently, clinical studies have suggested favorable results about the neuroprotective effects of Epo in newborns. However, many questions still remain unanswered. More information is needed regarding the optimal treatment regimens (dose, dosing frequency and length of treatment).

A concern unique to the preterm population remains whether Epo might increase the risk or severity of ROP. Recent clinical trials did not show any apparent side effects of systemically administered Epo but the long-term data are not sufficient and the studies are ongoing. Recent data in neonatal rat model suggested that early high-dose Epo did not exacerbate ROP. To avoid the possible adverse effects of Epo, other non-erythropoietic variants may hold great promise for the future treatments of focal and global cerebral injury. Further research involving longer follow-up of

the neuroprotective effects after Epo administration is required prior to routine clinical application.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

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