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Use of stem cells in perinatal asphyxia: from bench to bedside

Simone de Paula,¹ Samuel Greggio,¹ Jaderson Costa DaCosta²

Abstract

Objectives: To present recent scientific evidence on the effects of stem cell transplantation in animal models of neonatal hypoxic-ischemic brain injury and address the translational relevance of cell therapy for clinical application in this context.

Sources: The PubMed and Scopus databases were used to select articles. The selection criterion was the specificity of articles regarding the subject studied, preferably articles published from 2000 onward. We also reviewed classic articles from previous years that were applicable to this review.

Summary of the findings: Stem cells from different exogenous sources may exhibit neuroprotective properties in experimental models of neonatal hypoxia-ischemia. In most animal experiments, the morphological and functional benefits observed were independent of neural differentiation, suggesting associated mechanisms of action, such as the release of trophic factors and inflammatory modulation.

Conclusions: Based on the experimental studies analyzed, cell therapy may become a promising therapeutic approach in the treatment of children with hypoxic-ischemic encephalopathy. However, further studies are warranted to elucidate potential mechanisms of action of these cells and to define safe and effective clinical strategies.

J Pediatr (Rio J). 2010;86(6):451-464: Hypoxic-ischemic encephalopathy, stem cells, asphyxia, cell therapy.

Introduction

Neonatal hypoxic-ischemic (HI) brain injury is a major cause of neurological morbidity and mortality in infants. Statistical data suggest an incidence of asphyxia of 2-4 per 1,000 full-term births. In Brazil, neonatal asphyxia is estimated to occur in approximately 2% of live births.¹ Furthermore, 20-50% of asphyxiated newborns die within the neonatal period, and up to 25% of survivors may

exhibit permanent neuropsychological impairments, such as mental retardation, cerebral palsy, epilepsy, and learning disability.²

The most frequent cause of HI encephalopathy is severe intrauterine asphyxia, and the main pathogenic mechanism attributed to its neuropathology is impaired cerebral blood flow.³ Associated neurotoxic events, such as

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energy failure, membrane depolarization, excitatory amino acid release, accumulation of free radicals, and apoptosis, occur simultaneously and contribute to cellular dysfunction and neuronal death after HI insults.⁴

Despite the technological and scientific advances in perinatal care of at-risk newborns, the clinical management of asphyxiated infants has been limited to maintenance of oxygenation, control of blood pressure and homeostasis, treatment of seizures, and control of intracranial hypertension.⁵

New neuroprotective strategies have been investigated in experimental studies and clinical trials due to the clinical significance and socioeconomic impact generated by neonatal brain damage. Calcium blockers, inhibitors of excitatory amino acids and free radicals, use of nitric oxide, growth factors, neuropeptides and hypothermia are some of the current therapeutic approaches that aim to interrupt the cascade of neurochemical events triggered by hypoxia-ischemia.⁴ Except for hypothermia, which shows satisfactory outcomes only in infants with moderate HI injury, these therapies have limited results.⁶

In this context, cell therapy has been explored because it is an up-to-date and promising approach for treatment of severe neurological diseases. Stem cells represent a natural unit of embryonic development and tissue repair, characterized as a subset of immature, undifferentiated and unspecialized cells that have the capacity for self-renewal and differentiation into specific cell lineages.⁷ Such cells have been found in all postnatal organs and tissues, including the central nervous system (CNS), previously known by the lack of progenitor cells and regenerative potential.⁸ Recent discoveries have revolutionized the field of stem cell biology by demonstrating the clinical potential of these cells in a variety of human diseases. Initially used in the treatment of hematologic malignancies and autoimmune disorders, transplantation of immature and undifferentiated cells is being currently proposed as a potential source of new cells and trophic factors to minimize cell damage and regenerate necrotic tissues resulting from CNS injury.⁹ Experimental studies have shown that stem cell transplantation can improve functional recovery in experimental models of cerebral ischemia, Parkinson's disease, Huntington's disease, epilepsy, and spinal cord injury.¹⁰⁻¹⁴

Basically, stem cells that have been used in experimental studies of neonatal HI injury fall into two categories: neural stem cells from neuronal embryonic or adult tissue; and somatic stem cells of non-neural origin, particularly those isolated from bone marrow and umbilical cord blood.

The objective of this review was to present the state of the art of experimental studies of cell therapy in animal models of neonatal HI brain injury (Table 1), addressing potential mechanisms of action of this therapeutic resource and the translational relevance of stem cell transplantation for clinical application in HI encephalopathy.

Transplantation of fetal neocortical tissue

In 1996, Elsayed et al.¹⁵ conducted the first study on the use of cellular resources in the treatment of experimental HI brain injury. In that investigation, the authors assessed the effects of intracerebral transplantation of fetal neocortical tissue blocks performed 7 days after induction of HI brain injury in neonatal rats. Although successful transplants were observed in 63% of cases, the study failed to demonstrate significant therapeutic effects on brain atrophy. Additionally, that study did not conduct a functional assessment of transplanted animals. Another research group also performed intracerebral transplantation of fetal neocortical tissue 3 days after induction of HI brain injury.¹⁶ Instead of using tissue blocks, the authors used cell suspensions to facilitate the transplantation procedure. The results showed improvement in motor function and asymmetry in treated animals. However, although transplants were identifiable in 72% of the animals 10-12 weeks after implantation, the authors observed absence of cortical cytoarchitecture recovery.

Neural stem cell transplantation

Neural stem cells (NSC) have the capacity for self-renewal and limited capability to generate cells of neuronal and glial lineages. Such cells can be isolated from different regions of the embryonic nervous system or harvested from two specific regions of the adult brain: the subventricular zone of the lateral ventricles and the subgranular zone of the hippocampal dentate gyrus.^{7,9} Studies have shown that NSC can migrate and survive in injured brain areas and can be induced to differentiate *in vivo* and *in vitro* into neurons, oligodendrocytes and astrocytes, indicating a potential alternative to replacement of cell types affected in HI brain injury.^{9,17}

Once isolated, NSC can proliferate *in vitro* in response to the presence of specific growth factors, generating cell clusters called neurospheres. Basically, these structures are composed of multipotent NSC and neural progenitors with more impaired development.^{9,18,19} Some studies have shown that subventricular zone astrocytes that express a glial protein may also be considered as NSC.²⁰

In this context, Zheng et al.²¹ demonstrated that multipotent astrocytic stem cells from the subventricular zone of mice migrate to the cortical and periventricular ischemic region and show signs of neuronal and astrocytic differentiation in HI animals subjected to intracerebral transplantation. In contrast, when transplantation was performed in healthy animals, the cells remained at the site of transplantation and maintained their astrocytic profile. These data indicate that HI brain tissue shows physiological processes that can attract astrocytic stem cells to the site of injury, as well as induce neuronal differentiation.

Table 1 – Review of publications on stem cell therapy in experimental hypoxia-ischemia in the neonatal period

Reference	HI model (animal, age, and duration of hypoxia)	Cell type	Transplantation (concentration, route, timing, and immunosuppression)	Functional outcomes*	Morphological, cellular, and molecular outcomes*	Cellular Migration	Cellular Differentiation
Elsayed et al. ¹⁵	Long-Evans black-hooded rats; P7-8; 60, 120 or 120-150 min	Murine fetal neocortical tissue (embryonic day 13)	1-2 mm ³ block transplant; ic (IT); 7 days after HI; NI	NA	No effect on brain atrophy. Presence of axonal connectivity between graft and adjacent cortical areas of host tissue	Transplants with satisfactory (63%) and poor (19%) development, located in the cortex or adjacent areas 6 weeks pT	NA
Jansen et al. ¹⁶	Wistar rats; P7; 150 min	Murine fetal neocortical tissue (embryonic day 16)	5 x 10 ⁴ cells/μL; ic (IT); 3 days after HI; NI	Improvement of motor function (3-8 weeks pT) and asymmetry (9 weeks pT)	Absence of cortical cytoarchitecture recovery; transplants stained for neurochemical markers and absence of glial labeling; presence of astrocytes surrounding the transplant (9-11 weeks pT)	Identification of 72% of transplants located in areas adjacent to the corpus callosum of the sensorimotor cortex 9-11 weeks pT	NA
Park et al. ²⁷	CD-1 mice; P7; 120 min	Murine NSC (clone C17.2) in PGA scaffold (NSC-PGA)	1 or 2 NSC-PGA complexes (1 x 10 ⁷ cells/mL; 100-200 μL); ic (IT); 7 days after HI; NI	↓ unilateral rotation	Reduction of brain damage; formation of interconnections between NSC/PGA and host tissue; neovascularization; ↓ monocyte infiltration and astroglial scar; restoration of long-distance neuronal projections	Transplant showed satisfactory uptake in the infarcted brain cavity 2-6 weeks pT	Transplanted cells exhibited markers of neurons (5%) and oligodendrocytes, in the cortical penumbra of injury, 2 weeks pT
Imitola et al. ²³	C57Bl/6 mice; P7; 120 min	Five strains of human and murine NSC	5 x 10 ⁵ cells/mL; ic (CT); 3 days after HI; cyclosporine	NA	NSC express CXCR4; ↑ expression of SDF-1α in the infarcted brain region (astrocytic and endothelial cells); interaction of SDF-1α/CXCR4 pathway	Positive correlation between the presence of transplanted cells in the ischemic region and SDF-1α expression	Transplanted cells with neuronal labeling in the cortical penumbra of injury
Katsuragi et al. ³²	Wistar rats; P14; 120 min	Encapsulated BHK-GDNF cells	1 capsule (1 x 10 ⁸ cells/mL); ic (IT); 2 days pre-HI; NI	NA	↑ serum GDNF; ↓ incidence and severity of neuronal damage (7 days pT)	BHK-GDNF cells remained viable 7 days pT	NA
Katsuragi et al. ³³	Wistar rats; P9; 120 min	Encapsulated BHK-GDNF cells	1 capsule (1 x 10 ⁸ cells/mL); ic (IT); 2 days pre-HI; NI	Improvement of cognitive performance after 6 weeks pT	Reduction of brain injury 17 weeks pT	NA	NA
Zheng et al. ²¹	Sprague-Dawley rats; P7; 120 min	Multipotent astrocytic stem cells from murine subventricular zone	5 x 10 ⁴ cells/μL; ic (IT); 24 h after HI; NI	NA	NA	Detection of transplanted cells in the cortical and periventricular ischemic region within 21 days pT	Transplanted cells exhibited markers of astrocytes and neurons 3-21 days pT
Meier et al. ³⁶	Wistar rats; P7; 80 min	HUCBSC	1 x 10 ⁷ cells/500 μL; ip; 24 h after HI; NI	Improvement of locomotor function 21 days pT	No effect on brain atrophy within 21 days pT	Presence of transplanted cells surrounding brain injury 21 days pT	Absence of transplanted cells with markers of astrocytes and neurons 21 days pT

BDNF = brain-derived neurotrophic factor; BHK-GDNF = baby hamster kidney cells (BHK) transfected with glial cell line-derived neurotrophic factor (GDNF) complementary DNA; MAPC = multipotent adult progenitor cells; ChABC = chondroitinase ABC; HUCBSC = human umbilical cord blood stem cells; MSC = mesenchymal stem cells; NSC = neural stem cells; CXCR4 = CXC chemokine receptor 4; FGF-2 = fibroblast growth factor 2; GDNF = glial cell line-derived neurotrophic factor; HI = hypoxia-ischemia; ic = intracerebral route; ica = intracardiac route; icv = intracerebroventricular route; ip = intraperitoneal route; iv = intravenous route; NA = not analyzed; NGF = nerve growth factor; NT3 = neurotrophin 3; P = postnatal day; PGA = polyglycolic acid; pT = post-transplantation; SDF-1 = stromal cell-derived factor 1; T-PX = transplantation on postnatal day X; NI = no immunosuppression; CT = contralateral transplant; IT = ipsilateral transplant; VEH = vehicle.

* Treated animals vs. control animals.

↑ = increase.

↓ = reduction.

Table 1 – Review of publications on stem cell therapy in experimental hypoxia-ischemia in the neonatal period (*continuation*)

Reference	HI model (animal, age, and duration of hypoxia)	Cell type	Transplantation (concentration, route, timing, and immunosuppression)	Functional outcomes*	Morphological, cellular, and molecular outcomes*	Cellular Migration	Cellular Differentiation
Yasuhara et al. ⁵⁸	Sprague-Dawley rats; P7; 150 min	MAPC from murine bone marrow	2 x 10 ⁵ cells/3 µL; ic (IT); 7 days after HI; cyclosporine A	Improvement of asymmetry, but not motor function, 14 days pT	NA	Detection of transplanted cells in hippocampal CA2 and CA3 regions 14 days pT	NA
Park et al. ²²	CD-1 mice; P7; 138 min	Murine NSC (clone C17.2)	0.4-1.6 x 10 ⁵ cells/4 µL; ic; 1 (IT), 3 (IT or CT), 7 (IT), 14 (IT), or 35 (IT) days after HI; NI	NA	NA	CT: cell migration across the corpus callosum and other interhemispheric commissures to the infarcted area; IT: cells remained at injury site, with better uptake when transplanted 3 to 7 days after HI	IT vs. CT (3 days after HI): transplanted cells showed labeling of neurons (~5 vs. 0%), oligodendrocytes (~4 vs. ~1%), astrocytes (~23 vs. ~15%), and neural progenitor cells (~18 vs. ~6%) in the neocortex (1 month pT)
Park et al. ²⁹	CD-1 mice; P7; 120-180 min	Murine NSC (clone C17.2) with overexpression of NT-3 (NSC-NT3)	3 x 10 ⁵ cells/8 µL (ic, IT at 2 sites) and 1.0 x 10 ⁵ cells/2 µL (icv, CT); 3 days after HI; NI	NA	Overexpression of NT-3	Presence of cells at transplantation sites 2-4 weeks pT	Transplanted cells with neuronal labeling (cholinergic, GABAergic and glutamatergic) in the penumbra (81.4%) and infarcted area (10-20%), and labeling for oligodendrocyte (0.4%) and glia (1%); transplanted cells with neuronal labeling in the contralateral hemisphere (~90%) (2-4 weeks pT)
Ma et al. ²⁴	C57Bl/6 mice; P7; 90 min	Murine embryonic stem cells after neural differentiation <i>in vitro</i>	1 x 10 ⁴ cells/µL; icv (IT); 2-3 days after HI; NI	Improvement of spatial memory 2 and 8 months pT	↑ neuronal cells in the hippocampal CA1 region 8 months pT	Detection of transplanted cells in the hippocampus and cerebral cortex 2 and 8 months pT	Transplanted cells exhibited markers of neurons, but not astrocytes, 8 months pT
Dayer et al. ³⁰	Wistar rats; P7; 30 min	Murine neural progenitor cells with overexpression of FGF-2	4 x 10 ⁴ cells/0.5 µL; ic (IT); 1 and 3 days after HI; NI	NA	Preservation of an immature and proliferative state is inversely proportional to FGF-2 expression in transplanted cells	Detection of transplanted cells in the infragranular ischemic cortex and in the margin between cortex and corpus callosum 2 weeks pT	The vast majority of transplanted cells exhibited markers of immature neurons; in smaller numbers, there was labeling for astrocyte, oligodendrocyte and neuron
Yasuhara et al. ⁵⁷	Sprague-Dawley rats; P7; 150 min	MAPC from murine bone marrow	2 x 10 ⁵ cells/3 µL; iv or ic (IT); 7 days after HI; NI	Improvement of motor function and asymmetry 14 days pT (iv and ic)	Reduction of neuronal death in the hippocampal CA3 region 14 days pT (iv and ic)	Detection of transplanted cells in ischemic hippocampal CA2 and CA3 regions 14 days pT (iv and ic). The cells were also located in blood vessels in the hippocampus (iv)	Transplanted cells showed neuronal labeling 14 days pT (iv and ic)
Sato et al. ²⁸	Sprague-Dawley rats; P7; 120 min	Murine NSC (embryonic day 14) or NSC-ChABC (25 mU)	2.5 x 10 ⁵ cells/5 µL; icv (IT); 24 h after HI; NI	NA	↓ brain damage (NSC+ChABC < NSC = VEH); ↑ brain weight (NSC+ChABC > NSC = VEH) (7 days pT)	Presence of transplanted cells in the ischemic injury and adjacent areas 7 days pT	NA

BDNF = brain-derived neurotrophic factor; BHK-GDNF = baby hamster kidney cells (BHK) transfected with glial cell line-derived neurotrophic factor (GDNF) complementary DNA; MAPC = multipotent adult progenitor cells; ChABC = chondroitinase ABC; HUCBSC = human umbilical cord blood stem cells; MSC = mesenchymal stem cells; NSC = neural stem cells; CXCR4 = CXC chemokine receptor 4; FGF-2 = fibroblast growth factor 2; GDNF = glial cell line-derived neurotrophic factor; HI = hypoxia-ischemia; ic = intracerebral route; ica = intracardiac route; icv = intracerebroventricular route; ip = intraperitoneal route; iv = intravenous route; NA = not analyzed; NGF = nerve growth factor; NT3 = neurotrophin 3; P = postnatal day; PGA = polyglycolic acid; pT = post-transplantation; SDF-1 = stromal cell-derived factor 1; T-PX = transplantation on postnatal day X; NI = no immunosuppression; CT = contralateral transplant; IT = ipsilateral transplant; VEH = vehicle.

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Reference	HI model (animal, age, and duration of hypoxia)	Cell type	Transplantation (concentration, route, timing, and immunosuppression)	Functional outcomes*	Morphological, cellular, and molecular outcomes*	Cellular Migration	Cellular Differentiation
de Paula et al. ³⁸	Wistar rats; P7; 120 min	HUCBSC	1 x 10 ⁷ cells/100 µL; iv; 24 h after HI; NI	No effect on spatial memory deficit 3 weeks pT	No effect on brain atrophy 3 weeks pT	Detection of few transplanted cells 24 h, 1 and 3 weeks pT	NA
Yasuhara et al. ⁴⁰	Sprague-Dawley rats; P7; 150 min	HUCBSC or HUCBSC+M (mannitol 1.1 mol/L)	1.5 x 10 ⁴ cells/200 µL; iv; 7 days after HI; NI	Improvement of motor function and asymmetry 7 and 14 days pT (HUCBSC < HUCBSC+M)	↑ brain levels of GDNF, BDNF and NGF 3 days pT (HUCBSC < HUCBSC+M); ↑ dendritic density in the hippocampal CA1 region 14 days pT	Presence of few transplanted cells in the ischemic hippocampus 14 days pT	NA
Pimentel-Coelho et al. ³⁷	Lister-Hooded rats; P7; 90 min	HUCBSC	2 x 10 ⁶ cells/200 µL; ip; 3 h after HI; NI	Improvement of sensorimotor reflexes 4 days pT	↓ neuronal death and caspase 3 expression in the ischemic striatum 2 days pT; ↓ microglial activation in the cortex 7 days pT	Presence of few transplanted cells in the ischemic cortex and striatum 2 days pT	NA
Jenny et al. ³¹	Wistar rats; P7; 30 min	Murine neural progenitor cells with overexpression of FGF-2	2-5 x 10 ⁴ cells/1 µL; ic (IT); 4 days after HI; NI	NA	Formation of cell clusters with overexpression of FGF-2 in perivascular areas	Presence of transplanted cells in areas of cortical injury close to blood vessels 7 days pT	Transplanted cells showed labeling of immature neurons 7 days pT
van Velthoven et al. ⁵⁶	C57Bl/6 mice; P9; 45 min	MSC from murine bone marrow	1 x 10 ⁵ cells/2 µL; ic (IT); 3 days (T-P12) or 10 days (T-P19) after HI; NI	Improvement of motor asymmetry 7 and 18 days pT (T-P12) or 11 and 18 days pT (T-P19)	T-P12: ↓ brain injury (18 days pT); ↑ neuro- and oligodendrogenesis, and ↓ microglial proliferation in the hippocampus and ischemic cortex (7 and 18 days pT); ↑ astrocyte proliferation in the hippocampus, and ↓ in the ischemic cortex (7 days pT). T-P19: ↓ brain injury (18 days pT)	Less than 1% of proliferating cells in the ischemic hemisphere derived from transplanted cells	NA
Lee et al. ⁵⁵	Sprague-Dawley rats; P7; 210 min	MSC from human bone marrow	1 x 10 ⁶ cells/µL; ica; 3 days after HI; NI	Despite no effect on motor function deficit, there was ↓ motor asymmetry 20 days pT	No effect on brain atrophy 6 weeks pT	Detection of transplanted cells equally distributed in both hemispheres 6 weeks pT	Transplanted cells showed labeling of astrocyte and microglia (> number), neuron and oligodendrocyte (< number), equally distributed in both hemispheres 6 weeks pT
Xia et al. ⁴¹	Sprague-Dawley rats; P7; 150 min	MSC from human umbilical cord blood	5 x 10 ⁴ cells/µL; ic (IT); 3 days after HI; cyclosporine	Improvement of neurological function 14, 21 and 28 days pT	↓ brain injury 28 days pT	Presence of transplanted cells in the cortex and dispersion into the hippocampus 7 days pT	Transplanted cells exhibited markers of astrocytes, but not neurons, 7 days pT

BDNF = brain-derived neurotrophic factor; BHK-GDNF = baby hamster kidney cells (BHK) transfected with glial cell line-derived neurotrophic factor (GDNF) complementary DNA; MAPC = multipotent adult progenitor cells; ChABC = chondroitinase ABC; HUCBSC = human umbilical cord blood stem cells; MSC = mesenchymal stem cells; NSC = neural stem cells; CXCR4 = CXC chemokine receptor 4; FGF-2 = fibroblast growth factor 2; GDNF = glial cell line-derived neurotrophic factor; HI = hypoxia-ischemia; ic = intracerebral route; ica = intracardiac route; icv = intracerebroventricular route; ip = intraperitoneal route; iv = intravenous route; NA = not analyzed; NGF = nerve growth factor; NT3 = neurotrophin 3; P = postnatal day; PGA = polyglycolic acid; pT = post-transplantation; SDF-1 = stromal cell-derived factor 1; T-PX = transplantation on postnatal day X; NI = no immunosuppression; CT = contralateral transplant; IT = ipsilateral transplant; VEH = vehicle.

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Daadi et al. ²⁵	Sprague-Dawley rats; P7; 90 min	Human NSC from embryonic stem cells	5 x 10 ⁴ cells/μL; ic (IT at 3 sites); 24 h after HI; NI	Improvement of motor function and asymmetry 28-30 days pT	No effect on brain atrophy; overexpression of genes involved in neuro-/gliogenesis and neurotrophic support in the ischemic hemisphere; ↑ axonal sprouting into the ipsilateral hemisphere; ↑ axon terminals in the sensorimotor cortex, corpus callosum, and ipsilateral striatum/thalamus; ↑ microglial cells in the ischemic striatum, but not in the cortex (1 month pT)	Good survival of transplanted cells, with dispersion into the ischemic striatum, 1 month pT	Transplanted cells showed labeling of neural stem cells, neurons and astrocytes 1 month pT

BDNF = brain-derived neurotrophic factor; BHK-GDNF = baby hamster kidney cells (BHK) transfected with glial cell line-derived neurotrophic factor (GDNF) complementary DNA; MAPC = multipotent adult progenitor cells; ChABC = chondroitinase ABC; HUCBSC = human umbilical cord blood stem cells; MSC = mesenchymal stem cells; NSC = neural stem cells; CXCR4 = CXC chemokine receptor 4; FGF-2 = fibroblast growth factor 2; GDNF = glial cell line-derived neurotrophic factor; HI = hypoxia-ischemia; ic = intracerebral route; ica = intracardiac route; icv = intracerebroventricular route; ip = intraperitoneal route; iv = intravenous route; NA = not analyzed; NGF = nerve growth factor; NT3 = neurotrophin 3; P = postnatal day; PGA = polyglycolic acid; pT = post-transplantation; SDF-1 = stromal cell-derived factor 1; T-PX = transplantation on postnatal day X; NI = no immunosuppression; CT = contralateral transplant; IT = ipsilateral transplant; VEH = vehicle.

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In an elegant study, Park et al.²² presented interesting observations on the behavior of NSC when transplanted into the brain of neonatal mice subjected to HI injury. When transplanted into the contralateral hemisphere of the HI brain injury, NSC migrated across the corpus callosum and other interhemispheric commissures to the infarcted area. However, when transplanted into the ipsilateral hemisphere, the cells remained at the site of injury. In both cases, NSC differentiated in the ischemic site, exhibiting labeling for neurons, oligodendrocytes, astrocytes, and neural progenitor cells. In addition, that same research group elucidated the inflammatory mechanisms involved in the migration of NSC in response to HI injury.²³

Embryonic stem cells from the inner cell mass of blastocyst-stage embryos also have the ability to differentiate into neurons by specific culture protocols and may be considered as alternative cell sources for treatment of brain injury. Two studies used embryonic stem cells differentiated into neurons in HI injury models. In the first study, transplantation of embryonic stem cells differentiated into neurons *in vitro* resulted in improved spatial memory in HI rats.²⁴ However, although the number of neuronal cells increased in the ischemic

hippocampus, there is no clear correlation between the outcomes and neuronal repopulation, differentiation or release of trophic factors by the delivered cells. In another recently published study, restoration of motor function and symmetry was observed in HI rats after transplantation of human embryonic stem cells differentiated into neurons. However, the authors found no restorative effects on the structural brain damage.²⁵

The remaining ischemic environment can influence tissue repair due to the severity of the HI process. HI injuries may associate with massive tissue necrosis, resulting in a porencephalic cyst. Such situation provides an impaired microenvironment for homing of transplanted cells due to lack of blood supply and adequate extracellular matrix. Through studies in the field of tissue engineering, a new treatment approach has been proposed based on the use of biodegradable scaffolds within the infarcted cavity in order to foster the formation of connections between graft and host tissue.^{9,26} Using this therapeutic approach, Park et al.²⁷ demonstrated successful implantation of scaffolds associated with reduction in infarction cavities and motor deficit in rats subjected to HI brain injury. Surprisingly, the authors observed the formation of interconnections

between graft and host tissue, in addition to restoration of long-distance neuronal projections previously damaged by the HI process.

Other compounds may also be associated with cell therapy in order to provide support to NSC development in HI injury models. An example is the use of chondroitinase ABC (ChABC) combined with intracerebroventricular NSC transplantation in a neonatal HI rat model, which resulted in a significant reduction of brain damage.²⁸ Due to the ability to promote brain plasticity, the authors suggest that ChABC would have facilitated NSC adhesion and migration to ischemic areas.

Considering NSC as possible cellular vehicles of trophic factors, some studies have used genetically engineered stem cells to express increased levels of these substances. Using NSC overexpressing neurotrophin-3 (NSC/NT-3) in the HI rat brain, the researchers observed an increase in neuronal differentiation of 5 to 10-20% in the infarcted area and > 80% in the penumbra, when compared to non-genetically engineered NSC lines. The authors suggest that the production of NT3 by NSC may have functioned in an autocrine/paracrine fashion by binding to adjacent cells of the host tissue and stimulating endogenous neurogenesis.^{22,29} Other studies have also shown that NSC overexpressing specific trophic factors migrate to the injured brain site and generate niches of immature and proliferative cells available for brain repair.³⁰ Additionally, the perivascular environment has been shown to be critical to maintaining these cells in an active immature and proliferative state.³¹ Studies have also demonstrated that the use of cellular vehicles to release certain trophic factors can reduce the incidence and extent of brain damage and cognitive impairment in HI experimental studies.^{32,33}

Umbilical cord stem cell transplantation

Human umbilical cord blood stem cells (HUCBSC) have been used in experimental studies of HI brain injury due to their ability to differentiate into neurons and glial cells *in vitro* and *in vivo*. However, there are only few studies using non-human umbilical cord blood cells in animal models of neurological diseases, with conflicting results concerning the concomitant use of immunosuppressants.^{34,35}

The first study to test the effect of HUCBSC transplantation in HI rats was performed by Meier et al.³⁶ The authors demonstrated that intraperitoneal injection of HUCBSC 24 h after HI injury in neonatal rats resulted in improved walking pattern in the animals. The authors also found that the stem cells migrated to the injured brain region, but no reduction in brain atrophy or transdifferentiation of transplanted cells was observed. Using the same route of delivery, a group of researchers demonstrated that intraperitoneal transplantation of HUCBSC 3 h after HI injury can improve primitive reflexes in these animals.³⁷ In addition, the

authors demonstrated reduced cell death in the striatum and an anti-inflammatory effect in the cortex. However, the presence of few transplanted cells could be observed in the ischemic striatum and cortex of treated animals.

That same year, our research group published a study on the effects of intravenous delivery of HUCBSC in an experimental model of HI brain injury.³⁸ However, our results demonstrated only a trend toward reduction of behavioral and morphological deficits in treated rats 30 days after transplantation. Moreover, few transplanted cells were identified in the brain of treated animals. Aspects such as dosage, length of follow-up for evaluation and route of delivery may have influenced the outcomes of the study.

In parallel, we also developed an experimental model of hypoxia-ischemia in newborn piglets for the study of cell therapy in medium-sized animals.³⁹ Newborn pigs that received HUCBSC via the common carotid artery showed better neurological scores compared to animals subjected only to the HI injury model or the group transplanted via the umbilical artery. Additionally, the presence of few transplanted cells could be observed only in some animals in the group transplanted via the carotid artery.

An interesting study showed that intravenous injection of HUCBSC 7 days after HI brain injury, improved motor coordination and asymmetry in treated rats.⁴⁰ Furthermore, increased levels of growth factors were detected in the ipsilateral hemisphere 3 days after transplantation. In that study, the authors also included a group of HI animals receiving the same dose of HUCBSC combined with mannitol, a substance that increases blood-brain barrier permeabilization. The results of this experimental group showed that mannitol enhanced further the functional effects and expression of trophic factors, compared to the group that received HUCBSC alone.

The potential therapeutic feasibility of mesenchymal stem cells derived from human umbilical cord blood has been recently analyzed in an experimental model of HI brain injury.⁴¹ Transplantation was performed 3 days after HI injury by intracerebral route. Treated animals showed a progressive improvement of neurological function, associated with reduction of brain tissue injury. In addition, the authors verified the presence of transplanted cells in the cortex, with dispersion into the hippocampus, 7 days after transplantation, showing astrocytic, but not neuronal differentiation.

In the clinical setting, researchers at the Duke University have initiated a phase I study with autologous umbilical cord blood grafts in full-term newborns with moderate-to-severe HI encephalopathy (NCT00593242). Children will be followed for assessment of neurodevelopmental outcomes and subjected to magnetic resonance imaging. Currently, the clinical trial is in the process of recruiting patients who meet the selection criteria of the study.

Bone marrow stem cell transplantation

Studies of bone marrow stem cells have also been widely used in animal models of cerebral ischemia, such as adult rats,⁴²⁻⁴⁵ and, more recently in clinical trials, showing promising results.^{46,47}

Bone marrow-derived blood contains, at least, two cell populations with great potential for clinical use: hematopoietic stem cells and mesenchymal stem cells (MSC).³⁴ The finding that hematopoietic stem cells also have the ability to transdifferentiate into neuronal lineage has expanded the scope of use of this source of immature cells.^{48,49} Specifically, MSC derive from bone marrow stromal cells and are defined according to the presence of specific cell-surface markers, *in vitro* behavior, and differentiation potential.⁵⁰ This cell type has an increased capacity to express neuronal phenotypes *in vitro*^{51,52} and *in vivo*.^{53,54}

Recently, two research groups have presented their results using bone marrow-derived MSC in an experimental model of HI brain injury. Lee et al.⁵⁵ used the intracardiac route for transplantation 72 h after injury. The authors observed the presence of transplanted cells equally distributed in both hemispheres 6 weeks after transplantation. Such cells expressed more frequently markers of astrocyte and microglia, in addition to markers of neurons and oligodendrocytes, in smaller numbers. Furthermore, motor asymmetry improved in HI rats 40 days after transplantation, although repair of damaged ischemic tissue was not observed.

In another study, intracerebral injection of MSC 72 h after injury, increased proliferation and neuronal differentiation in the ischemic hemisphere.⁵⁶ Histological and motor outcomes were also favorable 10 and 21 days after treatment. However, less than 1% of new neurons derived from the transplanted cells. The study suggests that MSC have favored endogenous formation of new neuronal cells via suppression of microglial inflammatory activity.

Using a purified fraction of MSC, known as multipotent adult progenitor cells (MAPC), Yasuhara et al.⁵⁷ demonstrated that intracerebral or intravenous delivery of MAPC derived from the mouse bone marrow, 7 days after HI injury, resulted in improved motor function in transplanted animals. Interestingly, recovery of motor deficit was similar between animals that received allogeneic transplants (genetically different animals, but of the same species) and those that received syngeneic transplants (genetically identical animals). Associated with decreased neuronal loss, the authors also detected the presence of MAPC in ischemic hippocampal regions and neuronal labeling 14 days after transplantation.^{57,58}

Mechanisms of action involved in cell therapy in experimental hypoxia-ischemia

Understanding the mechanisms of action involved in the process of stem cell-induced neuroregeneration is essential to optimize the clinical benefits of cell therapy. Several factors (summarized in Table 2) may be responsible for the positive results shown in recent studies of experimental HI brain injury. However, despite the significant benefits demonstrated in these studies, the variables responsible for the successful transplantation of stem cells in HI brain injury have yet to be established.

Cell transdifferentiation

The first hypothesis examined to justify the beneficial effects of cell therapy in neurological diseases was the direct transformation of stem cells into mature neurons, a process known as transdifferentiation. However, studies of HI brain injury have shown that functional recovery in treated animals occurs independently of the presence of stem cells with expression of neuronal labeling in ischemic regions. The transdifferentiation of adult stem cells of mesodermal origin

Table 2 - Main mechanisms of action of stem cells in perinatal asphyxia

Mechanism of action	Description
Cell transdifferentiation	Direct transformation of stem cells (various sources) into mature neurons
Release of trophic factors	Stem cells may act as cellular vehicles for the production and/or release of neurotrophic factors
Stimulation of endogenous neurogenesis	Transplanted stem cells are believed to potentiate these intrinsic mechanisms of repair
Modulation of the inflammatory process	Stem cells may inhibit the activation of various immune cells and thereby increase neurogenesis and production of trophic factors
Stimulation of angiogenesis	Transdifferentiation of stem cells into new blood vessels (angiogenesis), increased vascularization in the penumbra and indirect formation of new blood vessels by the release of growth factors
Induction of neuroplasticity	Increase in afferent and efferent connections between the site of injury and brain regions, restoring local synaptic activity by synaptogenesis

into cells derived from the ectodermal layer is still under debate. In *in vitro* studies, transdifferentiation is detected by the positivity of specific markers of neurons and glial cells. However, the characterization of electrophysiological properties of these transdifferentiated cells is as yet poorly documented.⁵⁹⁻⁶¹ Due to these findings, other mechanisms have been proposed.⁶²

Release of trophic factors

Trophic factors are a family of polypeptides essential for the survival and differentiation of normal developing neurons and also play an important role in neuroprotection of mature neurons under pathological conditions.⁶³ Stem cells can serve as vehicles for specific molecules, acting as vectors for the production and/or release of neurotrophic factors.^{29,64} By interacting with their receptors, stem cells may release growth factors and cytokines, thus inhibiting apoptosis, increasing angiogenesis, and/or stimulating the differentiation of endogenous precursor cells.^{12,40} Data from the study by Borlongan et al.⁶⁵ reported a 15% increase in the production of trophic factors in the circulating blood of adult rats subjected to cerebral ischemia after HUCBSC transplantation. Recently, the same group has reported similar results after stem cell transplantation in a neonatal HI injury model in rats.⁴⁰ Although endogenous release of trophic factors by neuronal tissue occurs in response to ischemic injury, this compensatory mechanism is insufficient to promote tissue regeneration and/or functional recovery. Therefore, the authors of these studies suggest that the increase of specific trophic factors occurs probably due to the transplantation of stem cells.

Stimulation of endogenous neurogenesis

Several studies have demonstrated increased endogenous neurogenesis in response to neonatal HI injury.^{66,67} However, transplanted stem cells are believed to potentiate these intrinsic mechanisms of repair. In the study by van Velthoven et al.,⁵⁶ transplantation of stem cells into HI animals was shown to reduce microglial proliferation and increase neurogenesis. The authors suggest that the transplanted cells had the ability to modulate post-HI injury inflammatory response, thus facilitating the process of endogenous neurogenesis.

Modulation of the inflammatory process

While beneficial to the recruitment and migration of stem cells to the site of injury, inflammatory process determined by brain damage appears to be restrictive for cell differentiation.²³ In this context, recent studies have shown that different sources of stem cells can inhibit the activation of various immune cells. Pluchino et al.⁶⁸ showed that NSC promoted neuroprotection through anti-inflammatory cytokines and immunomodulatory molecules.

In another study, intravenous injection of HUCBSC increased the survival of neuronal tissue and reduced leukocyte infiltration and expression of proinflammatory proteins in the ischemic brain.⁶⁹

In the CNS, microglial cells are the first line of immune defense and comprise a major inflammatory process that contributes to brain damage. This cell population releases a variety of cytokines and growth factors that react rapidly in the injured brain tissue to promote phagocytosis and present antigens to T cells. However, microglial activation is commonly associated with decreased production of trophic factors and reduced neurogenesis.^{9,63,70} Two studies of cell therapy in experimental HI injury reported that transplantation of stem cells reduced microglial expression in the ischemic cortex and hippocampus and, consequently, improved functional outcomes in treated animals.^{37,56} In contrast, Daadi et al.²⁵ found increased proliferation of microglial cells in the striatum of HI animals that received NSC transplants. These data support the idea that microglial cells play a pro- and anti-inflammatory role, depending on their activation state and functional phenotype.⁷¹

Stimulation of angiogenesis

Some studies of ischemic injury in adult rats have shown increased vascularization in the penumbra within a few days after transplantation of stem cells.^{72,73} Furthermore, transdifferentiation of transplanted stem cells into new blood vessels (angiogenesis) has been observed.^{34,74} It is also likely that indirect cellular events occur in blood vessel formation. Chen et al.⁷⁵ showed that stem cells from the bone marrow stroma promoted angiogenesis in the ischemic region by increasing the endogenous levels of vascular endothelial growth factor. To date, there is only one study indicating neovascularization of brain tissue in HI animals that received NSC transplants.²⁷ The authors suggest that post-transplantation angiogenic signals were generated, thus allowing the formation of a new and vascularized parenchyma at the site of the porencephalic cyst.

Induction of neuroplasticity

Neuroplastic events include an increase in afferent and efferent connections between the site of injury and brain regions, restoring local synaptic activity by synaptogenesis.³⁴ The expression of synaptic proteins has been recently observed in adult rats with ischemic brain injury undergoing stem cell transplantation, which indicates the formation of synaptic contacts.^{76,77} Confirming this possibility, a recent study showed that intracerebral transplantation of NSC in HI rats increased the number of axon terminals in the sensorimotor cortex, corpus callosum, and ipsilateral striatum/thalamus as well as enhanced axonal sprouting into the injured hemisphere.²⁵

Translational aspects of cell therapy in perinatal asphyxia

Translational research can be defined as a process which leads from evidence-based medicine to sustainable solutions for public health problems.⁷⁸ Preclinical studies are the first step in developing new therapies. However, the transfer of knowledge acquired through studies *in vitro* and in animal models of HI brain injury to the clinical use of cell therapy requires attention to differences between species and critical analysis of experimental results. Moreover, aspects still poorly understood (Table 3), such as the route of delivery, the number of transplanted cells, the cell type used, and the timing of intervention after injury, are important variables that might significantly influence clinical response. Therefore,

after clarifying key issues concerning safety of treatment, it is possible that phase I clinical studies may be initiated to evaluate this novel therapeutic approach.

The choice of delivery route may be a crucial factor for the success of cell therapy when considering that the benefits of treatment depend on migration and location of transplanted cells in the brain region affected. The best route for stem cell transplantation in the treatment of neurological disorders has yet to be defined. However, there is a consensus that the optimal route should provide high efficiency and specificity, with minimal adverse effects. Studies in animal models of HI injury have used a variety of delivery methods, including intraperitoneal, intravenous and intracerebral routes.

Table 3 - Relevant aspects to the clinical use of cell therapy in perinatal asphyxia

Aspect	Advantages	Disadvantages
Route of delivery		
Intravenous	Less invasive and safer	Need for chemotactic signals Cells may migrate to other organs Need to assess adverse effects
Intra-arterial	Increased targeting of stem cells to injured sites	Risk of microembolism and ischemia Routine technique
Intracranial	Facilitates migration of stem cells at the site of ischemic injury	More invasive, risk of injury
Intraperitoneal	Practical, simple and less invasive technique	Cells need to travel long distances Few studies
Cell source and type		
Embryo or fetus	Increased capability to differentiate and proliferate	Ethical and religious barriers Difficulty in obtaining samples Impractical for use in the acute phase of injury
Umbilical cord blood (mononuclear cells)	Ease of obtaining samples Large amounts of cells Possibility of autologous transplantation	Little characterization of transplanted cells
Bone marrow (mononuclear cells)	Large amounts of cells Possibility of autologous transplantation	Little characterization of transplanted cells Painful technique and impractical for newborns
Neural stem cells	Cells of the same embryonic origin Widely studied Gold standard for treatment of brain injury	Restricted migration Cell death Difficulty in obtaining samples and immediate use
Post-transplantation timing		
Acute	Plays a neuroprotective role Presence of chemotactic signals and vascular permeability Consistent preclinical results	Limited to the use of little processed cell types (culture) Impractical for established cases
Chronic	Possibility of use in patients with established sequelae	Absence of appropriate chemotactic signals Absence of vascular permeability Few studies

By intracerebral transplantation, it is possible to reach directly a specific brain area. Despite the precision of the technique, this is an invasive procedure and several applications are needed to cover an ischemic injury, resulting in further brain impairment.⁷⁹ Intracerebroventricular injections represent another intracranial approach to deliver stem cells. However, despite allowing a diffuse cellular distribution, this route is not capable of reaching lesions distant from the ventricles and faces the same issues that are common to the intracerebral transplantation. In addition, transplanted cells may adhere to the ventricular wall and cause obstructive hydrocephalus.^{79,80} Although most experimental studies of cell therapy in neonatal HI brain injury use intracerebral transplantation, other less invasive delivery routes are more likely to be established for clinical practice. In the experimental context, intravenous and intracerebral transplantation of stem cells showed similar extent of motor and morphological recovery in an animal model of neonatal HI brain injury.⁵⁷

A recent meta-analysis demonstrated that intravenous cell delivery can improve outcomes in animal models of neurological disorders, and apoptosis inhibition is the main molecular change in the brain after transplantation.⁸¹ Intravenous cell delivery is a less invasive, simple and safe procedure, allowing widespread distribution of transplanted cells to HI brain areas.^{38,40,57} In addition, this procedure enables exposure of cells to chemotactic signals originating from the brain injury, which can selectively accumulate cells within the target tissue.^{79,82} However, only a small amount of stem cells may be able to reach the site of brain injury, since they can be trapped in the lungs, kidneys, liver, and spleen. Therefore, ectopic growth and toxicity in other organs need to be assessed before clinical use.⁸⁰

An alternative approach to avoid the systemic circulation system has been to deliver stem cells intra-arterially. Recent data show equally distributed migration and favorable neurological outcomes using this therapeutic approach in a rat model of HI brain injury.⁵⁵ This route bypasses the uptake by the systemic organs, allowing a large number of stem cells to reach the ischemic site, once vessels are reperfused. In the case of a permanent vascular occlusion, the transplanted cells will be distributed only in the penumbra region.⁷⁹ However, recent studies have reported high mortality rates with intra-arterial delivery in HI transplanted animals, suggesting the formation of microvascular occlusion and ischemia.^{79,80,83} In medical practice, this delivery method is commonly used in interventional vascular catheterization techniques. In a Brazilian clinical trial of cell therapy in patients with chronic ischemic stroke (NCT00473057), promising results were observed after intra-arterial transplantation of autologous bone marrow stem cells.⁴⁶

The intraperitoneal route has also been used for cell transplantation in neonatal HI injury, demonstrating cell migration from the peritoneal cavity into the injured brain

regions.^{36,37} The study findings suggest the presence of precise chemotactic signals and the need to break the blood-brain barrier so that cell migration can occur over such long distances. Despite being a practical procedure, further studies are needed to support this alternative route of transplantation.

The choice of time interval between the establishment of HI injury and intervention is also important, since the brain environment changes dramatically over time after HI injury. As a result, most studies using systemic administration of stem cells in HI injury are conducted in the acute stages of injury. Early injection of stem cells may exert an effect on the preservation and survival of neuronal tissue. Additionally, van Velthoven et al.⁵⁶ suggest that cell transplantation be performed between 2 and 3 days after HI injury, at which time the brain has greater capacity for cell proliferation and activation of endogenous repair mechanisms. In the study by Park et al.,²² the therapeutic window for effective cell transplantation covered 3-7 days following HI injury, due to high metabolic, biochemical and molecular activity in this period which could facilitate the migration of transplanted cells. In the case of transplantation at later time intervals, stem cells might stimulate the release of trophic factors to restore lost function.⁸⁰ However, despite the functional improvement observed in adult rats that received stem cells one month after cerebral ischemia,⁸⁴ there are no studies demonstrating therapeutic benefits in animal models of chronic HI brain injury, thus limiting clinical applicability to HI injury established due to the absence of appropriate chemotactic signals and vascular permeability.

Blood-brain barrier permeabilization may also facilitate CNS entry of stem cells or neurotrophic factors, especially when peripheral routes are used for transplantation. However, it has been recently shown that the number of intravenously delivered stem cells in the ischemic hippocampus of HI transplanted animals did not differ significantly when comparing animals that received stem cells alone to those that received a combination of mannitol and stem cells.⁴⁰ In contrast, transplanted animals that were administered mannitol showed increased brain levels of some trophic factors. There was also a marked improvement in behavioral and histological outcomes with the combined use of stem cells and mannitol, possibly through a paracrine effect of these cells. This evidence points out the importance of blood-brain barrier permeabilization in cell therapy, but also questions whether neuroprotection is really dependent on cell migration and differentiation.

The cell type and source most suitable for treatment of neonatal HI injury have yet to be defined.³⁴ The use of NSC is considered the gold standard for treatment of neurological diseases. However, the results of several studies show that cell death, restricted migration and rapid differentiation into glial cells limit the applicability of NSC in neuronal repair. In addition, a major obstacle to clinical

use of this cell type in neonatal brain injury is the difficulty in obtaining samples. Biopsy tissue or postmortem tissue specimens may be insufficient to produce significant amounts of these cells.⁸⁵ Ethical and religious issues involved in the harvesting of embryonic and fetal tissue and concerns about the risks of immune response or formation of malignant tumors also inhibit the clinical applicability of these cells. *In vitro* neuronal differentiation for future use of these cells in newborns with HI injury raises further concerns about potential risks of contamination, immune reaction to the factors added to the cell culture medium, and difficulty of use in acute transplantation. Similar disadvantages related to safety and difficulties of immediate use are also presented by MSC from bone marrow and umbilical cord blood, widely used in experimental HI brain injury.

In contrast, the processing of mononuclear stem cells derived from umbilical cord blood and bone marrow is relatively simple and rapid. In the neonatal context, mononuclear stem cells derived from umbilical cord blood offer some advantages over bone marrow aspiration. Obtaining stem cells from umbilical cord blood offers no risk or discomfort to the newborn, and cells can be transplanted after autologous collection. In addition, umbilical cord blood can be used therapeutically in the perinatal period or cryopreserved for later use.^{62,86} However, further studies are needed to characterize cell populations and evaluate effectiveness and safety.^{80,87}

Dose-response analysis of stem cells in animal models also needs to be performed to provide a basis for their clinical use. According to Janowski et al.,⁸¹ there is a dose-response association between the number of cells injected and the effects of the treatment of neurological diseases in experimental studies. However, the optimal dose of stem cells is partly dependent on the delivery route as well as on the cell type used, the timing of intervention, and the amount of transplanted cells that reach the brain injury. From a clinical standpoint, outcomes related to safety of cell transplantation, such as ectopic tissue formation and behavioral abnormalities, should be incorporated in the methodology of investigations and patient follow-up. It is also important to consider safe and effective methods for the noninvasive monitoring of migration of transplanted cells, thus establishing a proper understanding of patients' responses to the proposed treatment.

Conclusions

Basic research has shown promising results in the field of neonatal brain injury. Despite the variety of methods employed, most studies cited in this review indicate that stem cells may have neuroprotective properties, resulting in improved functional outcomes in treated animals. Certainly, the success of cell therapy in rodents can be regarded as the first step in developing a therapeutic approach for

future clinical use. However, it is noteworthy that human brain diseases have more complex mechanisms of damage and regeneration than in experimental animals. Therefore, issues related to dose, cell type, route of delivery and proper timing of intervention need to be defined. Although not crucial to clinical application, further investigations on the mechanisms of action of stem cells in HI injury are also an important tool in establishing the safety and effectiveness of transplantation.

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