Promoting neuroregeneration after perinatal arterial ischemic stroke: neurotrophic factors and mesenchymal stem cells

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Newborns suffering from perinatal arterial ischemic stroke (PAIS) are at risk of neurodevelopmental problems. Current treatment options for PAIS are limited and mainly focus on supportive care, as presentation of PAIS is beyond the time window of current treatment strategies. Therefore, recent focus has shifted to interventions that stimulate regeneration of damaged brain tissue. From animal models, it is known that the brain increases its neurogenic capability after ischemic injury, by promoting neural cell proliferation and differentiation. However, neurogenesis is not maintained at the long term, which consequently impedes full repair leading to adverse consequences later in life. Boosting neuroregeneration of the newborn brain using treatment with neurotrophic factors and/or mesenchymal stem cells (MSCs) may be promising novel therapeutic strategies to improve neurological prospects and quality of life of infants with PAIS. This review focuses on effectiveness of neurotrophic growth factors, including erythropoietin, brain-derived neurotrophic factor, vascular endothelial growth factor, glial-derived neurotrophic factor, and MSC therapy, in both experimental neonatal stroke studies and first clinical trials for neonatal ischemic brain injury.

50–75% of infants, neonatal stroke leads to abnormal motor and neurodevelopmental outcome, including cerebral palsy, cognitive dysfunction, behavioral disorders, and epilepsy (5). Current treatment options for PAIS mainly focus on supportive care, such as controlling hypoglycemia and treatment of (subclinical) seizures. As these treatments offer limited protection, additional therapeutic strategies for PAIS are urgently needed. Early neuroprotective treatments mainly aim at preventing production of free radicals and apoptotic cell death. For example, therapeutic hypothermia, started early after birth, has shown benefit in newborns with hypoxic–ischemic (HI) encephalopathy (HIE) (7). However, hypothermia is only beneficial when applied within 6 h after a well-documented HI insult followed by moderate-to-severe encephalopathy (8). As cerebral abnormalities evoked by PAIS often do not present themselves within this short therapeutic timeframe, these neuroprotective treatment strategies are not applicable to PAIS. Owing to the relative late diagnosis, therapeutic focus has rather shifted to interventions that stimulate repair of the damaged newborn brain (9). In most recent years, preclinical research has aimed to develop additional therapies that boost endogenous regenerative pathways, as these will be critical for improving outcome in severely affected PAIS patients.

Perinatal arterial ischemic stroke (PAIS) is an important cause of perinatal morbidity. It occurs in 1 in 2,500–4,000 live births, forming a large burden for patients and society worldwide (1,2). PAIS manifests itself most often with one-sided seizures in the first week after birth, often accompanied with (asymmetric) hypotonia, lethargy, and apnea (1,3,4). Using neuro-imaging, it is commonly identified as a focal wedge-shaped cerebral lesion that leads to loss of all cell types in that part of the brain. PAIS is most often detected in the middle cerebral artery (MCA) with a predilection for the left MCA (3–5). The exact underlying pathology is unknown, but the most important risk factors are intrapartum or peripartum complications, such as prolonged rupture of membranes, thick meconium, and abnormal cardiotocography (6). In

NEUROREGENERATION

Neural stem cells (NSCs) residing in the subventricular zone and subgranular zone of the hippocampus are self-renewing and are capable of differentiating into neurons, astrocytes, and oligodendrocytes. These processes of neurogenesis and gliogenesis continue throughout life but decrease with age. After (hypoxic) ischemic brain injury, the brain enhances its neurogenic capability by promoting proliferation of young precursors not only in the subventricular zone and subgranular zone but also in the striatum, cortex, and hippocampus (10,11). Growth factors, which regulate several cellular processes including apoptosis, inflammation, angiogenesis, cell differentiation, and proliferation, are increasingly expressed following exposure to HI and thereby aid in

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Table 1. Neurotrophic factor therapy in animal models of neonatal stroke

Paper	Growth Factor	Animal model	Type of ischemic injury	Dosing and timing of treatment	Administration route	Effect
Dixon and Sherrard (40)	BDNF	Neonatal rat: P15 or P30	Unilateral transection of olivocerebellar pathways	1 dose of 1 µl of rh-BDNF (4 µmol/l) at 24 h after injury	Intracerebellar injection	BDNF induced axonal growth by transcommissural olivocerebellar reinnervation at 7 days after injury
Husson <i>et al.</i> (42)	BDNF	Neonatal mouse: P5	Focal excitotoxic lesions: periventricular leukomalacia	1 dose of 0.5, 5, or 50 ng rh-BDNF simultaneously with excitotoxic drugs	Intracerebral (intraparenchymal)	BDNF reduced 36% and 60% of cortical and white matter lesions, involving TrkB receptors, MAPK pathway and reduced apoptosis at 120 h after injury. At P0, BDNF exacerbated neuronal death. At P10, BDNF did not have an effect
Bemelmans <i>et al.</i> (127)	BDNF	Neonatal mouse: P5	Focal excitotoxic lesions: periventricular leukomalacia	1 dose of 10 ng or 25 ng of LV-BDNF (with BDNF level reaching 0.086–0.118 pg/mg protein) 3 days before inducing lesions (P2)	Intraventricular delivery of BDNF-expressing lentiviral vector	BDNF induced protection against ischemic injury at 5 days after injury. Viral-mediated gene transfer was more efficient for neuroprotection than the intraparenchymal route
Sola <i>et al.</i> (26)	EPO	Neonatal rat: P7	Permanent MCAO	1 dose of 1,000 U/kg rh-EPO at 15 min after injury and 2 doses of 100, 1,000, or 5,000 U/kg once a day for 2 days	Intraperitoneal	EPO reduced the number of apoptotic cells and decreased infarct area and volume at 3 days after injury providing significant neuroprotection. 1,000 U/kg rh-EPO for 3 days was the most effective dose
Chang <i>et al.</i> (29)	EPO	Neonatal rat: P10	Transient MCAO: 45 min	1 dose of 5 U/g rh-EPO immediately upon reperfusion	Intraperitoneal	EPO preserved hemispheric brain volume and improved functional outcome (by decreasing forelimb asymmetry) at 2 weeks after injury
Gonzalez <i>et al.</i> (27)	EPO	Neonatal rat: P10	Transient MCAO: 45 min	1 dose of 5 U/g rh-EPO immediately upon reperfusion	Intraperitoneal	EPO preserved hemispheric brain volume, increased neurogenesis and decreased gliogenesis at 6 weeks after injury
Gonzalez <i>et al.</i> (28)	EPO	Neonatal rat: P10	Transient MCAO: 45 min	1 dose of 5 U/g rh-EPO immediately upon reperfusion or 1 dose of 1 U/g immediately upon reperfusion followed by 1 U/g at 24 h and 7 days after injury	Intraperitoneal	EPO increased brain volume and improved functional outcome (spatial learning and memory performance) at 2–4 months after MCAO
Larphaveesarp <i>et al.</i> (31)	EPO	Neonatal rat: P10	Transient MCAO: 180 min	3 doses of rh-EPO 1,000 U/kg started one week after injury	Intraperitoneal	Delayed EPO treatment improved histological and functional outcome at 4 weeks after MCAO
Wen <i>et al.</i> (128)	EPO	Neonatal rat: P7	Permanent MCAO	3 doses of rh-EPO 1,000 U/kg started 15 min. after injury and repeated once a day for 2 days	Intraperitoneal	EPO reduced infarct volume and improved sensorimotor function recovery at 6–12 weeks after MCAO. The effect was more beneficial in female compared with male rats
Dzietko <i>et al.</i> (51)	VEGF	Neonatal rat: P10	Transient MCAO: 90 min	1 dose of 1.5 µg/kg rh-VEGF at day 8 after injury (P18)	Intracerebroventricular	VEGF enhanced angiogenesis, endothelial proliferation, and vessel volume leading to improved brain injury recovery at 1 week after injury

Table 1 Continued

Paper	Growth Factor	Animal model	Type of ischemic injury	Dosing and timing of treatment	Administration route	Effect
Matheson et al. (60)	GDNF	Neonatal rat: P1	Sciatic nerve axotomy	Pretreatment: 1 dose of 25 µl of GDNF (1 mg/ml). Postinjury: Gelfoam soaked in 1 mg/ml GDNF immediately after injury; and reinjection with 5 µl (1 mg/ml) on P4, P5, and P6	Pretreatment: subcutaneous. Postinjury: implantation of GDNF-soaked Gelfoam into nerve stump	GDNF improved neuronal survival in dorsal root ganglions to nearly 100% at 7 days after injury
Morcuende et al. (41)	GDNF and BDNF	Neonatal rat: P0 (6–24 h old)	Extraocular motoneuron axotomy	BDNF and GDNF both 1 dose of 5 µg immediately after injury	Implantation of BDNF or GDNF-soaked Gelfoam into orbit	BDNF and GDNF both rescued extraocular motoneurons at 30 days after injury. GDNF was more potent for survival than BDNF. Combining GDNF and BDNF was not more effective

BDNF, brain-derived neurotrophic factor; EPO, erythropoietin; GDNF, glial-derived neurotrophic factor; LV-BDNF, lentiviral-mediated gene transfer of BDNF; MAPK, mitogen-activated protein kinase; MCAO, occlusion of the middle cerebral artery; P, postnatal day; TrkB, tyrosine kinase receptor B; VEGF, vascular endothelial growth factor; rh, recombinant human.

boosting neurogenesis (12). However, long-term neurogenesis is not maintained after HI brain injury, which consequently impedes full repair. It is therefore crucial to assist the brain during regeneration of injured areas. Stimulation of neurogenesis by exogenous administration of neurotrophic factors has been studied in the context of neonatal brain injury, especially in preclinical research. Furthermore, boosting endogenous neuroregeneration by administration of stem cells has also gained considerable attention in the treatment of neonatal brain injury. Therefore, this review will provide an up-to-date evaluation of preclinical and clinical evidence for potential future neuroregenerative therapies for neonatal stroke comprising neurotrophic factors and stem cells.

ANIMAL MODELS

When investigating neonatal brain injury, the use of highly reproducible and clinically relevant animal models is crucial. The most commonly used rodent model for neonatal brain injury is the Rice–Vannucci model, which combines permanent unilateral carotid artery ligation with exposure to systemic hypoxia in newborn pups (13,14). This model, however, contains a systemic hypoxic component that better reflects HIE caused by perinatal asphyxia in human neonates rather than PAIS. Therefore, specific models of neonatal stroke have been designed in newborn rodents that use permanent or transient occlusion of the MCA (MCAO) leading to (more) focal ischemia. Studies have shown contrasting variability between HI and stroke models and also the pathophysiological underlying mechanisms differ between HIE and stroke (15). As this review aims at covering specifically PAIS, the experimental data discussed in this paper will focus on regenerative therapies in animal models of neonatal stroke only.

PROMOTING NEUROREGENERATION: ANIMAL MODELS OF NEONATAL STROKE

Erythropoietin

Erythropoietin (EPO) is originally known for its role in erythropoiesis and it has long been used to treat anemia in premature infants (16,17). However, non-hematopoietic effects of EPO have also been shown. EPO is produced in the developing brain by multiple cell types, including neurons, astrocytes, oligodendrocytes, and microglia, and it promotes growth of the central nervous system (18,19). EPO is upregulated after cerebral injury, a process regulated by transcription factor hypoxia-inducible factor-1, a factor stabilized by hypoxic conditions (20,21). *In vitro*, EPO has been proven to exert neuroprotection against neuronal injury as it can reduce free radical formation, inflammation, and apoptosis in neuronal cultures (22). *In vitro* and *in vivo* studies have demonstrated that EPO not only prevents ischemia-induced cell death (i.e., acts neuroprotective) but also stimulates neuronal differentiation of neural progenitor cells (23–25). Experimental rodent studies using neonatal stroke models have shown that treatment with EPO substantially reduces infarct volume (26,27) as well as improves motor and cognitive function (28,29). A review from our group

summarized neuroregenerative effects of EPO in neonatal experimental *in vivo* studies, including MCAO models (30). In general, EPO administration after MCAO in postnatal day (P) 7–10 rat pups was found to improve neurogenesis, as measured by increased brain volume up to 70% (Table 1) (30). More recently, it was shown that delayed EPO treatment, up to 1 week after the onset of neonatal stroke, improved histological as well as functional outcome, which underlines the involvement of EPO as a trophic factor stimulating neurogenesis (31). Gonzalez *et al.* (32) also demonstrated that EPO treatment after neonatal stroke in rats stimulated neural progenitor cells' proliferation in the subventricular zone and migration of these progenitors to the site of the injury, again emphasizing the neuroregenerative effects of EPO.

Brain-derived neurotrophic factor

Brain-derived neurotrophic factor (BDNF) is a neurotrophic factor known to promote neural cell proliferation and survival in the developing human brain (33,34). BDNF protein expression is especially high in the hippocampus, but BDNF can affect survival and proliferation of several neural cells, including cerebellar and cortical neurons (33,35). The neurogenic effects of BDNF are mediated via activation of two different receptor pathways: the p75 neurotrophin receptor and the tyrosine kinase receptor B, that activates mitogen-activated protein kinase pathways. BDNF levels rapidly increase in response to brain injury in neonatal rats (36,37), leading to reduced neuronal apoptosis and increased neuronal survival (37). *In vitro*, BDNF improves survival of hypoxic–hypoglycemic hippocampal neurons by reduction of apoptosis through tyrosine kinase receptor B (38). Other *in vitro* studies demonstrated that BDNF enhanced neurite outgrowth of neonatal cortical neurons in the presence of astrocytes (39).

To the best of our knowledge, BDNF has never been studied in neonatal rodent models of PAIS. However, in neonatal rat models of focal cerebellar injury, BDNF treatment stimulated axonal regrowth leading to re-innervation of the cerebellum (Table 1) (40). In addition, a neonatal rat model for motorneuron axotomy demonstrated the beneficial effect of BDNF on motor neuron survival up to 30 days after injury (41). A neonatal mouse model mimicking periventricular leukomalacia in preterm newborns showed that intraparenchymal injections of BDNF (at P5) reduced cortical gray and white matter lesions with 36% and 60%, respectively. Protective effects of BDNF treatment in this excitotoxic mouse model were associated with tyrosine kinase receptor B receptor/mitogen-activated protein kinase pathway activation and reduced apoptosis (42). However, timing of BDNF administration seemed crucial, as BDNF treatment at P0 exacerbated neuronal death but BDNF treatment at P10 did not have any effects on periventricular leukomalacia in neonatal mice (42). Therefore, more preclinical evidence for beneficial effects of exogenous BDNF administration is needed to determine the exact treatment regimen and possible risks for neonatal stroke.

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is a factor produced by neurons and astrocytes in the developing brain. Experimental data indicate that VEGF is involved in several stages of neurodevelopment, including migration, differentiation, synaptogenesis, and myelination (43). Furthermore, VEGF stimulates vascular processes such as angiogenesis and vasculogenesis by stimulating endothelial cell proliferation and migration via Flk1 and by improving vascular stabilization via Ang1 (44,45). VEGF also enhances blood–brain barrier maintenance. These vascular processes together contribute to an optimal microenvironment for NSCs to drive neuronal regeneration, thereby demonstrating an important role for VEGF in brain repair (46,47). Experimental studies demonstrate that VEGF expression is increased in the brain after neonatal stroke *in vivo* (48). Interestingly, increased VEGF expression after neonatal cerebral ischemia is associated with NSC proliferation and differentiation (49). Furthermore, it has been shown that inhibition of the VEGF receptor-2 after neonatal stroke worsened injury, increased cell death, and reduced endothelial cell proliferation in 10-day-old rats, indicating a role for VEGF signaling in recovery and repair after ischemic brain injury (50).

Dzietko *et al.* (51) have shown that intracerebroventricular VEGF treatment enhanced angiogenesis, endothelial proliferation, and vessel volume, leading to improved recovery of brain injury after MCAO in P10 rats (Table 1). Experimental adult stroke models have demonstrated that timing of VEGF administration is crucial: early administration leads to brain edema, whereas late application has desired neuroprotective effects (52). In contrast, in newborn rats with HI early VEGF administration (i.e., at 5 min to 3 days after injury) resulted in decreased brain damage, possibly via reduced neuronal apoptotic cell death in the cortex and hippocampus (53,54). These studies indicate that in the neonatal brain VEGF does not only support neuroregeneration after brain injury via angiogenesis but may also exert neuroprotective properties by reducing apoptosis via the Akt/extracellular signal-regulated kinase signaling pathway. Overall, late administration of VEGF seems most favorable in neonatal stroke, as diagnosis is usually late after assumed onset of PAIS. However, the exact timing of VEGF for neonatal stroke remains to be studied.

Glial-derived neurotrophic factor

Glial-derived neurotrophic factor (GDNF) is a member of the transforming growth factor β superfamily, produced by glial cells and neurons, and has an important role in neuronal differentiation during normal development (55). *In vitro*, GDNF was found to increase the number of surviving neonatal rat corticospinal motor neurons (56). After central or peripheral nervous system injury, GDNF also promotes survival and recovery of several types of mature neurons, including motor and dopaminergic neurons (57,58). In neonatal rats with HI brain injury, GDNF levels in the serum and brain were upregulated by 48 h and returned to normal by 7 days (59). Increased GDNF levels were associated with

reduced neuronal apoptosis, indicating that GDNF may reduce neonatal brain injury (59).

Although GDNF has not been studied in the treatment of neonatal stroke in rodents (Table 1), GDNF treatment induced nearly 100% neuronal survival of dorsal root ganglions after sciatic nerve axotomy in newborn rats (60). In neonatal rats, GDNF was also found to rescue extraocular motoneurons from axotomy-induced cell death at 30 days after injury (41). To translate GDNF treatment to the clinic for the treatment of PAIS, more preclinical research is needed to overcome several potential hurdles. For example, in contrast to EPO and BDNF, GDNF does not cross the blood–brain barrier, which makes exogenous administration of GDNF more difficult (61). Additionally, GDNF exerts only transient effects, so repeated administration into the cerebral or ventricular space would be required (62).

Mesenchymal stem cell therapy

Overall, the levels of growth factors that are naturally available during brain development and that are upregulated after an ischemic insult are unable to accomplish full repair of the injured neonatal brain. As described above, boosting neuroregeneration after neonatal brain injury by exogenous administration of single growth factors has been shown to be beneficial in many experimental studies. These factors can stimulate repair of the neonatal brain by promoting neurogenesis and stimulating neural cell survival (Figure 1). Moreover, numerous studies have been performed to explore the potential of multipotent stem cells as a therapy for neonatal brain injury. Experimental data strongly indicate that stem cells secrete a plethora of trophic factors that can boost neuroregenerative processes in the injured neonatal brain.

Multipotent stem cells are capable of self-renewal and can commit to differentiate into cell types of a discrete lineage. For example, hematopoietic stem cells give rise to several blood cell types, such as erythrocytes, lymphocytes, and neutrophils. Other multipotent stem cell types include NSCs and mesenchymal stem cells (MSCs). MSCs can differentiate into cells of the mesoderm, such as bone, cartilage, or fatty tissue, but it has been demonstrated that MSCs are also capable of developing into neuronal cells, given specific conditions (63). MSCs display various characteristics that can be favorable as a regenerative therapy for neonatal brain injury. First, MSCs are found in several birth-related tissues, including the placenta, umbilical cord, and Wharton's Jelly (64). These resources seem ideal for easy and non-invasive isolation of MSCs and allow them to be used in an autologous manner if collected at the time of birth. Moreover, MSCs do not express MHC class II, making them excellent candidates for allogeneic transplantation, as they do not cause immune responses/graft-vs.-host disease (65). Given their potent neuroregenerative properties and favorable immunological profile, MSCs seem to be promising for neuroregenerative medicine in neonatal brain injury (64). In animal models of neonatal brain injury, MSC therapy has been shown to improve motor function and cognitive behavior by stimulating neurogenesis, gliogenesis,

and axonal remodeling as will be discussed below. Therefore, MSCs could function as miniature factories of a mixture of growth factors to repair neonatal brain injury in a tailor-made way.

MSCs have been shown to be effective in repairing brain tissue after neonatal stroke (Table 2). Kim *et al.* (66) treated P10 rats intracerebroventricularly with umbilical cord-derived MSCs at 6 h after permanent MCAO. At day 28, they observed that MSC transplantation had significantly attenuated brain infarct volume measured by magnetic resonance imaging and had improved functional motor performance (66). Other studies have focused on non-invasive routes of MSC administration, such as intranasal MSC treatment, which specifically targets the brain (67). It has been shown that MSCs migrate rapidly toward the lesioned brain area, i.e., within 2 h, after intranasal administration (68,69). Wei *et al.* (70) intranasally administered bone marrow-derived MSCs to P7 rat pups at 6 h and 3 days after induction of stroke. At P24, MSC treatment had significantly reduced infarct volume and blood–brain barrier disruption and increased angiogenesis, leading to neurovascular repair and improved cerebral blood flow. MSC treatment also stimulated neurogenesis, leading to better sensorimotor and social functions (70). In addition, van Velthoven *et al.* (71,72) showed that intranasal MSC treatment at 3 days after neonatal stroke also significantly reduced infarct size and white and gray matter loss in newborn rats, leading to improved motor performance at 28 days after the infarct.

From a mechanistic point of view, it is important to note that the number of MSCs at the lesion site drastically decreases at 12 h after intranasal administration and the majority of cells does not survive >72 h (69,73). These findings indicate that the regenerative effects of MSCs in the neonatal brain are not caused by integration of transplanted MSCs themselves but rather by their paracrine effects. It was demonstrated that MSC administration after neonatal HI injury in mice specifically regulates cerebral expression of genes regulating both proliferation and survival (73). *In vitro*, MSCs that promote axon growth in developing rats were shown to express BDNF and VEGF (74). Furthermore, MSCs that were cultured in the presence of ischemic vs. control brain extracts show a specific upregulation of several growth factors, indicating that MSCs can adapt their secretion profile according to the tissue milieu (75). We hypothesize that the secretion of several neurotrophic factors by MSCs modulates the neurovascular niche to promote endogenous repair of the injured neonatal brain.

ANIMAL MODELS OF ADULT STROKE

In parallel to neonatal stroke, quite extensive preclinical and clinical research has been performed to study neuroregenerative therapies for the treatment of adult ischemic stroke. It is important to realize that results from adult trials cannot be directly translated to neonatal care for several reasons. First of all, the newborn brain has much greater plastic capacity than the brain of older children and adults. This causes the

Figure 1. The effects of neurotrophic factors on neurogenesis and gliogenesis. Neural stem cells from the subventricular zone differentiate into two lineages of progenitor cells: glial and neural progenitor cells, resulting in gliogenesis and neurogenesis respectively. Erythropoietin (EPO) stimulates neuronal differentiation of neural progenitor cells. Glial-derived neurotrophic factor (GDNF) is also involved in neurogenesis by increasing the number of neurons but additionally promotes survival and recovery of several types of mature neurons. Brain-derived neurotrophic factor (BDNF) is involved in neural cell proliferation and survival. Vascular endothelial growth factor (VEGF) mainly stimulates vascular processes, such as angiogenesis and vasculogenesis.

newborn to recover relatively more easily after brain injury than an adult (76,77). Second, effectiveness of neuroregenerative treatments reduces with growing age, because endogenous neurogenesis capacity declines with age. In other words, the neonatal brain has more potential to regenerate damaged tissue than the adult brain (78–81). Lastly, PAIS is a different disease than adult stroke, with different pathology and symptomatology (82). Despite these differences, the results from experimental adult stroke studies provide biological plausibility support for potential effectiveness of interventions in PAIS and will therefore be discussed shortly below.

Neurotrophic factors

Preclinical studies in rodent models of adult stroke have also shown potential effectiveness of administration of the discussed

factors EPO, BDNF, VEGF, and GDNF in stroke recovery (83–87). However, neuroregenerative effects of many more (hematopoietic) growth factors, such as granulocyte colony-stimulating factor, epidermal growth factor, fibroblast growth factor-2, and insulin-like growth factor were described for adult stroke as well. All these factors are upregulated after focal cerebral ischemia and potentially have a neuroregenerative role by stimulating neurogenesis and angiogenesis and inhibiting neuronal death (88). A review from 2011 provides an overview on experimental studies of acute ischemic stroke in adult rodents using hematopoietic growth factors and neurotrophins. The review summarizes results of studies which demonstrate that these factors both reduce infarct size, brain edema, and apoptosis, as well as stimulate cell proliferation, survival of new mature neurons, and neovessel formation, subsequently leading to improved clinical outcome (88). The authors conclude that

Table 2. Mesenchymal stem cell therapy in animal models of neonatal stroke

Paper	Therapy	Animal model	Type of ischemic injury	Dosing and timing of treatment	Administration route	Effect
Kim et al. (66)	Human umbilical cord blood-derived MSCs	Neonatal rat: P10	Transient MCAO: 60 min	1 dose of 1×10^5 MSCs in 10 μ l of PBS at 6 h after injury	Intraventricular	MSC transplantation decreased brain infarct volume (by MRI), improved histological abnormalities and improved functional motor outcome
Wei et al. (70)	Rat hypoxic-preconditioned bone marrow-derived MSCs	Neonatal rat: P7	Permanent MCAO	2 doses of 1×10^6 MSCs in 100 μ l of PBS at 6 h and 3 days after injury	Intranasal	MSCs reduced infarct size, promoted angiogenesis, neurogenesis, neurovascular repair, and improved local blood flow. Additionally, MSCs improved sensorimotor function at 17 days after injury
Van Velthoven et al. (72)	Rat MSCs (GIBCO, bone marrow-derived?)	Neonatal rat: P10	Transient MCAO: 90 min	1 dose of 1×10^6 MSCs in 20 μ l PBS (as 2 doses of 5 μ l per nostril) at 3 days after injury	Intranasal	MSCs reduced infarct volume, white and gray matter loss, and improved motor deficits until 28 days after injury
van Velthoven et al. (71)	Rat MSCs (GIBCO, bone marrow-derived?)	Neonatal rat: P10	Transient MCAO: 90 min	1 dose of 1×10^6 MSCs in 20 μ l PBS (as 2 doses of 5 μ l per nostril) at 3 days after injury	Intranasal	MSCs attenuate white matter injury (on MRI) and enhance somatosensory function 28 days after injury

MCAO, occlusion of the middle cerebral artery; MRI, magnetic resonance imaging; MSC, mesenchymal stem cell; P, postnatal day; PBS, phosphate-buffered saline.

these growth factors were shown to be potentially effective in experimental models of adult stroke, but the therapeutic potential of many of these growth factors still needs to be investigated in experimental neonatal stroke. Although a few reports are available on the beneficial effects of granulocyte colony-stimulating factor (89–91), basic fibroblast growth factor (92) and insulin-like growth factor-I (93,94) in neonatal HI brain injury, evidence is limited to date and therefore beyond the scope of this review.

MSC therapy

A large meta-analysis from Vu *et al.* (95) identified 46 studies that report on the use of MSC treatment in animal models of adult stroke. Of these, 44 reported that MSCs significantly improved neurological outcome. Effect sizes varied significantly with administration route and species and had a median of 0.9 for reduction of infarct volume to 1.8 for Neurological Severity Score. The authors concluded that the effect of MSC therapy was very robust and consistent over different studies, species, routes, and treatment protocols, and translation of MSC treatment in ischemic stroke in (adult) humans should be further enhanced (95).

PROMOTING NEUROREGENERATION IN NEONATAL CLINICAL TRIALS

Neurotrophic factors

EPO has been studied most often clinically in the context of neonatal brain injury. Our group recently performed a clinical study in 20 full-term infants with PAIS, who were treated with three doses of recombinant human erythropoietin (rhEPO) 1,000 U/kg intravenously (96). Most importantly, no adverse effects of rhEPO were observed. Furthermore, volumetric magnetic resonance imaging measurements of the stroke area were smaller in 10 rhEPO-treated infants compared with 10 non-treated PAIS historical controls, but this effect did not reach significance (96). A randomized controlled trial (RCT) from our group is currently undertaken to show the effect of darbepoetin on stroke tissue loss and neurodevelopmental outcome in PAIS patients (ClinicalTrials.gov: NCT03171818). Other groups have described the use of rhEPO or darbepoetin in the context of HIE (97–100). A few RCTs are currently studying full-term infants with perinatal asphyxia who are receiving hypothermia with rhEPO as an add-on therapy and the results are promising (101,102). Most importantly, it was concluded that repeated rhEPO treatment regimens were well tolerated without any serious adverse events (97,99–103).

Currently, there is no evidence from human studies on treatment of PAIS with BDNF, VEGF, or GDNF. However, altered levels of neurotrophic factors (including EPO, VEGF, and BDNF) in serum, cerebrospinal fluid, and/or cord blood have been described for neonates with perinatal asphyxia, hydrocephalus, and intraventricular hemorrhage, which were, in some studies, related to higher severity of brain injury (104–109). Other types of neonatal brain injury, including PAIS, are also very likely to alter the levels of neurotrophic factors in serum as a response to brain damage, but this

remains to be studied. Studies in adult humans have also found correlations between neurotrophic factors and progenitor cells in serum and severity and outcome of ischemic stroke (110,111), suggesting that neurotrophic factors may serve as predictive parameters in clinical care. This also seems a potential field of interest when setting up future studies in neonatal stroke.

MSC therapy

At present, no results are available of clinical trials on the treatment of neonatal stroke with MSCs. There is only one clinical trial describing the use of MSCs in treatment of neonates (112). Chang *et al.* (112) showed in a phase I dose-escalation trial the safety and feasibility of intratracheal administration of allogeneic umbilical cord-derived MSCs in nine preterm infants with high risk for bronchopulmonary dysplasia compared with historical case-matched controls. Importantly, no serious adverse events or toxicity related to a higher dose were observed in this study. The same study group is currently performing a phase I study in preterm neonates with severe intraventricular hemorrhage using intracerebroventricularly administered umbilical cord-derived MSCs (ClinicalTrials.gov: NCT02274428).

In addition, the group of Cotten *et al.* (113) has treated neonates with HIE with autologous umbilical cord blood cells, and first results are promising as no safety concerns were observed. Although the exact cell types in the cord blood were not specified, it is hypothesized that cord blood contains MSCs among other progenitor cells (114).

A large meta-analysis reported on a total of 1,012 adult and pediatric patients with various pathological conditions, including ischemic stroke, who were treated with either autologous or allogeneic MSCs and did not show any evidence for severe adverse effects due to MSC transplantation (115). Including eight RCTs, the only significant side effect was an increased risk of transient fever after MSC administration compared with the control group (115). We hypothesize that systemic complications such as fever are probably more common when MSCs are administered systemically (e.g., intravenously) in comparison to local applications.

CLINICAL TRIALS IN ADULT STROKE

In adult humans, several groups studied EPO as a treatment strategy for acute ischemic stroke; however, results are conflicting (116–118). Treatment with other neurotrophic factors has not been studied for adult stroke yet. A Cochrane review on stem cell transplantation for adult ischemic stroke identified three very small published RCTs, of which two only reported subgroups of patients (119,120). Currently, more clinical trials studying efficacy and safety of MSCs for ischemic stroke are on their way, and safety reports until now are reassuring (121). Results from adult stroke studies may provide supportive evidence for the feasibility and safety of interventions for PAIS. However, as described above, we feel that results from adult trials cannot be directly translated to neonatal care and detailed information on regenerative

medicine for adult ischemic stroke therefore goes beyond the scope of this review.

FUTURE PERSPECTIVE

Current data indicate that MSCs may improve neurological outcome after neonatal stroke by secretion of several growth and/or neurotrophic factors that boost neuroregenerative processes. However, as only few study groups have focused specifically on neonatal stroke (as opposed to HIE), replication of preclinical study results seems mandatory. These studies should mainly focus on optimizing dosing regimens and finding the optimal time window and route of administration for regenerative treatment after neonatal stroke. When these experimental results become available, clinical trials should first address safety issues regarding the use of neurotrophic factors and/or MSCs in neonatal stroke specifically. With respect to rhEPO, these steps have already been undertaken, and our current RCT will show the potential effect of erythropoiesis-stimulating agents on stroke recovery in newborns in the near future (ClinicalTrials.gov: NCT03171818).

Most recently, research has focused on combining MSC therapy with additional neurotrophic factors by administration of MSCs that overexpress a neurotrophic factor. For example, BDNF-overexpressing MSCs were intranasally administered to newborn rats with transient MCAO and were found to potentially reduce infarct volume, white and gray matter loss, and improve motor deficits compared with vehicle-treated rats (72). Even though BDNF-overexpressing MSCs were not significantly better than ‘normal’ MSCs in improving neonatal stroke injury in this study, additional experiments are required to further elucidate the possible additional benefits of MSCs transduced with neurotrophic factors. Results of studies assessing the effects of modified MSCs in other animal models of, e.g., adult stroke or neonatal HI may serve as an example for future preclinical testing in neonatal stroke (Table 3). For instance, in an adult acute ischemic stroke model administration of MSCs transduced with the EPO gene decreased infarct volume and improved neurological function to a significant larger extent when compared with treatment with either vehicle, normal MSCs, or a combination of MSCs+rhEPO (122). Another rodent study showed that treatment with GDNF-modified MSCs for adult stroke was effective in reducing apoptotic cell numbers and improving functional outcome more potently than normal MSCs (123). In line, other studies have shown that administration of VEGF-overexpressing NSCs improved functional outcome after HI brain injury in neonatal rats significantly more than normal NSCs (47,124). We hypothesize that future MSC therapy for neonatal brain injury may be improved by manipulating these cells to produce enhanced levels of growth factors or by combining administration of several types of MSCs with specific neurotrophic factors. For example, it was shown that combining NSC transplantation with exogenous BDNF administration improved the nervous function recovery after HI injury in neonatal rats more than NSCs alone (125). As opposed, Ahn *et al.* (126) demonstrated

Table 3. Modified stem cells in animal models of stroke

Paper	Stem cells	Animal model	Type of ischemic injury	Dosing and timing of treatment	Administration route	Effect
Ahn <i>et al.</i> (126)	MSCs+BDNF knockdown	Neonatal rat: P4	Intraventricular hemorrhage	1 dose of 1×10^5 MSCs at 2 days after injury	Intraventricular	After BDNF knockdown, MSCs were no longer effective. BDNF has a pivotal role in MSC working mechanism
Wang <i>et al.</i> (125)	BDNF+NSCs	Neonatal rat: P1	Hypoxic–ischemic injury: carotid ligation+hypoxia	Unknown dose ^a at 7 days after injury	Intracerebral ^{2a}	BDNF stimulated survival of NSCs. BDNF stimulated proliferation and differentiation of NSCs. Functional recovery was also more improved with BDNF at 4 weeks after injury
Velthoven <i>et al.</i> (72)	BDNF-overexpressing MSCs	Neonatal rat: P10	Transient MCAO: 90 min	1 dose of 1×10^6 MSCs in 20 μ l PBS (as 2 doses of 5 μ l per nostril) at 3 days after injury	Intranasal	BDNF-overexpressing MSCs reduced infarct volume, white and gray matter loss, and improved motor deficits. However BDNF-overexpressing MSCs were not significantly better than normal MSCs at 28 days after injury
Cho <i>et al.</i> (122)	EPO-modified MSCs	Adult rat	Transient MCAO: 120 min	1 dose of 6×10^5 EPO-MSCs in 5 μ l at 2 weeks after injury	Intracerebral (intraparenchymal)	MSCs transduced with the EPO gene decreased infarct volume and improved neurological function at 20–35 days, even to a larger extent than normal MSCs
Zheng <i>et al.</i> (47)	VEGF-transfected NSCs	Neonatal rat: P7	Hypoxic–ischemic injury: carotid ligation+hypoxia	1 dose of 1×10^5 NSCs in 2 μ l buffer at 3 days after injury	Intracerebral (intraparenchymal)	VEGF-overexpressing NSCs improved functional outcome more than NSCs alone
Yao <i>et al.</i> (124)	VEGF-transfected NSCs	Neonatal rat: P7	Hypoxic–ischemic injury: carotid ligation+hypoxia	1 dose of 1×10^5 NSCs in 2 μ l buffer at 3 days after injury	Intracerebral (intraparenchymal)	VEGF-overexpressing NSCs improved histopathological changes of HI and improved learning and memory abilities more than unmodified NSCs
Wang <i>et al.</i> (123)	GDNF-modified MSCs	Adult rat	Transient MCAO: 120 min	1 dose of 500 μ l of MSC-suspension (5×10^6 cells/ml) with or without GDNF-gene expression vector at 72 h after injury	Intravenous	MSC and GDNF-modified MSCs both improved behavioral data after three days. At 14 days after injury, GDNF-modified MSCs were more effective in reducing apoptotic cells than normal MSCs

BDNF, brain-derived neurotrophic factor; EPO, erythropoietin; GDNF, glial-derived neurotrophic factor; HI, hypoxia–ischemia; MCAO, occlusion of the middle cerebral artery; MSC, mesenchymal stem cell; NSC, neural stem cell; P, post-natal day; PBS, phosphate-buffered saline; VEGF, vascular endothelial growth factor.

^aFull-text only available in Chinese.

that MSC therapy in combination with knockdown of BDNF was ineffective in improving outcome after intraventricular hemorrhage in neonatal rats, indicating that BDNF has a pivotal role in MSC therapy for neonatal brain injury. The interplay between several neurotrophic factors, MSCs, or the manipulation of MSCs to stimulate neurotrophic factor production needs to be further elucidated in preclinical studies to optimize regenerative treatment strategies for neonatal brain injury.

CONCLUSION

In conclusion, owing to their neuroregenerative properties, growth factors and stem cells have a relative large therapeutic window, making them excellent candidates for novel treatment strategies to improve neurological prospects and quality of life of infants with PAIS.

Although experimental neonatal stroke studies and first clinical trials show clear benefits, large promising potential, and safety of therapies using, e.g., VEGF, (rh)EPO, or MSCs, their effectiveness in neonatal PAIS needs to be confirmed. The current hypothesis is that MSCs can improve neurological outcome after neonatal stroke by functioning as miniature factories secreting a wide array of growth and/or neurotrophic factors that boost neuroregenerative processes. Recent studies indicate that modification of MSCs, e.g., by overexpression of specific neurotrophic factors might be even more beneficial to treat neonatal brain injury. More research is needed to determine the safety, therapeutic window, and dosage of modified MSCs and to compare the potential of overexpression of the specific growth factors. An important issue for optimization of MSC-based repair treatments is to determine the potential of overexpression of one specific growth factor vs. combinations of growth factors or even combinations of different overexpressing MSCs at different times after the insult for effective tailor-made treatment of neonatal stroke to eventually combat the devastating consequences of PAIS.

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