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Review Article

Time to reconsider extended erythropoietin treatment for infantile traumatic brain injury?

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ABSTRACT

Pediatric traumatic brain injury (TBI) remains a leading cause of childhood morbidity and mortality worldwide. Most efforts to reduce the chronic impact of pediatric TBI involve prevention and minimization of secondary injury. Currently, no treatments are used in routine clinical care during the acute and subacute phases to actively repair injury to the developing brain. The endogenous pluripotent cytokine erythropoietin (EPO) holds promise as an emerging neuroreparative agent in perinatal brain injury (PBI). EPO signaling in the central nervous system (CNS) is essential for multiple stages of neurodevelopment, including the genesis, survival and differentiation of multiple lineages of neural cells. Postnatally, EPO signaling decreases markedly as the CNS matures. Importantly, high-dose, extended EPO regimens have shown efficacy in preclinical controlled cortical impact (CCI) models of infant TBI at two different, early ages by independent research groups. Specifically, extended high-dose EPO treatment after infantile CCI prevents long-term cognitive deficits in adult rats. Because of the striking differences in the molecular and cellular responses to both injury and recovery in the developing and mature CNS, and the excellent safety profile of EPO in infants and children, extended courses of EPO are currently in Phase III trials for neonates with PBI. Extended, high-dose EPO may also warrant testing for infants and young children with TBI.

1. Introduction

Moderate and severe pediatric traumatic brain injury (TBI) remains a leading cause of childhood mortality and morbidity worldwide (Thurman, 2016). TBI is typically caused by a blow or other traumatic injury to the head or body, with the degree of damage dependent on the nature of the injury and the force of the impact. Common events that cause brain injury in children and infants comprise falls, motor vehicle collisions (MVC), penetrating injuries and abusive head trauma (AHT). In the United States, unintentional injuries are the primary cause of death and disability for children and adolescents < 20 years old (Centers for Disease, C. and Prevention, 2012). The leading cause of TBI-related death among those < 4 years, however, is assault, including AHT (Taylor et al., 2017). Over the past two decades, the incidence of TBI among children and adolescents in high income countries has significantly diminished with the implementation of greater safety and preventive measures, such as increased and proper use of helmets, appropriate use of child restraints in motor vehicles, avoidance of high-risk activities, and the emergence of regionalized, dedicated pediatric trauma centers. Despite this improvement, TBI continues to inflict a significant burden on infants and children, especially in middle- and low-income countries where resources for public health initiatives dedicated to recognition and prevention strategies are scarce, and pediatric trauma centers are few (Taylor et al., 2017). Infant TBI represents a serious challenge to clinical treatment due to the rapid periods of brain development superimposed on heterogenous pathophysiological injury mechanisms.

The high incidence of AHT contributes to the prevalence of infant TBI. Depending on the geographic region, AHT accounts for about one third of infant TBI (Dashti et al., 1999). In the United States, AHT varies by socioeconomic status (Berger et al., 2011; Huang et al., 2011). AHT is most common in children under 5, with the highest incidence occurring in children under 1 year (Nuno et al. 2019, Keenan et al., 2019). For infants with AHT, 30-day mortality approaches 5%, and reaches 50% mortality by 21 years of age due to the associated long-term morbidity (Miller et al., 2014). Long-term follow-up of infants with

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AHT shows that only 15% have a “good” outcome, and only 30% participate in a typical school curriculum (Miller et al., 2014). Indeed, chronic neurological deficits in long-term survivors of AHT are pervasive and common. Specifically, attention deficits are observed in 80%, and behavior issues are present in 53%. Speech deficits occur in 50%, with vision deficits in 45%, motor deficits in 45%, and epilepsy in 38% (Lind et al., 2016). Infants with noninflicted, moderate to severe TBI, suffer similar cumulative deficits (Ewing-Cobbs et al., 2006; Prasad et al., 2017). Given that these young children suffer from a multitude of additive deficits, and that there is a strong influence of age on mortality that differs by type of trauma (abusive *versus* unintentional) (Nuno et al. 2019), therapies that are clinically appropriate for infants with heterogeneous brain injury are desperately needed. This is especially true in the context of infants, as they manifest worse neurodevelopmental outcomes compared to toddlers (Keenan et al., 2019). Although daunting, the overwhelming prevalence of these injuries necessitates the development of neuroreparative strategies that specifically support the injured developing brain and reduce long-term deficits in survivors of TBI.

The pathophysiology of TBI involves a primary injury that culminates in structural pathology, as well as, the initiation of molecular, cellular and ultrastructural physiologic events that dictate the degree of injury evolution and secondary injury to the developing brain (Davanzo et al., 2017). Indeed, despite the initiating insult (MVC, AHT, fall), the secondary injury with associated hemodynamic instability, hypoxia and post-traumatic seizures, can have an ancillary, yet profound, impact on functional outcome. While all efforts to reduce the incidence and severity of pediatric TBI should be continued, and resources should be invested in preventative measures, novel pharmacological therapies are needed to enhance brain repair after injury occurs. Presently, the only effective interventions available to infants with TBI are supportive care to reduce secondary injury, including management of airway, breathing and circulation, cerebral edema, intracranial pressure, seizures, hemorrhagic progression and systemic complications such as infection and anemia. New therapies targeted to effectively repair the injured brain are required, and ideally would involve a cocktail of exogenous agents aimed at combating the typical developmental arrest that appears after moderate to severe infant TBI, and limiting the numerous resultant cognitive and behavioral difficulties (Huh and Raghupathi, 2019).

The dominant mechanism of molecular and cellular injury after TBI in the developing brain likely varies depending on the cerebral microenvironment. This local microenvironment reveals the combination of primary and secondary insults, and the child's inherent neuro-reparative resilience. This inherent individual resilience (or vulnerability) to brain injury reflects baseline genetic and epigenetic influences. For example, the tendency to develop post-traumatic epilepsy, a common problem in children with TBI, is dependent on genetic predisposition (Ritter et al., 2016; Sun et al., 2017; Kumar et al., 2019). Specific genotypes have been associated with outcomes after TBI, including Glasgow outcome score (Osier et al., 2018), hippocampal volume (Hayes et al., 2018) and cognition (Merritt et al., 2018). Thus, the multi-faceted suppression of damage and enhancement of repair from agents with pleiotropic effects over a sustained period, such as EPO, may prove to have superior utility compared to therapies specifically targeted to time-limited mechanisms. To this end, here we review the evidence to consider the emerging intervention, extended high-dose erythropoietin (EPO) as a mechanistically and age-appropriate, clinically-viable therapeutic agent for infantile TBI. The developmental regulation of EPO signaling, excellent safety profile, and recent progress in clinical trials for perinatal brain injury (PBI) suggest recombinant human EPO may be appropriate to reconsider as a treatment for repair in the acute and subacute phase after infant TBI.

Table 1
Targets of Erythropoietin (EPO) Neurorepair.

▪ Neurogenesis	Yu et al. (2002), Tsai et al. (2006), Iwai et al. (2007)
▪ Neuronal differentiation	Park et al. (2006)
▪ Neuroblast migration	Tsai et al. (2006)
▪ Reduce apoptosis	Yu et al. (2002)
▪ Oligodendrogenesis	Iwai et al. (2010), Jantzie et al. (2013), Kaneko 2013
▪ Oligodendrocyte maturation	Sugawa et al. (2002), Jantzie et al. (2013)
▪ Myelination	Mazur et al. (2010)
▪ Axon health	Hellewell et al. (2013), Jantzie et al. (2014)
▪ Reduce oxidative stress	Genc et al. (2002). Kumral 2005
▪ Reduce ferroptosis	Wu et al. (2019)
▪ Reduce mitochondrial dysfunction	Xiong et al. (2009), Millet et al. (2016)
▪ Reduce neuroinflammation	Mazur et al. (2010), Hellewell et al. (2013)
▪ Reduce excess calpain activity	Jantzie et al. (2016)
▪ KCC2 levels	Jantzie et al. (2014)

2. EPO in neural cell development

Erythropoietin is a 30.4 kDa endogenous glycoprotein secreted postnatally by the kidney peritubular interstitial cells (Fisher, 2010). In 1961, as a young faculty member, Robert Fisher and his medical student, BJ Birdwell, initially provided the first direct proof that in the mature animal, *de novo* synthesis of EPO occurs in the kidneys (Fisher, 2010). Sixteen years later, in 1977, Wasserman and colleagues showed that fetal EPO is made in the liver, and that production switches from the liver to the kidneys after birth (Zanjani et al., 1977), with the transition complete by 40 days postpartum (Zanjani et al., 1977; Fisher, 2010). That same year, Miyake, Kung and Goldwasser first isolated human EPO from urine (Miyake et al., 1977). In 1989, D'Andrea, Lodish and Wong cloned EPO receptors (EPOR) in mice (D'Andrea et al., 1989), opening the path to investigate EPOR's signaling mechanisms. The same year, the FDA (Food and Drug Administration) first approved EPO for use in anemia (Fisher, 2010). Almost 30 years later, EPO is routinely used to treat anemia in patients of all ages.

It was not until the 1990s that the critical role of EPO signaling in the development and maintenance of the CNS was recognized (Table 1), similar to many other cytokines and chemokines that were first known for their non-CNS functions. EPO/EPOR signaling is essential for multiple aspects of neurodevelopment, including stimulation of neural progenitors and prevention of neural apoptosis (Yu et al., 2002). Most types of neural cells express EPOR, including neurons, oligodendroglial lineage cells, astrocytes and microglia (Masuda et al., 1993; Digicaylioglu et al., 1995; Bernaudin et al., 1999; Sugawa et al., 2002; Marti, 2004; Ott et al., 2015). In the CNS, astrocytes are primarily responsible for secreting EPO.(Chavez et al., 2006) In mice, lack of EPORs affects brain development as early as E10.5, resulting in reduced neural progenitor numbers and increased apoptosis (Yu et al., 2002). Cortical cells from EPOR null mice exhibit decreased neurogenesis and have increased sensitivity to hypoxia (Yu et al., 2002). Interestingly, in wild type animals with the appropriate compliment in EPORs, *in vitro* hypoxia increases EPO expression and precipitates a tenfold increase in EPOR expression, whilst increasing cell survival and decreasing apoptosis (Yu et al., 2002). In 2000, Brines and colleagues showed that EPOR was expressed abundantly on brain capillaries facilitating the transport of EPO across the blood-brain barrier (Brines et al., 2000). This study also revealed that systemic administration of EPO ameliorated brain injury after focal brain ischemia, concussion, experimental autoimmune encephalomyelitis and kainite toxicity (Brines et al., 2000). In a complimentary investigation, Tsai et al., confirmed reduced cell proliferation in the subventricular zone and impaired post-stroke neurogenesis when cells lacked EPOR expression. This brain specific deletion of EPOR thus lead to impaired migration of neuroblasts to the

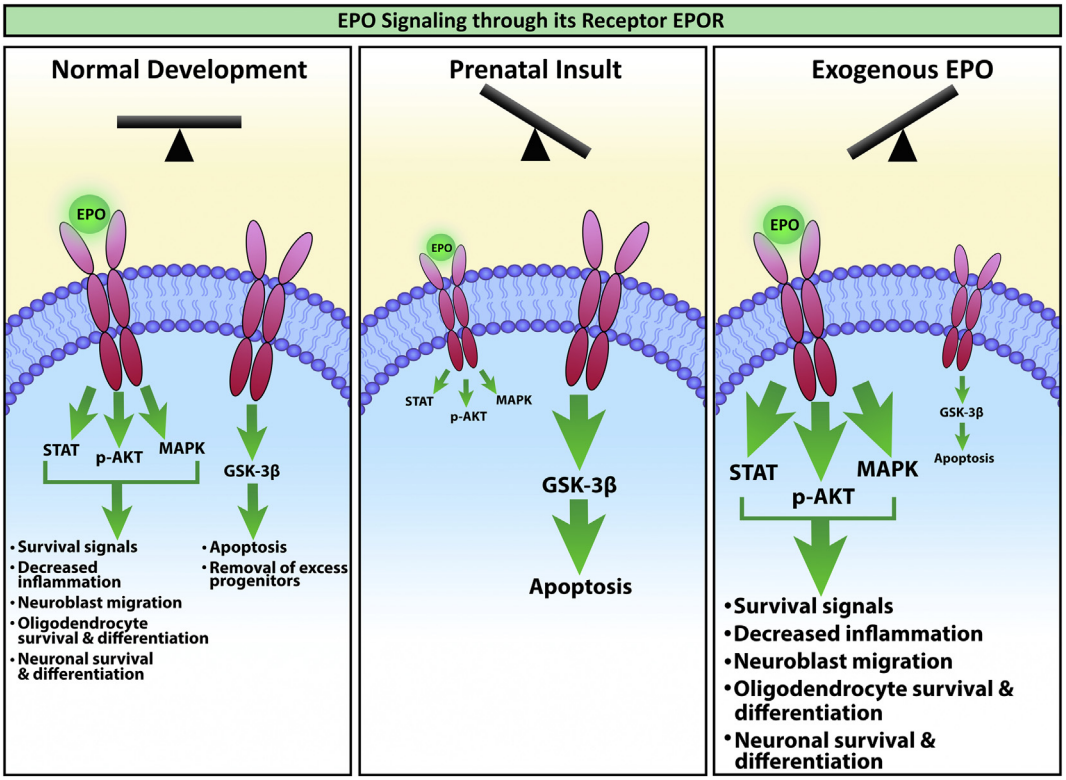

Fig. 1. Summary diagram representative of the equilibrium of erythropoietin (EPO) and its receptor in development, injury, and in response to exogenous EPO treatment.

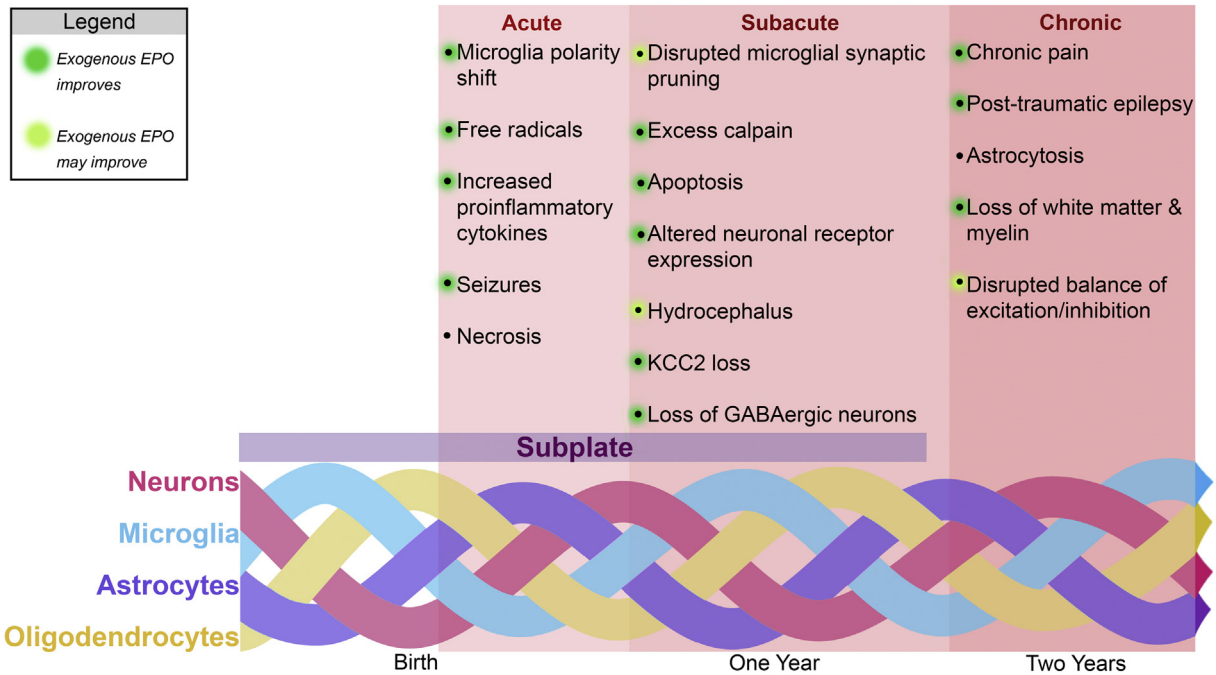

Fig. 2. Erythropoietin (EPO) has numerous beneficial mechanisms of action for neural repair. These mechanisms differ as brain injury progresses through acute, subacute and chronic injury phases. These processes are superimposed upon complex and interconnected developmental trajectories, including rapid periods of circuit formation involving the subplate and active myelination that require multiple cell lineages in a homeostatic microenvironment.

peri-infarct cortex following stroke in adult mice (Tsai et al., 2006). Sanchez and colleagues confirmed that the role of EPO/EPOR in neural cell health is approximately 10-fold greater prenatally, and gradually diminishes as the CNS matures, highlighting the importance of EPO/EPOR homeostasis and downstream signal transduction in the context of the developing brain (Sanchez et al., 2009). Significantly, during development the amount of EPO present dictates neural cell survival, including the number and maturation of neurons (Knabe et al., 2004; Knabe et al., 2005). If EPO ligand binds EPOR, the neural cell survives, whereas unbound EPORs trigger cell death through upregulation of

Table 2
Perinatal brain injury phase III clinical trials with extended regimens of rhEPO.

Acronym		Injury	Cumulative dose	Outcome interval	Results	Refs.	Safety
BRITE	NCT01207778	Preterm	mean ~9600 IU/kg	1.5 & 4 yrs	↓ CP, ↓ NDD, cognition	78,79	none
HEAL	NCT02811263	HIE	5000 IU/kg	2 yrs	pending	77	none
Song et al.	NCT02036073	Preterm	3500 IU/kg	1.5 yrs	↓ NDD & death	80	none
PENUT	NCT01378273	Preterm	~9600 IU/kg	2 yrs	pending	81	pending
EpoRepair	NCT02076373	Preterm IVH	10,000 IU/kg	5 yrs	pending	82	pending

Abbreviations: CP-cerebral palsy, HIE- hypoxic-ischemic encephalopathy, IVH-intraventricular hemorrhage, Preterm- brain injury associated with prematurity/encephalopathy of prematurity, NDD- neurodevelopmental disability.

GSK-3β which facilitates the initiation of apoptosis through formation of the mitochondrial permeability transition pore (Fig. 1) (Knabe et al., 2004; Knabe et al., 2005). These studies have been replicated in numerous preclinical models, and confirm that EPO/EPOR signaling regulates the genesis, survival and differentiation of both neuronal and oligodendroglial lineages during development (Fig. 2) (Sugawa et al., 2002; Park et al., 2006; Iwai et al., 2007; Ransome and Turnley, 2007; Iwai et al., 2010; Jantzie et al., 2013). EPO/EPOR signaling in the normal development of astrocytes and microglia has yet to be investigated in depth *in vivo*.

3. Mechanisms of EPO repair

In addition to pleiotropic effects during neurodevelopment, EPO/EPOR signaling has an essential role in endogenous neurorepair. Elevated endogenous EPO levels have been noted after perinatal brain injury (Spandou et al., 2004; Teramo and Widness, 2009; Logan et al., 2014; Sweetman et al., 2017; Teramo et al., 2018). While the biologic function of elevated endogenous EPO levels is not yet fully understood, it may reflect an innate response to prenatal and perinatal stressors, and an attempt to restore metabolic equilibrium and microenvironmental adaptation to hypoxia (Teramo et al., 2018). Similar to increased EPO ligand after injury, there is a sustained upregulation of EPOR on many neural cells, which often extends beyond 72 h post-injury (Spandou et al., 2004; Assaraf et al., 2007; Mazur et al., 2010; Messier and Ohls, 2014; Robinson et al., 2016). However, while EPOR levels are amplified after injury (Spandou et al., 2004; Keller et al., 2006), the magnitude of concomitant EPO ligand upregulation is insufficient to protect or repair neural cells after brain injury. Specifically, these endogenous elevations of EPO are several magnitudes below the plasma and CSF levels achieved with high dose regimens of exogenous EPO for neurorepair (Frymoyer et al., 2017). Thus, the central principle of EPO repair is to supplement this existing endogenous signaling cascade with administration of exogenous EPO (Fig. 1).

A complimentary principle of EPO therapy is to capitalize on the multiple, beneficial mechanisms of enhanced EPO/EPOR signal transduction (Table 1). Importantly, the numerous mechanisms of action for EPO/EPOR signaling after injury address the multiple, complex mechanisms of injury implicated in pediatric TBI (Kochanek et al., 2017). Notably, the incongruity between ligand and receptor balance is exacerbated at the extremes of the lifespan of the organism (Mazur et al., 2010; Lourhmati et al., 2013; Hernandez et al., 2017), supporting the concept that EPO therapy needs to be tailored specifically to the age of the patient population in question. Exogenous EPO crosses the blood-brain barrier (Brines et al., 2000; Juul, 2004; Xenocostas et al., 2005), and replenishes deficits in EPO ligand after injury to counterbalance injury-induced EPOR expression. Supplementation of EPO/EPOR signal transduction is implicated in the genesis, survival and maturation of all neural cells. EPO protects neurons and oligodendrocyte precursor cells after injury (Mazur et al., 2010; Kato et al., 2011; Jantzie et al., 2013). Furthermore, EPO induces both neurogenesis and oligodendrogenesis after injury to the brain (Zhang et al., 2012; Gonzalez et al., 2013; Jantzie et al., 2013). Significantly, the protective effect of EPO on specific neural cell types varies by the type of insult, the developmental age of the animal, and maturational stage of cells within their lineage (Chavez et al., 2006; Mazur et al., 2010; Jantzie et al., 2013; Bond and Rex, 2014; Pathipati and Ferriero, 2017). EPO therapy has also been shown to mitigate apoptosis (Yu et al., 2002), ferroptosis (Wu et al., 2019), caspase activation (Yu et al., 2002; Mazur et al., 2010), neuroinflammation (Mazur et al., 2010; Hellewell et al., 2013), oxidative stress (Genc et al., 2002; Kumral et al., 2005), endoplasmic reticulum stress and mitochondrial dysfunction (Xiong et al., 2009; Millet et al., 2016), and ubiquitin proteasome system imbalance (Table 1). EPO/EPOR signaling modulates apoptosis as well as autophagy *via* activation of mTOR (mechanistic target of rapamycin) (Maiese, 2016). Additionally, EPO/EPOR signaling has been shown to reduce neuroinflammation, including microglial activation, gliosis, and to reduce pro-inflammatory cytokines (Mazur et al., 2010; Hellewell et al., 2013). With respect to TBI in particular, controlled cortical impact (CCI) in EPOR-null mice aggravates functional deficits, and impairs cortical neurogenesis (Xiong et al., 2008). Administration of EPO reduces acute brain edema following TBI (Blixt et al., 2018), and damage from activated caspases and calpain are mitigated by EPO/EPO signaling in pediatric TBI (Table 2) (Schober et al., 2014a, 2014b; Robinson et al., 2016). After TBI, EPO/EPOR signaling also reduces endoplasmic stress and mitochondrial dysfunction (Xiong et al., 2009; Millet et al., 2016).

4. Therapeutic window

Extended periods of EPOR upregulation following injury suggests that a broad window for the administration of exogenous EPO exists (Messier and Ohls, 2014). In both PBI and pediatric TBI, the discrepancy between ligand and elevated EPOR levels appears to persist for weeks (Schober, 2010), and thus an extended course of EPO is likely necessary. Studies in a preclinical model of stroke have supported the concept of an extended window, by demonstrating that systemic EPO administration commencing one week after middle cerebral artery occlusion improved motor function when assessed 4 weeks post injury (Larpthaveesarp et al., 2016). Significantly, across the time courses of damage and recovery, exogenous EPO can shift its predominant mechanisms of action from reduction of oxidative and inflammatory stress, to promotion of neural cell development and regeneration of lost neural cells. Moreover, action by exogenous EPO can prevent the developmental arrest that is often observed after pediatric TBI. Thus, extended dosing regimens with repeated high doses have typically shown the most beneficial for repair (Mazur et al., 2010; Meng et al., 2011). For example, after early injury to the developing brain, a single dose of EPO had no effect on histology or function, whereas a moderate dosing regimen (1000 U/kg × 3 daily doses) improved histology but not function. High dose, extended therapy (2000 U/kg × 5 daily doses) mitigated both histological damage and functional deficits (Mazur et al., 2010). High dose, extended therapy, improved inhibitory neuron number, oligodendrocyte development and myelination, while also improving motor skills such as bar holding, and gait (Mazur et al., 2010). Similarly, after adult CCI, multiple doses provided both functional and histological improvement and mitigated hippocampal cell

Table 3
Preclinical infant CCI treatment with extended erythropoietin therapy

Reference	Histology	Biochemistry	Function	Imaging	Fluid biomarkers
Schober et al. (2014a) (CCI P17 & EPO 5000 U/k ip 1 h, 24 h, 48 h and 7d after CCI	-Increased CA3 neuron fraction with EPO treatment	-rhEPO present in rat pup brain -Changes in Bad, Bax, Bcl2, and caspase activity with EPO treatment	-EPO improved NOR at PID 14		
Schober et al., 2018 (CCI P17 & EPO 5000 U/kg ip 1 h, 24 h, 48 h and 7d after CCI)	-EPO prevented hippocampal volume loss	-EPO improved hippocampal BDNF mRNA expression	-EPO improved NOR at PID 50		
Robinson et al., 2016 (CCI P12 & EPO 3000 U/kg at 24 h, 48 h, 76 h, 96 h, 6d and 8d after CCI)		-Increased cortical EPOR expression 72 h post-injury -Increased alpha 2 spectrin ratio, 90 kDA KCC2 fragment, GFAP total and GFAP degradation products in cortex after CCI	-EPO reversed CCI induced abnormalities in gait, including stance and metrics in brake and propel phases	-EPO treatment improved MD, AD and RD in cortex, capsular white matter	-EPO attenuated increases in total GFAP, GFAP degradation products, tumor necrosis alpha and CXCL1 in serum following CCI.
Robinson et al. (2018b) (CCI P12 & EPO3000 U/kg at 24 h, 48 h, 76 h, 96 h, 6d and 8d after CCI))			-EPO reversed deficits in reversal learning, including perseveration on touchscreen cognitive testing -Correlation between poor cognitive flexibility and MD, AD and RD in multiple gray and white matter brain regions	- EPO treatment repaired MD, FA, AD and RD abnormalities at P30 induced by CCI in the medial frontal cortex, lateral frontal cortex, ventral frontal cortex, striatum ad corpus callosum. - EPO repaired CCI-induced abnormalities in FA at P90 in corpus callosum, external capsular white matter, lateral frontal cortex and medial frontal cortex.	

AD- axial diffusivity; CCI- controlled cortical impact; CXCL1- (C-X-C Motif) ligand 1; FA- fractional anisotropy; GFAP- glial fibrillary acidic protein; MD- mean diffusivity; NOR- novel object recognition; P- postnatal day; PID- post-injury day; RD- radial diffusivity;

loss, while enhancing angiogenesis and neurogenesis in the cortex and hippocampus (Xiong et al., 2010). Together, these studies highlight the importance of a therapeutic window and the benefits of early and late dosing. They also emphasize that while early administration may spare tissue and is reflected by histological improvement, late dosing is essential for functional improvement and thus potentially essential for the preservation of motor and cognitive function after TBI. These data also suggest that the addition of other endogenous therapies to EPO may be beneficial to counteract the complex, multifaceted pathophysiology of TBI. For example, the addition of an extended dosing regimen of melatonin in addition to EPO has demonstrated improved outcomes in conditions of complex preterm brain injury precipitated by chorioamnionitis compared to EPO alone (Jantzie et al., 2018; Robinson et al., 2018a).

5. Evidence from other CNS injuries: The mechanistic overlap between infant TBI and PBI

EPO has been used extensively in pediatric patients of all ages to treat anemia, and EPO has an excellent safety profile (Juul, 2012). Doses of rhEPO are administered in international units of biological activity (IU). The EPO doses for neurorepair are typically several-fold higher than those used for anemia. Extremely preterm infants with intraventricular hemorrhage (IVH) who received EPO treatment for anemia of prematurity had better outcomes at 10–13 years of age than those who were untreated (Neubauer et al., 2010). Interestingly, no difference in outcomes emerged in those extremely preterm infants who did not suffer IVH (Neubauer et al., 2010), emphasizing that the additional injury associated with IVH may have widened the discrepancy between EPO ligand and receptor, and the responsiveness to a relatively low dose exogenous EPO. In support of this concept, a short dosing regimen of postnatal EPO in very preterm infants failed to improve outcomes at 2 years corrected age (NCT#00413946) (Natalucci et al., 2016). However, because of the promising efficacy of extended courses of EPO in preclinical models of PBI (Juul, 2004; Mazur et al., 2010), and the excellent safety profile illustrated by early clinical trials (Fauchere et al., 2008; Juul et al., 2008), high-dose, extended EPO regimens have progressed to Phase III trials in neonates with brain injury (Table 2) (Leuchter et al., 2014). Specifically, extended moderate or high dose EPO regimens are being tested for efficacy in term infants with hypoxic-ischemic encephalopathy in the HEAL (High-Dose Erythropoietin for Asphyxia and Encephalopathy) trial (NCT02811263) (Juul et al., 2018). The small multi-center BRITE (Brain Imaging and Developmental Follow up of Infants Treated with Erythropoietin) trial (NCT01207778) of erythropoiesis-stimulating agents in very preterm infants has shown reduction of cerebral palsy (Ohls et al., 2014), and improvement in preschool age cognition and behavior.(Ohls et al., 2016). The much larger EpoRepair trial (NCT02036073) compared 330 infants who received a low-dose EPO regimen for an extended course (500 IU/kg every other day for 7 doses) to placebo, and found a significant reduction ($p < 0.001$) in death and moderate/severe neurological disability at 18 months (Song et al., 2016). The results from the PENUT (Preterm Erythropoietin Neuroprotection Trial) Phase III trial (NCT01378273) that enrolled > 940 extremely preterm infants are anticipated in 2019 (Juul et al., 2015). The EpoRepair study (NCT02076373) has randomized very preterm infants with intraventricular hemorrhage to 5 doses of EPO over an extended course, and results with 5 year outcomes are anticipated in a few years (Naulaers, 2018). Importantly, no adverse events have emerged with high-dose, extended EPO regimens, and EPO appears to have an excellent safety profile thus far in neonatal trials (Fauchere et al., 2008; Juul et al., 2008; Benders et al., 2014; Fauchere et al., 2015; Song et al., 2016). Together, these results from the closely related field of PBI emphasize that extended courses of EPO can impact long-term outcomes of neonates with early CNS injury.

6. Preclinical studies of exogenous EPO in infantile TBI

Numerous preclinical studies by independent research groups using extended, high dose, mechanistically-relevant EPO regimens using preclinical models of pediatric TBI have shown acute (Schober et al., 2014a; Robinson et al., 2016), subacute (Schober et al., 2014a; Robinson et al., 2016; Robinson et al., 2018b; Schober et al., 2018), and chronic recovery (Table 3) (Robinson et al., 2018b; Schober et al., 2018). Notably, the timing of corresponding neurodevelopmental processes in rodents and humans varies depending on the molecule, cell and circuit under study (Semple et al., 2013). In general, a human term infant is equivalent to a postnatal day 7 (P7) to P10 rat, whereas P20 is equivalent to a 2–3 year old toddler (Semple et al., 2013). Using a rat postnatal day 17 (P17) CCI model, equivalent to 1–2 year old infants, Schober and colleagues demonstrated that four doses of EPO (5000 U/kg/dose i.p.) over 7 days reduced early caspase activation in hippocampal neurons, and improved novel object recognition subacutely at two weeks following injury (Schober et al., 2014a). The same four dose regimen over 7 days of EPO after P17 CCI also improved novel object recognition 50 days after injury at P67, equivalent to young adulthood (Table 3) (Schober et al., 2018).

Using a preclinical model to recapitulate brain injury at an even younger time point, P12 rats, approximately equivalent to a few-months-old human infant, were administered six doses of high dose EPO (3000 U/kg/dose i.p.) over 8 days following CCI (Table 3) (Robinson et al., 2016; Robinson et al., 2018b). Acutely, vehicle-treated injured rats had elevated cortical expression of EPOR (Robinson et al., 2016). Additionally, signs of excess cerebral calpain activity were present, with increased levels of degradation products for neuronal αII-spectrin, KCC2 (potassium chloride cotransporter 2) and GFAP (glial fibrillary acidic protein) consistent with fragmentation of essential neural networks (Robinson et al., 2016). In the subacute period, juvenile vehicle-treated P12 CCI rats exhibited complex gait abnormalities, including dysfunction in stance width and the propel phase, that were prevented with extended EPO treatment (Robinson et al., 2016). To test cognition with clinically relevant outcome measures, non-aversive touchscreen operant chamber platforms were used to assess visual discrimination and reversal learning consistent with paradigms in the Cambridge Neuropsychological Test Automated Battery (CANTAB) (Bussey et al., 2012; Oomen et al., 2013). The assessment of visual discrimination and reversal learning used in this platform specifically examined multiple pillars of cognition. Adult rats from all three groups (sham, vehicle-treated P12 CCI, and EPO-treated P12 CCI) were able to successfully complete visual discrimination, demonstrating that the rats had the visual, motor and baseline cognitive skills to interact with the touchscreen and receive a reward from the dispenser for a correct response (Table 3) (Robinson et al., 2018b). Reversal learning was then tested to quantify the executive function of cognitive flexibility (Mar et al., 2013). Vehicle-treated CCI rats, but not sham or EPO-treated CCI rats, had significant difficulty with reversal learning (Robinson et al., 2018b). Specifically, the vehicle-treated CCI rats showed an inability to adapt to the new paradigm, and perseverated on the incorrect response (Robinson et al., 2018b). Together, these results show that extended, high dose EPO treatment administered after early CCI can prevent long-term cognitive deficits.

These findings satisfy the criteria listed by Huh and Ragupathi, and the consensus guidelines from the recent Moody Symposium for Translational Research in TBI. Specifically, the Moody Symposium guidelines state that agents for pediatric TBI should be tested with long-term, functionally-relevant outcomes, specific pathophysiological targeting, use of well-characterized experimental TBI models and improvement in outcome in more than one TBI model (DeWitt et al., 2018; Huh and Raghupathi, 2019). Because few preclinical models accurately capture the complexity and nuances of severe pediatric TBI, the concept of testing potential agents in multiple preclinical models of TBI is attractive. Testing a single dose of potential agents in adult TBI models

(Bramlett et al., 2016), however, fails to account for the specific molecular mechanisms of action for each agent, and more importantly, the developmental regulation of both the injurious mechanisms of pediatric TBI and the developmental differences in EPO/EPOR signaling during repair (Juul and Ferriero, 2014; Messier and Ohls, 2014; Huh and Raghupathi, 2019). More recently, preclinical studies of PBI that add extended dosing of melatonin to EPO show additional benefits with the combination therapy compared with EPO alone (Jantzie et al., 2018; Robinson et al., 2018a), suggesting that EPO may be one component of a neuro-reparative cocktail.

7. Differences between the developing and mature brain

Thus far, no clinical trials have tested the efficacy of high dose, extended EPO regimens after acute pediatric TBI. In particular, EPO has not been tested in the setting of infant TBI, where perhaps the molecular mechanisms hold the most promise due to the developmental regulation of EPO/EPOR signaling. More than thirty intervention trials for TBI have failed to demonstrate efficacy, including pediatric TBI trials (DeWitt et al., 2018). Not surprisingly, these trial failures have diminished enthusiasm for further TBI clinical trials, although numerous potentially modifiable problems have been identified (Bell et al., 2017; DeWitt et al., 2018). The ADAPT TBI (*A*pproaches and *D*ecisions in *A*cute *P*ediatric *T*BI *T*rial) trial aims to provide a better data set for future trial design (Bell et al., 2017), and to utilize machine learning to predict and address intracranial pressure elevations, which may also address the inherent variability of TBI patients, and make it easier to detect changes in outcome above the inherent clinical variation in severe TBI populations (Lazaridis et al., 2019). While there are some similarities between severe adult and pediatric TBI, the numerous differences in the etiology and pathophysiology strongly suggest that the developing brain is more susceptible to apoptosis and oxidative stress than the adult brain (Semple et al., 2013), and that EPO treatment may be worth considering in pediatric TBI to aid neurorepair. Long-term neurological responses to treatment after TBI is a dynamic process, and this is even more pronounced in the context of the developing CNS. Indeed, adult clinical trialists have recognized that failure to capture individual heterogeneity in recovery may impact findings from signal randomized controlled trials(Benoit et al., 2019). Thus, there is value in taking a comprehensive view of recovery as a temporally dynamic process shaped by both treatment and injury severity, thereby highlighting the importance to appropriately time outcome measure evaluations. In adults, effects of EPO treatment have varied as a function of injury severity and time (Benoit et al., 2019). Just as adult treatment regimens may not work effectively in pediatric TBI, the recent positive findings in perinatal brain injury may or may not carry over to success in clinical trials of infants and toddlers with TBI. As noted by Huh and Ragapathi, minocycline was found to be effective in adult TBI, but not pediatric TBI (Huh and Raghupathi, 2019), which serves as another example of how the developing brain differs from the mature brain after injury.

With respect to the adult EPO trials that have failed, dosing regimens were likely too low or of insufficient dosing intensity to produce neurological improvement. In addition to incomplete dosing regimens, single endpoints with differential timing of primary outcome measures, and lack of sensitive outcome measures despite severe injury at initiation of treatment (death, GOS-E level 1–4 improvement) (Nichol et al., 2015; DeWitt et al., 2018; Lazaridis et al., 2019), have contributed to trial failures (Benoit et al., 2019). Three meta-analyses of adult TBI trials of EPO treatment for TBI have recently been published (Li et al., 2016; French et al., 2017; Liu et al., 2017). All three reports reached the same conclusion that erythropoiesis-stimulating agents reduce mortality of patients with severe TBI compared to placebo, but that trials thus far have not shown any improvement in functional neurological outcome (French et al., 2017; Liu et al., 2017; Lee et al., 2018). Importantly, no adverse events were evident compared to placebo treatment (French et al., 2017, Liu et al., 2017, Lee et al., 2018). Both the Australian EPO-TBI trial (NCT00987454) and the US EPO trial (NCT003131716) tried to balance the perceived risks of complications in adults, including increased hematocrit and thromboses, with the need to achieve high-dose, extended therapy, which may have led to under dosing and lack of efficacy (Nichol et al., 2015). Li and colleagues randomized 159 adults with isolated severe TBI to receive either extended EPO dosing (post-injury day 1, 3, 6, 9 and 12 at a low dose of 100 U/kg/dose) or placebo, and found good recovery (GOS, Glasgow Outcome Score) at 3 months in 33% of the EPO-treated patients, compared with 12.6% of the control group (Li et al., 2016). In the Australian EPO-TBI study, post-hoc analysis found that EPO treatment significantly improved outcomes for a subgroup of patients with diffuse TBI that did not require a neurosurgical operation prior to admission to the intensive care unit (Skrifvars et al., 2017), presumably those who had diffuse TBI and not a mass-occupying lesion that required immediate craniotomy or decompressive craniectomy. Also, in those patients with multi-system trauma with extensive extracranial injury, EPO provided significantly reduced mortality (Skrifvars et al., 2017). Together, these findings emphasize the need for careful clinical trial design. Given that infants and young children with TBI who often have diffuse and widespread CNS injury, frequently also have extensive extracranial systemic injury and a high rate of mortality, EPO treatment may offer a promising adjunct to other advances in pediatric TBI care.

8. Clinically-relevant outcomes in infantile TBI: imaging biomarkers

Another challenge in conducting TBI trials in infants and young children is the lack of sensitive, reliable outcome measures. For example, while the GOS-E pediatric version was validated in children (Beers et al., 2012), and recommended in the Common Data Elements for pediatric TBI trials (McCauley et al., 2012), it is possible that the GOS-E may not to be sensitive enough to detect individual, clinically-relevant improvements in infants and toddlers at 30 days or 6 months after TBI in the context of their complex clinical course (SR, personal communication). Most detailed scales to assay infant development are designed for application at consistent developmental ages across a study, such as at two years equivalent after a neonatal injury. However, recent advances in imaging and fluid biomarkers may provide sensitive, reproducible representative indicators of functional outcomes. Advanced radiographic techniques offer an opportunity to quantify outcomes with heightened sensitivity than functional scales in infants and toddlers. As noted at the Moody Project for Translational TBI Research Symposium, imaging may provide more sensitive assessment of outcomes (DeWitt et al., 2018). Indeed, preclinical *ex vivo* imaging after infant CCI showed widespread microstructural abnormalities in multiple diffusion tensor imaging (DTI) parameters at 2.5 weeks after injury, and at 6 months after injury, and that this injury was prevented by treating with an extended course of post-injury EPO treatment (Robinson et al., 2016; Robinson et al., 2018b). Moreover, this improvement correlated with improved gait and cognition (Robinson et al., 2016, Robinson et al., 2018b). After unilateral CCI injury at P12, fractional anisotropy (FA) color maps show widespread loss of white matter at P90, an effect that is prevented by an extended rhEPO course (Fig. 3). These preclinical findings align well with the clinical literature. Lower FA in the corpus callosum and internal capsule is associated with poor cognitive and behavioral outcomes after pediatric TBI (Wilde et al., 2006; Wozniak et al., 2007; Yuan et al., 2007; Ewing-Cobbs et al., 2008; Tasker et al., 2010; Konigs et al., 2018). In addition to reduced FA, higher radial diffusivity (RD) is also associated with worse functional outcomes after significant pediatric TBI (Wilde et al., 2006; Yuan et al., 2007; Ewing-Cobbs et al., 2008; Ewing-Cobbs et al., 2016; Ressel et al., 2017). Widespread microstructural abnormalities in DTI also correlated with poor social cognitive outcomes (Ryan et al., 2018). One limitation of using DTI, is the difficulty in comparing DTI data across

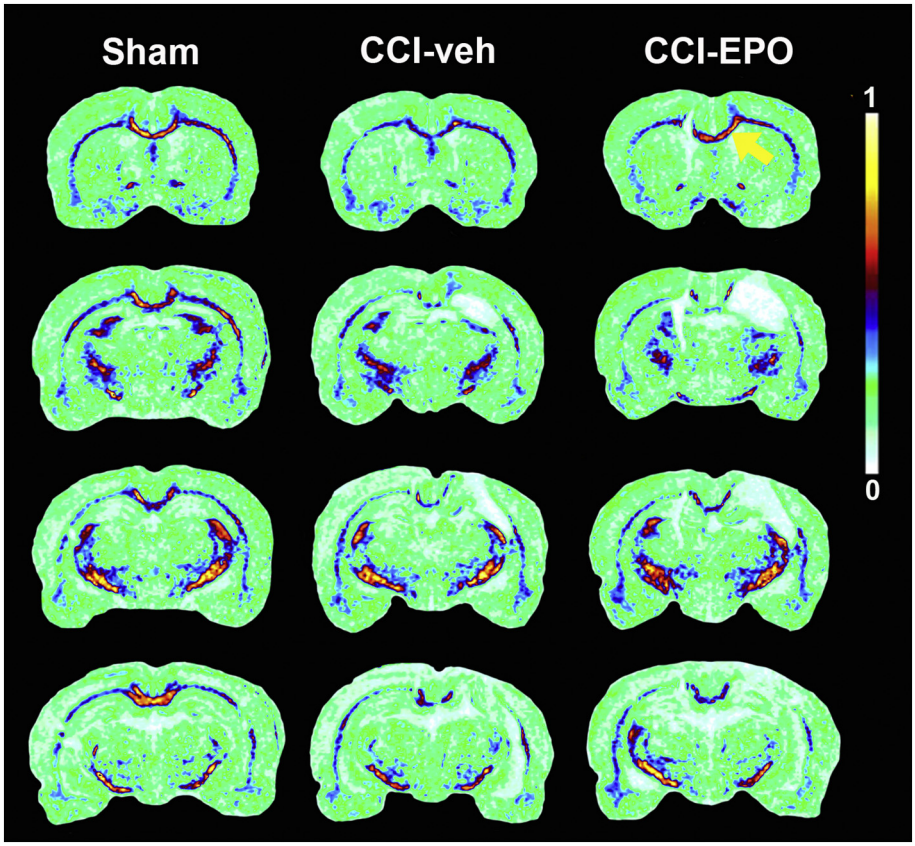

Fig. 3. Representative color coded fractional anisotropy (FA) maps at postnatal day 90 (P90) after P12 left controlled cortical impact injury show widespread, bilateral substantial loss of white matter that is largely prevented by an extended high dose rhEPO regimen. Most of the improvement after rhEPO treatment is in the corpus callosum (arrow) and white matter distant from the site of direct impact.

centers owing to the use of different scanners and imaging sequences and protocols specific to different manufacturers and research groups. With careful study design this challenge can likely be surmounted, as is being accomplished in the PBI field (Leuchter et al., 2014).

9. Clinically-relevant outcomes in infantile TBI: fluid biomarkers

Fluid biomarkers, particularly from serum or plasma, also offer a path toward identifying and tailoring interventions, including EPO therapy. One of the most promising biomarkers in TBI is GFAP, which can now be quantified in humans with an FDA-approved assay. Similarly, in preclinical studies, GFAP calpain degradation products (GFAP-DPs) have also been shown to be sensitive and specific after infant TBI (Robinson et al., 2016). Calpain is activated after pediatric TBI (Schober et al., 2014a; Robinson et al., 2016), and EPO is known to reduce calpain activity (Hempel et al., 2014; Jantzie et al., 2014; Jantzie et al., 2016; Robinson et al., 2016). In a preclinical study three days after CCI injury at P12, GFAP-DPs were elevated in both brain and serum (Robinson et al., 2016). Other promising fluid biomarkers are UCHL1 (ubiquitin carboxy-terminal hydroxylase L1), which is also now an FDA-approved assay for humans with TBI; pNF-H (phosphorylated neurofilament heavy chain); neuron specific enolase (NSE), neurogranin and S100β (Papa et al., 2013; Mondello et al., 2016; Abu Hamdeh et al., 2018). In the adult EPO-TBI clinical trial, not surprisingly, a single dose of EPO had no impact on early UCHL1 (ubiquitin carboxy-terminal hydroxylase L1) or pNF (phosphorylated neurofilament heavy chain) levels (Hellewell et al., 2018). By contrast, a study by Li et al. found a decrease in serum NSE and S-100β in EPO-treated patients compared to the control group (Li et al., 2016). While progress is being made in that more reliable, validated assays are available, we do not yet have a sensitive readout of injury, response to treatment, or repair in pediatric TBI.

10. Conclusions and future directions

Moderate to severe pediatric TBI, especially for infants and toddlers remains a vexing problem for these children, their families and for society, given the lifelong burden of chronic deficits. Extended regimens of high-dose rhEPO offer a promising candidate therapy option. Not only does rhEPO have an established, relatively safe profile for side effects, but the biological mechanisms of pediatric TBI and of rhEPO repair after injury are aligned. Clinically-relevant extended regimens have been tested in P12 and P17 preclinical CCI models, with biochemical, cellular signs of improvement (Schober et al., 2014b; Robinson et al., 2016; Schober et al., 2018), and preclinical studies in larger models of pediatric TBI are underway. Importantly, both groups using rodent models demonstrated long-term functional improvement, including improvement to cognition (Robinson et al., 2018a; Schober et al., 2018). Brain injury elucidated in preclinical models using advanced imaging with DTI, that shows chronic damage patterns reminiscent of children who underwent neuroimaging in late follow-up after TBI, was prevented by rhEPO treatment after the initial injury (Robinson et al., 2018b). The extensive use of extended high-dose EPO therapy in clinical trials in PBI, which likely closely mimics many mechanisms of pediatric TBI injury and repair in infants and toddlers, is yielding promising results in both safety and efficacy. Together, this body of existing literature fulfills many of the criteria recently suggested to advance the progress of preclinical research in pediatric TBI (DeWitt et al., 2018; Huh and Raghupathi, 2019). While the field of pediatric TBI research has not always advanced at a pace that allowed for immense progress in our daily experience of treating these children, our patients and their families deserve rigorous, mechanistically-sound research guided by an appreciation for the nuances of the developing brain.

Conflicts of interest

The authors have no conflicts of interest.

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