Editor's Choice

Repair of neonatal brain injury: bringing stem cell-based therapy into clinical practice

NIENKE WAGENAAR[1] | CORA H NIJBOER[2] | FRANK VAN BEL[1]

1 Department of Neonatology, Wilhelmina Children's Hospital, University Medical Centre Utrecht, Utrecht; **2** Laboratory of NeuroImmunology and Developmental Origins of Disease, University Medical Centre Utrecht, Utrecht, the Netherlands.

Correspondence to Frank van Bel at Wilhelmina Children's Hospital, University Medical Centre Utrecht, Internal mail no. KE 04.123.1, P.O. Box 85090, 3508 AB Utrecht, the Netherlands. E-mail: f.vanbel@umcutrecht.nl

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ABBREVIATIONS

HIE	Hypoxic-ischaemic encephalopathy
MSC	Mesenchymal stem cell

Hypoxic-ischaemic brain injury is one of most important causes of neonatal mortality and long-term neurological morbidity in infants born at term. At present, only hypothermia in infants with perinatal hypoxic-ischaemic encephalopathy has shown benefit as a neuroprotective strategy. Otherwise, current treatment options for neonatal brain injury mainly focus on controlling (associated) symptoms. Regeneration of the injured neonatal brain with stem cell-based therapies is emerging and experimental results are promising. At present, increasing efforts are made to bring stem cell-based therapies to the clinic. Among all progenitor cell types, mesenchymal stromal (stem) cells seem to be most promising for human use given their neuroregenerative properties and favourable safety profile. This review summarizes the actual state, potential hurdles and possibilities of stem cell-based therapy for neonatal brain injury in the clinical setting. An early version of this paper was presented at the Groningen Early Intervention Meeting which was held in April 2016.

Worldwide, 2.9 per 1000 term infants suffer from neonatal hypoxic-ischaemic brain injury, including both hypoxic-ischaemic encephalopathy (HIE) and perinatal arterial ischaemic stroke. These disorders are important causes of perinatal mortality and long-lasting neurodevelopmental problems and form a large burden for patients, their families, and society at large.[1,2] Current treatment options for neonatal hypoxic-ischaemic brain injury mainly focus on supportive care, such as controlling hypoglycaemia, treatment of convulsions, and associated infections. Therapeutic hypothermia, started within the first 6 hours after birth, has shown to be beneficial for newborn infants with HIE.[3] Pharmacological neuroprotective therapies have been explored in animal models and may be promising in improving outcome in newborn infants in the future.[4] However, current therapeutic possibilities are limited and no treatment is available that leads to restoration of hypoxic-ischaemic brain injury in newborn infants.

CELL THERAPY

Increasing experimental evidence shows that regeneration of the developing injured brain with stem cell-based therapies is promising and may serve as an effective treatment strategy. Stem cells have an intrinsic potential for self-renewal and can differentiate into several cellular phenotypes.[5]

Stem cell types

Embryonic stem cells are derived from the inner mass of the blastocyst and they are truly pluripotent: able to self-renew indefinitely and to give rise to cell types from each of the three germ layers (ectoderm, endoderm, and mesoderm).[6] Given their pluripotent capacity, embryonic stem cells seem the most obvious choice for repair of brain injury, but can induce formation of teratomas after transplantation. Their application therefore raises considerable ethical concerns.[7] Multipotent adult stem cell types arise from embryonic stem cells and are subdivided in hematopoietic, neural, and mesenchymal stem cells. A major advantage of multipotent neural stem cells is their possibility to derive all neural lineages, but their accessibility in humans is limited and they also carry a significant risk of tumour formation.[8] As a detailed discussion of the potential benefits and hazards of several types of stem cells is beyond the scope of this review, we refer to Fleiss et al.[5] who have published an overview of the safety aspects and therapeutic values of major stem cell types.

Among all progenitor cells, the mesenchymal stem cell (MSC) seems to be most promising for near-future use in humans given its potent neuroregenerative properties and favourable immunological profile.[9] MSCs can differentiate into mesodermal tissue cells (e.g. bone, cartilage, fat), but experiments have demonstrated that MSCs are also capable, given specific in vitro conditions, of developing characteristics associated with neuronal cells.[9,10] In vivo administration of MSCs is associated with increasing numbers of neurons, astrocytes, and oligodendrocytes, and it is hypothesized that MSCs stimulate this formation of new brain cells by paracrine effects (see below) rather than by

transformation into various cell types themselves.[11–13] This review will therefore focus on the use of MSCs to treat neonatal brain injury.

Physiological aspects of MSCs

MSCs are relatively easy to isolate, have neuroregenerative properties, and exert important immunomodulating and anti-apoptotic effects.[5,11,14,15] MSCs are a heterogeneous cell population and originate from birth-associated tissues including the placenta, umbilical cord blood, and umbilical cord stroma (Wharton's Jelly), but also from adult tissues including bone marrow, peripheral blood, and fat tissue.[16] MSCs can be characterized by their adhesion to plastic when cultured, specific surface markers, and lack of expression of major histocompatibility (class-II antigens).[17] An advantage of cell-therapy with MSCs is that both autologous and allogeneic transplantation is possible. Autologous transplantation is of special interest for neonatal brain injury as MSCs from the newborn infant's own umbilical cord (blood) can be used for transplantation. MSCs have also shown to be safe for allogeneic transplantation, given their favourable immunological profile as shown in experimental studies and clinical studies for various pathologies.[18,19] However, safety studies on autologous MSC administration in newborn infants with neurological disorders have not been performed yet.

For clinical application, MSCs can be harvested from different tissue sources, but MSCs from birth-associated tissues, especially from the placenta and the umbilical cord, may have some advantages.[16] For example, they can be obtained non-invasively and without ethical concerns. Additionally, MSCs from birth-associated tissues have higher proliferative potential and faster self-renewal compared to MSCs from adult tissues.[20] Another advantage of MSCs from birth-associated tissues is that they have more primitive properties, which resemble embryonic rather than adult stem cell characteristics, which could be beneficial as adult MSCs show reduced differentiation capacity with increasing age.[16,20] In summary, in contrast to adult MSCs, MSCs from birth-associated tissues can be obtained in large quantities without limitations, which make them excellent potential candidates for use in neonatal care.

MECHANISMS OF ACTION OF MSCs

From neonatal rodent models of brain injury, we have learned that hypoxic-ischaemic brain injury induces changes in the neurovascular environment that promote (transient) neurogenesis, that is increased cell proliferation and differentiation. However, hypoxia-ischaemia does not induce and maintain long-term neurogenesis, resulting in a residual cerebral lesion. It is crucial to assist the neonatal brain in regenerative processes after injury and to reinforce proper development of the maturating brain. It is hypothesized that aiding endogenous regenerative mechanisms will subsequently lead to improvement of functional outcome.

Rodent studies have shown that lesion size does no longer increase after 4 days after a neonatal hypoxic-ischaemic event.[21] Therefore the time window for neuroprotective strategies, that is prevention of cell death and inflammation, lies before 4 days after the insult. However, MSC transplantation at day 10 after hypoxia-ischaemia is still able to reduce brain damage.[22] This clearly indicates that MSC treatment rather stimulates endogenous brain repair than acting neuroprotective. The regenerative mechanism of MSC transplantation must therefore be based on paracrine effects of MSCs on the endogenous repair system, that is boosting a growth-promoting environment for neural stem cells in neurogenic niches, instead of integration or direct differentiation of transplanted MSCs themselves into new neuronal cells. This is supported by several studies that demonstrated that MSCs migrate to the ischaemic boundary zone, induce changes in brain environment, and support neurogenesis.[11,23] MSC treatment after hypoxia-ischaemia markedly induced cell proliferation in the hippocampus and cortex, stimulated neuronal cell differentiation and formation of new neuroblasts within the subventricular zone.[11–13,24]

Paracrine effects of MSCs are thought to include the production of a plethora of factors (the secretome) involved in reduction of apoptosis and neuroinflammation, promotion of neurogenesis, angiogenesis, and synaptogenesis, and reduction of scar formation after brain damage (Fig. 1). The secretome of MSCs includes several growth factors such as vascular endothelial growth factor, brain-derived neurotrophic factor, nerve growth factor, basic fibroblast growth factor as well as anti-inflammatory cytokines.[25] A study from our group demonstrated upregulation of gene expression profiles associated with cell proliferation (e.g. Spp1 and IL17), neurogenesis (e.g. NRCAm and NGF), migration (e.g. CXCR4), and neuronal survival (e.g. glial derived neurotrophic factor) in the damaged area of the brain after MSC treatment. On the contrary, genes involved in inflammation (e.g. IL1B) were downregulated.[23] Other studies have hypothesized that MSCs inhibit apoptosis by transferring mitochondria during hypoxia-ischaemia.[26] These findings together support the view that MSCs induce neurogenesis and reduce neuroinflammation.

Besides beneficial effects of MSCs on cellular level, several studies have shown that MSCs improved functional outcome after brain injury in newborn rodents. MSCs given at day 10 after hypoxic-ischaemia were effective in reducing white and grey matter loss at 28 days after hypoxia-ischaemia.[22] The effects on lesion size were associated with improved sensorimotor function of MSC-treated mice after hypoxic-ischaemia compared to vehicle-treated littermates.[22] Functional improvements after MSC administration were long lasting: motor and cognitive performance had further improved in MSC-treated mice compared to vehicle-treated littermates up to 14 months after

What this paper adds

- Stem cell-based therapy is a promising near-future treatment strategy for neonatal brain injury.
- Mesenchymal stem cells have most favourable characteristics among other stem cell types for clinical use.
- First safety results from clinical trials are reassuring.

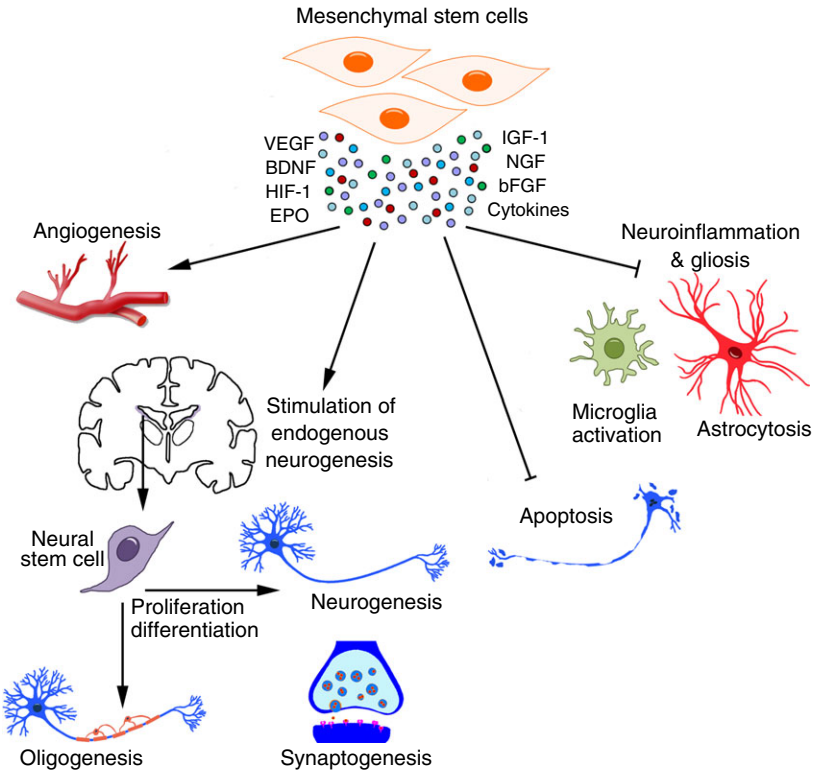

Figure 1: Potential mechanisms for mesenchymal stem cells to induce repair of neonatal brain injury, including induction of angiogenesis, stimulation (⟶) of neurogenesis in the subventricular zone, and reduction (⟞) of apoptosis, neuroinflammation, and gliosis. These pathways are mediated by the sectretome of mesenchymal stem cells, which consists of many growth factors such as vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), hypoxia-inducible factor 1 (HIF-1), erythropoietin (EPO), insulin-like growth factor 1 (IGF-1) nerve growth factor (NGF), basic fibroblast growth factor (bFGF), and anti-inflammatory cytokines. [Colour figure can be viewed at wileyonlinelibrary.com].

hypoxia-ischemia.[19] These experimental studies provide strong evidence for short- and long-term efficacy of MSC treatment after neonatal hypoxic-ischaemic brain injury.

Taken together, MSCs can exert beneficial effects via multiple paracrine routes that together create an environment that facilitates tissue regeneration, subsequently leading to improved functional outcome.

DELIVERY OF MSCS FOR THERAPY

Routes of delivery

MSCs can be delivered into the brain via different routes, using either systemic (e.g. into the bloodstream or peritoneal cavity), or local administration (e.g. intraparenchymal/intracranial, intracerebroventricular, intrathecal, intranasal). In experimental settings, several research groups have used systemic administration of MSCs for neonatal brain injury, as MSCs have proven to migrate to injured brain regions.[27] Intravenously administered MSCs migrated mainly to injured ischaemic brain areas and significantly improved functional outcome in a model of adult stroke.[12] A major disadvantage of systemic application of MSCs is loss of MSCs in other organs, which lowers effective cell numbers in the brain.[28] Especially in newborn infants suffering from systemic inflammation or birth asphyxia, the peripheral organs also undergo a hypoxic period, leading to multi-organ dysfunction. We hypothesize that this enhances loss of MSCs in these organs, which impedes delivery of MSCs to the brain. Therefore, local administration of MSCs seems more effective. As intracranial injection of MSCs (frequently used in rodents) is not feasible for clinical application; a less invasive local transplantation method is preferred. In clinical trials so far, MSCs or other stem cells (i.e. from autologous cord blood) have been administered mostly intravenously or intrathecally to treat cerebral palsy (Table I).

Intranasal

More recently, intranasal delivery is emerging as an effective administration method for several therapeutic substances, including insulin, oxytocin, orexin, growth factors, and neuro-peptides to treat central nervous system diseases.[29] Intranasal application might provide improvement over systemic routes as MSCs are targeted directly to the brain, preventing loss of MSCs in peripheral organs. Given the rapid distribution of MSCs from the nasal cavity towards ischaemic brain lesions within 2 hours,[24] migration of MSCs through the brain tissue using the rostral migratory stream seems unlikely. Therefore, in agreement

Table I: Recent clinical trials using stem cell therapy to treat newborn infants with neurological disorders

Study group	Indication	Control group	Age at inclusion	Cells used	Status	Efficacy	Adverse effects	Study period
Samsung Medical Centre, Korea NCT02274428	Preterm infants with grade 3–4 intraventricular haemorrhage (*n*=9)	None	23–34wks gestation	Pneumostem: Allogeneic human umbilical cord blood derived mesenchymal stem cells. Single intraventricular injection of low (5×10^6) or high (10×10^6) dose	Ongoing	Aim: acute adverse events and death or shunt operations after 1y	–	October 2014–December 2016
Duke University Medical Centre, Durham, NC, USA NCT00593242	Term newborn infants with HIE and hypothermia (*n*=23)	Concurrent cooled infants (*n*=33)	34–40wks gestation	IV infusion of autologous umbilical cord blood cells as soon as possible after birth and 24h, 48h and 72h postnatally. 1–4 doses of $1–5\times10^7$ cells/dose	Completed, results have been published[36]	Similar outcomes after 1y in both groups	None	January 2009–June 2012
Duke University Medical Centre, Durham, NC, USA	Infants with severe congenital hydrocephalus	None	6d–4.5y	IV infusion of autologous umbilical cord blood in 2–4 doses of a median of $0.1–13.3\times10^7$ cells/kg	Completed, results have been published[37]	All infants experienced developmental delays (as expected)	None	October 2006–August 2014
Duke University Medical Centre, Durham, NC, USA	Young children with acquired neurological disorders (*n*=184)	None	6d–9.5y	IV infusion of autologous umbilical cord blood cells in 1–2 doses of a median of $0.1–13.3\times10^7$ cells/kg	Completed, results have been published[38]	None described	1.5% acute anaphylactic reactions during infusion. No adverse events until 12mo	March 2004–December 2009
Duke University Medical Centre, Durham, NC, USA	Krabbe's disease: asymptomatic newborn infants (*n*=11) and symptomatic infants (*n*=14)	Matched untreated affected children (siblings)	Newborn infants: 12–44d Infants: 142–352d	IV infusion of allogeneic umbilical cord blood cells. Newborn infants received 22×10^7 cell/kg and infants received 17×10^7 cells/kg	Completed, results have been published[39]	In newborn infants very promising, but in infants minimal improvement	4/14 infants progressive disease, 2/14 infants GvHD, 2/14 infections	August 1998–August 2004
New York Medical College, Vallhala, New York, USA NCT02434965	Term newborn infants with severe HIE within 6h of birth (*n*=20)	None	≥36wks gestation	IV infusion of autologous cord blood and human placental derived stem cells (HPDSC). Collected after birth. Newborn infants received two doses of HPDSC at day 2 and day 7. Autologous cord blood was given in 3 doses: day 0, day 3, and day 7. Dosage depended on cell collection per participant	Ongoing	Aim: safety and tolerability, neurological condition at 2y	–	January 2016–June 2019
Hospital Universitario 'Dr Jose E. Gonzalez', Monterrey, Mexico NCT01506258	Term newborn infants with oxygen deprivation	None	37–42wks gestation	IV infusion of autologous hematopoietic stem cells/autologous cord/placental blood within 48h after birth (unknown dosage)	Completed, no results available	–	–	January 2012–April 2013
University of Texas, Houston, USA NCT01700166	Stroke in children	None	6wks–6y	IV infusion of autologous human umbilical cord blood-derived stem cell (unknown dosage)	Withdrawn before inclusion started	–	–	September 2012–December 2015

with literature, several possible routes are proposed for MSCs to reach the brain after intranasal application: by following the tracts of the olfactory or trigeminal nerve, the meningeal circulation, and/or via absorption into cerebrospinal fluid.[24,30]

Intranasal MSC treatment, when compared to vehicle treatment, improved sensorimotor and cognitive function and decreased grey and white matter loss in neonatal mice with hypoxic-ischaemic brain injury.[11,31] The beneficial effects of intranasal MSC treatment were similar to intracranial delivery of MSCs in the lesion area.[14] Therefore it was concluded that the nasal route is a rapid and efficient way for MSC delivery after brain injury in neonatal rodents.

As humans have a less developed olfactory bulb in comparison to rodents, our group has also demonstrated that intranasally administered MSCs have the potential to migrate to injured brain regions in a newborn primate model of hypoxic-ischaemic brain injury, in accordance with studies in rodents (unpublished observations). Although not investigated to date, it is expected that intranasally delivered MSCs will induce similar beneficial effects in the primate brain as observed in rodent models.

In summary, intranasal administration provides an effective and rapid alternative for MSC transplantation, allowing non-invasive MSC delivery for brain injury with minimal burden for patients.

Therapeutic window

Most available neuroprotective therapies for hypoxic-ischemic brain injury need to be started within a few hours (e.g. hypothermia) to be effective. In adult animal models, administration of MSCs at 3 to 24 hours after middle cerebral artery occlusion reduced the number of apoptotic cells in the ischaemic penumbra.[12,15] However, the therapeutic window of MSC treatment is much wider as it has been shown that MSCs improved functional outcome and lesion volume in neonatal hypoxic-ischaemic mice when administered at least until 10 days after induction of the insult.[22] In addition, it has been shown that administration of MSCs at 17 days after induction of brain damage was not effective any more as a result of lacking chemotactic signals within the brain lesion. So, the therapeutic window was determined to be at least 10 days but shorter than 17 days.[22] Although it might be hard to translate this window one to one to humans, as rodents of 2 to 3 weeks old correspond to a human child of approximately 0.5 to 2 years (depending on which parameter is used to relate age between the species), we hypothesize that the time window for MSC treatment after neonatal brain injury may be at least months in humans.[32]

Dosing

The appropriate effective MSC dose depends on several factors, including administration route, timing of injury, and treatment. In neonatal rodent models of stroke, the most effective dose of intranasally administered MSCs to improve functional outcome was $0.5–1.0 \times 10^6$ cells/pup (5–15g of bodyweight).[22,31] Other studies indicate that intracranial administration of 1.0×10^5 MSCs into the neonatal mouse brain at 10 days after the insult is sufficient to improve functional outcome.[11] Intravenously administered MSCs in adult rat models of stroke varied in dose, but were shown to improve functional outcome when given at 3×10^6 MSCs/rat.[12,15] In clinical studies for adult stroke, the intravenous dose varied between 0.6 to 1×10^8 MSCs/patient.[33,34] Taking into account an average weight of 75kg, these clinical studies have used dosages of 8 to 13×10^5 cells/kg, which seem rather low. Extrapolating experimental adult rat data (using a weight of around 300g per rat) to humans, an effective intravenous dose would be around 1×10^7 MSCs/kg. Intratracheally administered MSCs for bronchopulmonary dysplasia in infants born preterm were found to be safe and feasible when dosage was 1 to 2×10^7 cells/kg body weight, which was comparable to animal models.[35] Overall, it is hypothesized that higher doses of systemically administered MSCs are required compared to local MSC administration because of potential loss of MSCs in peripheral organs after systemic administration.

SAFETY ASPECTS

The most important potential risk factors of MSC treatment are thought to be inflammatory reactions when using allogeneic cells and formation of malignancies. These risks have been intensively elaborated on in preclinical studies and appear to be absent at follow up. Our research group has assessed long-term safety in a mouse model of neonatal hypoxic-ischaemic brain damage: at 14 months, no lesions or neoplasia were observed in the nasal turbinates, brains, or other peripheral organs of mice treated intranasally with MSCs.[19] Because animal studies have demonstrated that MSCs are hardly detectable in the brain at 3 days after transplantation,[24] the risk of Graft-versus-Host Disease or tumourigenicity seems low.

Additionally, human trials on MSC treatment in adults do not provide evidence for serious adverse events or risks. A large meta-analysis from 2012 on clinical trials for numerous diseases did not show any evidence for severe adverse effects.[18] This study used both adult and paediatric trials to report on a total of 1012 patients with various conditions, including neurological disorders, who were treated with either autologous or allogeneic MSCs. Including eight randomized control trials, MSC transplantation was not associated with acute infusional toxicity, organ system complications, infection, death, or malignancy. A significant fever was observed after systemic MSC treatment compared to the control group, but the fever was reported to be low and transient in all trials.[18]

In summary, no indications have been reported in experimental animal models that complications occur in a higher incidence after MSC transplantation as compared to vehicle-treated animals. Additionally, in clinical trials adverse events, that is transient fever, were only observed when

systemical MSC administration was used. Despite the benign safety profile of MSC therapy, safety and feasibility assessment of MSC treatment for neonatal brain injury should be confirmed in well-conducted clinical trials.

CLINICAL APPLICATION OF STEM CELLS IN NEWBORN INFANTS

Several clinical trials are recently being initiated using stem cell therapy to treat newborn infants with neurological disorders (HIE, intraventricular haemorrhage, stroke, hydrocephalus, or acquired neurological disorders) (Table I). There is large variety in terms of study design, type of cells, dosing, and timing of administration. Most studies use intravenous administration, but local intraventricular administration is used in an ongoing Korean trial for preterm infants with intraventricular haemorrhage (ClinicalTrials.gov registration number NCT02274428). Several studies use autologous cord blood or stem cells derived from cord blood, including the group from Duke University treating newborn infants with HIE, congenital hydrocephalus, and acquired neurological disorders including Krabbe's disease. Their results regarding safety seem promising: all infusions were well tolerated and no adverse events were observed.[36–39] However, efficacy of autologous umbilical cord blood therapy on improvement of outcome and survival was only found significant in asymptomatic newborn infants with proven Krabbe's disease.[39] Another study using autologous cord blood to treat HIE in newborn infants is registered, but to the best of the authors' knowledge, results have not been communicated so far (ClinicalTrials.gov registration number NCT01506258). The Korean study group has reported the intracheal use of allogeneic MSCs in preterm newborn infants with high risk for bronchopulmonary dysplasia.[35] Nine infants were included in this study: three patients were given 1×10^7 MSCs/kg and six were given 2×10^7 MSCs/kg. After 7 days, pro-inflammatory cytokines were decreased (e.g. interleukin-6 and interleukin-8) whereas the bronchopulmonary dysplasia severity score had improved in patients receiving MSCs compared to historical case-matched controls. No serious adverse events or toxicity related to a higher dose were observed.[35]

FUTURE PERSPECTIVES

The treatment of neonatal hypoxic-ischaemic brain injury with stem cell-based therapies is emerging and experimental and preliminary clinical trial results are promising. MSCs have various characteristics that are favourable to be used as a regenerative therapy in neonatal hypoxic-ischaemic brain injury: MSCs are present in several tissues, can be relatively easily harvested in large numbers, and have low immunogenicity which makes them excellent candidates for autologous and allogeneic transplantation. Preclinical research has shown that MSC therapy has potential to repair neonatal brain lesions by boosting the endogenous regenerative capacity of the immature brain, thereby improving functional outcome at the long term. Other approaches, such as manipulation of MSCs or use of MSC-derived exosomes, have recently been investigated aiming at improvement of MSC effectivity or the use of a cell-free approach in experimental settings. Additionally, first results from clinical trials regarding safety aspects from both autologous and allogeneic cell transplantation are reassuring. However, some consideration should be taken for application of MSCs in human newborn infants, as positive bias in many reports may fuel unrealistic expectations. Several clinical trials are underway to evaluate safety and efficacy of MSC therapy for neonatal brain injury, providing the first steps towards clinical application of MSCs for severely affected newborn infants, to ultimately improve their quality of life.

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