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Erythropoietin as a neonatal neuroprotectant: basic and clinical studies

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Neonatal brain injury

Although there are many mechanisms by which the neonatal brain can sustain injury, perinatal asphyxia and preterm delivery are two common mechanisms. To date, effective treatment strategies to improve neurodevelopmental outcome are not yet available. Perinatal asphyxia occurs in 2-4 of every 1000 live-born term infants,[1] and accounts for 23% of neonatal deaths world wide.[2] In recently published hypothermia trials, between 55 and 62% of infants diagnosed with perinatal asphyxia treated with conventional therapy died or survived with significant neurodevelopmental disability.[3,4] The cell death associated with perinatal asphyxia occurs in two phases: early necrosis followed by later apoptosis, the specific timing of which varies by location in the brain.[5] During acute hypoxia-ischemia, neuronal oxidative phosphorylation is diminished due to the lack of oxygen. The resulting energy depletion leads to an accumulation of extracellular glutamate, and activation of glutamate receptors, particularly the N-methyl-D-aspartate (NMDA) receptor. Excitotoxic stimulation of glutamate receptors leads to a massive influx of calcium, sodium and water into the cell, excessive free radical production, and subsequent impairment of mitochondrial function, cell swelling, and neuronal cell death.[6,7] A second wave of neuronal cell damage occurs during the reperfusion phase. During this period there is post-ischemic release of oxygen radicals, increased nitric oxide (NO) synthesis, inflammation and an imbalance between the excitatory and inhibitory neurotransmitter systems. The net result is often the stimulation of apoptotic cell death.[8-10] This deleterious cascade which evolves over many hours and persists for days following injury[5] can theoretically be abrogated even if interventions begin after the acute insult. Hence much of the effort to develop treatments to lessen the severity of injury is focused on this extended interval as a window of opportunity for intervention. Strategies have included interventions to reduce cerebral edema, glutamate toxicity, inflammation, and free radical damage.[11] Again, none of these strategies have effectively diminished death or disability. While early hypothermia is promising,[3,4] its effects are limited, and only appropriate for near-term to term infants, not preterm infants.

Preterm delivery and its sequelae is another important cause of brain injury in neonates. Neonatal mortality and morbidity is closely linked to gestational age and birth weight.[12] Since 1991, mortality rates for extremely low birth weight (ELBW) infants (preterm infants < 1000 gm) have improved significantly.[13] In fact, most ELBW infants now survive. Figure 1 shows the survival statistics for the University of Washington NICU over the years 2000-2004. These data are comparable to other level III units in the country. As survival improves in these tiny infants, a relatively constant proportion of survivors are significantly impaired, resulting in a larger number of impaired survivors.[14-16] Longterm survival (to 2 years of age) of ELBW infants is now between 60 to 70 percent.[16-18] These infants are particularly vulnerable to white matter injury, and sequelae include cerebral palsy, deafness, blindness, and mental retardation. When these abnormalities are considered together, neurodevelopmental impairment (neurosensory abnormality and/or MDI score <70) is present in 36 to 48 percent of survivors.[16-18] Correspondingly, these children have a disproportionately high use of special needs services due to their chronic conditions.[19]

Animal models of perinatal hypoxia-ischemia

Animal models of neonatal hypoxic-ischemic brain injury are essential for the development and testing of novel therapeutic approaches. Seven day old rat pups are frequently selected for study because this age corresponds neurodevelopmentally to a 32-34 week gestational age human fetus. At this age, cerebral cortex layering

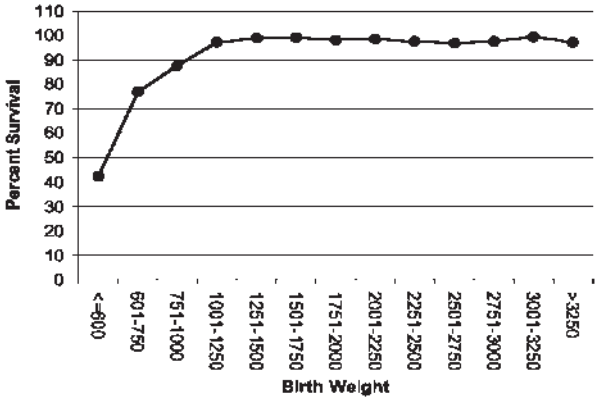

Figure 1. UW NICU survival 2000-2004.

is complete, but myelination is incomplete.[1] At an earlier age (P2), rat pups can be used to model extreme prematurity. Models of perinatal hypoxic brain injury vary from prolonged hypoxia alone,[20,21] to transient middle cerebral artery occlusion,[22,23] to unilateral ligation of the common carotid artery with subsequent hypoxia.[24] However, substantial morphological and functional differences between developing human and rodent brains exist, limiting the successful translation of neuroprotective strategies from laboratory studies to clinical use. To overcome these limitations, larger mammals including rabbits, dogs, piglets, sheep, and non-human primates have been used to model perinatal asphyxia.[25-28] The non-human primate is valuable because complex behavior more comparable to humans can be tested, but this model is expensive, and required specialized centers.[29-31] Ideally, new clinical therapies should be founded on data derived from a combination of *in vitro* and *in vivo* approaches, using small and large animal models, with non-human primates used as the penultimate pre-clinical step.

Erythropoietin

Erythropoietic effects of erythropoietin. Erythropoietin (Epo) is a 30.4 kD glycoprotein that regulates erythrocyte production.[32] Epo functions by binding to its specific cell surface receptor (Epo-R). Red cell regulation occurs by inhibiting apoptosis of erythroid progenitors, and by supporting their proliferation and differentiation into normoblasts. Since FDA approval, Epo has undergone many clinical trials in adults and children to test its safety and efficacy as an erythropoietic agent. It is now widely used to treat or prevent anemia due to a variety of causes including renal failure, cancer, and prematurity.

Neuroprotective effects of erythropoietin. Discoveries in our laboratory and others over the past thirteen years give ample rationale for the hypothesis that high-dose Epo administration can be neuroprotective after a central nervous system (CNS) injury. Indeed, in animal models of brain injury including asphyxia, hemorrhage, or trauma, high dose Epo treatment at the time of injury, and up to six hours following the event, can reduce the subsequent brain damage significantly.[33] The mechanisms by which Epo is neuroprotective remain an area of active investigation. Epo has modest protective effects both in the CNS[34,35] and the peripheral nervous system,[36] and these effects are seen in models that employ a wide range of mechanisms of injury.[33] This lack of specificity suggests that Epo may have multiple beneficial effects, some that affect neurons directly, and others that are likely secondary to Epo effects on other cells. To date, it has been shown that Epo effects include: direct neurotrophic effects,[37] decreased susceptibility to glutamate toxicity,[38,39] induction of anti-apoptotic factors,[40-45] decreased inflammation,[46-48] decreased NO-mediated injury,[49-51] direct antioxidant effects,[52-54] and trophic effects on glia.[55-57] *In vivo,* Epo also increases erythropoiesis, which stimulates increased iron utilization. Although under normal circumstances free iron is scarce, after hypoxic-ischemic injury, iron deposition is increased at the site of injury.[58] In the setting of hypoxia-ischemia, Epo-induced increased iron utilization might decrease the availability of free iron, thereby preventing or decreasing free iron accumulation and its associated consequences (oxidative injury). Epo may also provide neuroprotection by regulating blood flow to the brain following injury, as is suggested by Epo neuroprotection in the model of subarachnoid hemorrhage.[59,60] Long term beneficial effects of Epo treatment may include angiogenesis with improved blood flow to a damaged region,[61] and increased neurogenesis.[62,63] These non-erythropoietic effects of Epo may be mediated through a newly identified receptor made up of one Epo-R subunit, and the β common subunit, which is also part of the receptor complexes of interleukin (IL)-3, GM-CSF and IL-5.[64,65]

Epo in brain. Our group, and others, have established that Epo and its receptor are present in the developing fetal brain.[40,66-70] Epo and Epo-R mRNA and protein can be detected as early as 5 weeks human gestation, and persist throughout development. In the embryonic cerebral hemisphere at 5 to 6 weeks post conception, both Epo and Epo-R localize to undifferentiated neuroepithelial cells in the ventricular zone. As development proceeds, the diffuse staining of broad zones of developing neocortex seen in early gestation is replaced by more specific cellular staining of increasingly differentiated cells.[68] Epo immunoreactivity is present in multiple cell types of the developing human brain, including astrocytes, subpopulations of neurons, and microglia.[70] Although both neurons and astrocytes

produce Epo, the astrocyte is the primary source of brain Epo.[40,71] Brain Epo is smaller than circulating Epo, due to differences in sialylation.[66] In an elegant study of mouse neurodevelopment, Knabe *et al.* showed early diffuse Epo-R immunoreactivity of neuroepithelial cells, followed by more specific reactivity in radial glial cells from E11 onwards. Specific subpopulations of neurons expressed Epo as development progressed, and in the rostral midbrain and synencephalon, Epo-reactive neurons were associated with Epo-R-expressing radial glial cells, suggesting an important neurodevelopmental role for Epo and its receptor.[70]

Epo neuroprotection in neonatal models. Multiple investigators have now shown Epo to be neuroprotective in neonatal models of brain injury.[23,51,72-77] Neuroprotective dosing in these studies has ranged from 1,000 U/kg/dose to 30,000 U/kg/dose. To summarize these data, it can be concluded that Epo has anti-apoptotic and anti-inflammatory effects in the acute post injury period, with neurogenic and vasculogenic effects in the recovery period. Reported neuroprotective effects vary somewhat based on Epo dose, whether the mode of administration was intraperitoneal (IP) or subcutaneous (SC), and whether single or multiple doses were given. Ultimately, Epo treatment of neonatal brain injury results in improved short and longterm outcomes, with both structural and behavioral improvement. Both white and grey matter injury show improvement with Epo treatment. These effects are present when Epo is given immediately after the injury, or up to 24 hours following injury.[48]

Safety of high dose erythropoietin in neonates. Doses of Epo up to 1400 U/kg/week have been used safely to promote erythropoiesis in preterm neonates,[78] but potential long-term consequences of early high dose Epo have not been established in this population. We hypothesized that high dose Epo, given to normoxic or hypoxic animals in the neonatal period would be safe in a rat model.[79] Three experimental groups were assessed. Group 1 animals were given daily SC injections of 0, 2500, or 5000 U/kg Epo for the first five days of life (P1-P5). Group 2 animals were exposed to daily SC injections of 0, 2500, or 5000 U/kg Epo during P1-P5, plus 2 hours of hypoxia (8% O_2) daily from P1-P3. Group 3 animals underwent right carotid artery ligation followed by hypoxia (8% $O_2$2 x 90min) at P7, then injection of vehicle or Epo (2500U/kg daily x3). Short and long term physiologic and behavioral evaluations were made. Major organs were evaluated grossly and histologically. As expected, Epo treatment transiently raised the hematocrit in treated animals. It also prevented hypoxia-induced delays in geotaxis and growth as well as hypoxia-ischemia-induced learning impairment and substantia nigra neuron loss. No effect on adult blood pressure, and no histologic differences were found in brain, kidney, liver. We observed that repeated treatment of newborn rats with high dose Epo was safe in all conditions tested. Limitations of this study include the fact that more complex behavioral testing comparable to a human infant cannot be assessed in rats. Furthermore, the effect of high dose Epo on retinal vascular development was not tested.

Ongoing clinical studies. Several clinical studies testing the safety, pharmacokinetics, and efficacy of Epo as a neuroprotective agent are underway in Neonatal Units across the globe (Switzerland, Germany, United States). At this time, studies are focused primarily on possible protective effects of Epo in the very low birth weight or extremely low birth weight infant. If these studies show that Epo is safe, and has neuroprotective effects, then larger multicenter collaborative trials will be needed to confirm the findings. More information is needed regarding the optimal Epo dose and duration of therapy, and whether to target steady state vs peak serum concentrations. It is also unclear whether Epo will be more effective as a prophylactic or rescue treatment in this population. Similarly, for term infants with brain injury due to stroke, perinatal hypoxia-ischemia, or by other mechanisms, more data are needed. It is now possible to treat near term brain-injured infants with hypothermia. The question is, will additional adjunctive therapies further improve outcomes? This is promising research, and we anticipate that in the next decade, more progress will be made to answer these important questions.

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