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REVIEW ARTICLE

Baby STEPS: A Giant Leap for Cell Therapy in Neonatal Brain Injury

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ABSTRACT: We advance Baby STEPS or Stem cell Therapeutics as an Emerging Paradigm in Stroke as a guide in facilitating the critical evaluation in the laboratory of the safety and efficacy of cell therapy for neonatal encephalopathy. The need to carefully consider the clinical relevance of the animal models in mimicking human neonatal brain injury, selection of the optimal stem cell donor, and the application of functional outcome assays in small and large animal models serve as the foundation for preclinical work and beginning to understand the mechanism of this cellular therapy. The preclinical studies will aid our formulation of a rigorous human clinical trial that encompasses not only efficacy testing but also monitoring of safety indices and demonstration of mechanisms of action. This schema forms the basis of Baby STEPS. Our goal is to resonate the urgent call to enhance the successful translation of cell therapy from the laboratory to the clinic. **(*Pediatr Res* 70: 3–9, 2011)**

Why Do We Need Baby STEPS?

The need for standardized preclinical testing of neuroprotective drugs and cell therapy was recommended by Stroke Therapy Academic Industry Rountable (STAIR) and Stem cell Therapeutics as an Emerging Paradigm in Stroke (STEPS), both of which include support for the creation of a consortium of expert scientists and physicians, as well as solid representations from National Institutes of Health (NIH), U.S. Food and Drug Administration (FDA), and drug- and cell-based biotech companies (1–8). However, these preclinical criteria primarily target adult stroke. To this end, we advance a translational approach outlining the development of experimental therapeutics in neonatal brain injury requiring a unique set of guidelines and a consortium to advance the entry of therapeutic products from the laboratory into the clinical arena. Here, we focus on cell therapy for neonatal brain injury and propose Baby STEPS as a platform to establish guidelines and to solicit participation from academic, federal, and industry stakeholders to improve the translational potential of cell therapy in babies and/or young children.

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Patients Who May Benefit From Baby STEPS

There are several injuries or diseases that result in brain injury in the neonate that share pathophysiologic similarities with adult stroke and therefore would be potential candidates for cell-based therapies. These include neonatal encephalopathy, neonatal stroke, and periventricular leukomalacia (PVL). Neonatal brain injury can lead to a variety of neurodevelopmental problems including learning disabilities, mental retardation, hearing and visual impairments, and CP, a condition in which permanent damage to muscle coordination and body movement occurs. (http://www.ninds.nih.gov/disorders/cerebral_palsy/detail_cerebral_palsy.htm).

Neonatal encephalopathy occurs in about 20 of 1000 full-term infants and in nearly 60% of very LBW (premature) newborns (9,10). However, in the United States, as in other developed countries, the incidence of neonatal encephalopathy seems overstated, in that less than 10 per 1000 births each year succumb to neonatal encephalopathy. Because of concurrent injury to other organs, between 20 and 50% of babies with brain injury die during the newborn period (11). Of the survivors, up to 25% have permanent neuropsychological handicaps in the form of CP, with or without associated mental retardation, learning disabilities, or epilepsy (12,13). Neonatal brain injury may occur before delivery (placental abruption, toxemia, and maternal collagen vascular disease), during delivery (prolonged labor, difficult delivery, and abnormal presentation), or after delivery (sepsis, shock, and respiratory distress). The current state of the art treatment for neonatal encephalopathy is hypothermia (14–16). Although this therapy is an exciting evolution in the care of neonates with neonatal encephalopathy, only neonates with moderate encephalopathy seem to have the most favorable response to hypothermia with an improvement in neurodevelopmental outcomes (14,15). Moreover, the incidence of suboptimal neurodevelopmental outcomes in neonatal encephalopathy even after hypothermia is about 40–50% (15), suggesting the need for innovative treatments. Hence, although hypothermia

Abbreviations: HI, hypoxic-ischemia; **IPS,** ischemic perinatal stroke; **PVL,** periventricular leukomalacia; **STEPS,** Stem cell Therapeutics as an Emerging Paradigm in Stroke

may play a role in reducing the ongoing or escalating damage, repairing already damaged regions will require a cellular replacement approach that may be applicable for neonates with moderate to severe neonatal encephalopathy.

The perinatal period is the second highest risk group for developing cerebral stroke (17). Because ischemic perinatal stroke (IPS) is known to account for 30% of children with CP, IPS is labeled as the most common cause of CP (18). Thus, understanding the process and how to restore tissue damaged by IPS can significantly impact CP, which has an estimated lifetime cost of $11.5 billion (19). As such, it is vital to expand on basic science and clinical studies targeting the neonatal period as a potential treatment period to produce a clinical impact on neonates with IPS to reduce or eliminate this entity as a cause of CP.

PVL is cerebral white matter injury that occurs to some degree in 50% of neonates with birth weights less than 1500 g (20). PVL is associated with a decrease in volumes of the cortex, thalamus, and basal ganglia (21). This injury likely accounts for 90% of the neurologic deficits, including CP and cognitive, behavioral, and attentional deficits, that occur in surviving premature neonates (20). Because of a lack of current therapies for PVL, cell-based therapies offer promise as a potential treatment.

Neonatal Animal Models of Hypoxia-Ischemia and Stem Cell Therapy

Because of multiple neonatal pathologies resulting from hypoxic-ischemic (HI) injury to the brain, several animal models exist which attempt to mimic the various pathology seen in human neonates (22). Small animal neonatal models offer a very good platform to test proof of principle studies for cellular-based therapies and begin to understand the mechanism of injury because of their size, rapid and high-throughput testing, and the ability to perform functional outcomes. The rodent model developed by Vannucci (23–26) is one of the most widely accepted models of neonatal HI brain injury, involving the ligation of a unilateral carotid artery in a postnatal d 7 rat followed by exposure to systemic hypoxia (8% oxygen) for up to 3 h. The model produces injury to the cerebral cortex, subcortical and periventricular white matter, striatum, and hippocampus on the side of the ligation (23). This pattern of injury is similar to that seen in human neonates with neonatal encephalopathy. The Vannucci model has been adapted in mice (27), but the duration of hypoxic exposure varies because of diverse susceptibility of different mouse species to HI injury (28–31). With this in mind, the success of testing novel treatments for neonatal encephalopathy, such as cell therapy, seems highly dependent on the chosen species and strain that faithfully mimic the disease manifestations.

The age of the animal has also been implicated as playing a key role in animal modeling and translational research. The young age of the chosen neonatal animal allows brain plasticity to greatly influence the outcome of HI injury, which may exaggerate the therapeutic outcome of stem cell treatment. For example, animals between 1 and 2 postnatal days require a more severe hypoxia to produce the desired HI injury when compared with 7-d postnatal rat. Interestingly, postnatal d 1–2 rats show more damage to the ipsilateral subcortical developing white matter than the older rats (28). That these distinct neurodevelopmental pathological manifestations resulting from HI injury are dictated by age is exemplified by the observation of localized subplate neuronal death, which occurs concomitantly with increased oligodendrocyte progenitor cell proliferation following subcortical damage in young neonates (32–36). However, this limited extent of neurodegeneration and the compensatory endogenous cell repair mechanism seem to wane in older neonates when the HI insult encompasses both cortex and white matter (32–36). Accordingly, if cell therapy is initiated in HI injured rats of various postnatal ages, data interpretation should consider these dynamic levels of age-dependent neurodegeneration and neural repair. These results, altogether, suggest that the recognition of the age of neonatal animals is critical to producing a reliable HI injury model for testing experimental therapeutics related to the pathology of interest (*e.g.* PVL and neonatal encephalopathy).

The gender of the neonate also plays an important role in HI injury studies. In demonstrating the therapeutic benefits of erythropoietin in neonatal rats exposed to HI injury, the female animals displayed robust reduction in infarct volumes by 6 wk and maintained up to 12 wk postinjury, whereas the male animals exhibited only modest reduction in infarct volumes at 6 wk that worsened by 12 wk postinjury (37). These gender-dependent histological effects of erythropoietin were paralleled by similar differences in the resulting behavioral recovery during the same postinjury period, whereby females outperformed the males in a sensorimotor task. That gender affects the therapeutic outcome in neonatal HI injury model has been previously detailed in other experimental treatments (38,39) and should be a consideration in the design of clinical trials of cell therapy. Along this vein, stem cell transplants at childbirth may minimize the influence of gender differences by enhancing the fetomaternal stem cell trafficking that can increase the number of stem cells in the fetal circulation and afford immediate benefit to the baby in the event of newborn diseases such as neonatal encephalopathy (40). The delay in cord blood clamping may allow stem cell transplants to occur early on during childbirth and in a natural setting, thus this novel cell therapy approach may have a logistical advantage over hypothermia and other pharmacological treatments which likely can only be initiated after the baby is born.

Finally, when contemplating with animal modeling, a species closer to humans may approximate the disease and provide a better platform for testing potent treatments for neonatal encephalopathy. Larger animal models are a logical progression from small animal models, because they give the researcher the ability to use similar delivery approaches for cell therapy that would be encountered in a human neonate. However, these models have the disadvantage of being more difficult to work with postinjury often requiring resuscitation and sometimes intensive care. Several large species have recently been shown to have an impact on perinatal brain research such as fetal and neonatal nonhuman primate, sheep, lamb, puppy, piglet, and rabbit (22,41–46). A careful evalu-

ation of the rodent and the large animal models for HI injury should allow a much in-depth examination of the neurobehavioral pathology associated with the neonatal disease. Consideration should be given equally to the costs of proper animal handling for research and the neurostructural and behavioral manifestations produced by the experimental injury that should parallel the human condition to better assess the safety and efficacy of cell therapy for neonatal encephalopathy.

Review of Clinical Trials of Stem Cell Therapy for Cerebral Ischemia

Currently, there are eight stem cell products being evaluated in the clinic for adult stroke patients, with only one product in Phase III clinical trials. In general, the stem cells used for cerebral ischemic injury display mesenchymal or mesenchymal-like stem cell properties. Stem cells derived from patient's own tissues are also being investigated in some stem cell products. Embryonic-derived stem cells have not reached advanced preclinical testing in stroke (http://www.researchandmarkets.com/product/86f9c5/stem_cell_therapy_for_stroke). Our group has been associated with the preclinical testing of SanBio Inc.'s bone marrow-derived stromal cells in chronic stroke. We are also developing a pipeline for transplantation of umbilical cord blood cells for neonatal encephalopathy. There are at least two limited clinical trials in the United States testing the safety and efficacy of umbilical cord blood transplants in CP pediatric patients (Dr. James Carroll of Medical College of Georgia and Dr. Joanne Kurtzberg of Duke University). Careful clinical trial design and rigorous analysis of data should allow a critical assessment of the therapeutic potential of cell therapy in the clinic.

Identifying the Optimal Stem Cell Donor

Cell therapy for adult stroke has reached clinical trials (47–50). The effective donor cell type for stroke seemed to require a neuronal phenotype, and for that reason, many previous preclinical studies examined primary fetal neuronal cells and neuronal progenitor cells (50,51). However, the notion that stroke requires neuronal as well as glial and oligodendrocytic, cell replacement, in addition to trophic, vascuologenic, angiogenic, and synaptogenic, among other exogenous and endogenous neural repair mechanisms, facilitated the entry of novel cell graft donors including trophic factor secreting tissues, such as carotid body (52) and pineal gland (53), and embryonic, fetal, and adult sources of stem cells, such as umbilical cord blood (54,55), bone marrow (56–58), placenta/amnion tissue and fluid (59), and menstrual blood (60).

Stem cell researchers studying neonatal brain injury have similarly explored the need for identifying the optimal cell with neurogeneic, vascuologenic, angiogenic, and trophic support to afford therapeutic benefits in this setting. A major criterion related to demonstrating optimal cell type for neonatal brain injury requires the need to reveal the donor cell phenotype to allow cross-laboratory validation and replication, and phenotyping would need to occur by a uniform set of techniques, developed in concert with regulatory and consortium expertise, but equally important to create an off-the-shelf cell product that is readily available for transplantation in the clinic. To realize this cell characterization for clinical use, it will be most practical to conduct a quality control and assurance to ensure sterile condition of the cell product. Because a cell processing unit operating under strict good manufacturing practice (GMP) and good laboratory practice (GLP) is not routinely found in the clinic, the preferred approach is for all cell preparation done in an FDA-approved manufacturing facility, and that the envisioned cell product is frozen at this facility then delivered and thawed at the clinic for immediate use without additional manipulation. Among the many cell manipulation techniques include the basic phenotypic characterization of the donor cells, such as surface marker antigens and gene/protein expressions *via* immunocytochemistry and microarray/ELISA, respectively. In addition, if cell homogeneity is indicated for efficacy, flow cytometry should be considered.

Functional Outcome Measures

Appropriate behavioral and histological tests are extremely important for characterizing the HI injury and the therapeutic outcome of cell therapy in animal models. Similar to the guidelines proposed for adult stroke, the use of behavioral tests in neonatal HI injury should consider the clinical manifestation of the disease namely symptoms of motor (*e.g.* elevated body swing test, Rotorod, and general locomotor activity), somatosensory (*e.g.* neurological test, limb placement test, foot fault test, grip traction test, and postural reflex test), and cognitive functions (*e.g.* Morris water maze, plus maze, the eight-arm radial maze, and the choice reaction time task) seen in the clinic (61–64). Behavioral testing should also match the neuroanatomical damage produced by the HI injury. For example, cortical and hippocampal damage after HI should be reflected by impairments in motor function and learning and memory, behaviors that have been implicated as being mediated by these brain structures, respectively, which should prompt the investigator to use the corresponding behavioral tests (5–7,59,65–67). Because of the young age of the animals, the use of complex behavioral tests, including cognitive tasks, would be difficult to perform in the rodent model but may be possible in large animals. The behavioral testing should be performed over long-term after administration of therapy to reveal the onset and stable effects of the novel treatment (5–7,68). A key difference between adult stroke and neonatal brain injury is that neonatal brain injury presents with a considerable level of spontaneous recovery that accompanies the early stages of the experimental insult (69) and endogenous brain reorganization as the animal matures (70), requiring the need for a careful evaluation of the data especially when behavioral recovery is used as a major index of efficacy of therapeutic intervention. Based on our experience (unpublished results), increasing the complexity of the task (*e.g.* higher rod speed for Rotorod test) could reveal the subtle impairments in motor coordination even with the occurrence of spontaneous recovery in young, juvenile animals that received HI injury at postnatal 7 d of age. That plasticity of the neonatal brain after brain injury is similarly recognized in

pediatric patients (71). Note that although endogenous repair processes seem more robust in neonatal brain injury compared with adult stroke, long-lasting neurobehavioral deficits persists in the injured neonates that would require treatment interventions such as cell therapy. Equally noteworthy is that behavioral testing in large animal models of neonatal encephalopathy remains limited to nonhuman primates.

In addition to behavioral tests, histological assays of the host brain damage and the detection of the transplanted cells are extremely important. For determining host brain damage, the major focus until recently was the reduction in the core injury produced by the HI insult. However, in recent years, adjacent regions and even areas remote from the core injury have been the target of therapeutic interventions, including cell therapy (54,65). The targeted brain regions distant from the core injury include neurogenic sites, such as the subventricular zone and the dentate gyrus, and nonneurogenic sites, including the striatum and cortex, which have shown robust cell proliferation after HI injury and cell therapy (54,65). The other histological marker necessary to provide a link between grafted cells and the behavioral recovery relates to the assessment of the status of the grafted cells. Normally this evaluation of the grafted cells pertains to the cell fate, thus immunohistochemical assays *via* phenotypic markers are used to reveal maintenance of stemness or cell lineage commitment/differentiation (65,72). However, there is also compelling evidence that grafted cells' entry into the brain is not required for therapeutic effects and that their secreted factors or graft-stimulated growth factors from the host should be sufficient to afford functional recovery (54). Accordingly, markers of endogenous repair processes such as trophic effect, immunomodulatory response, neurogenesis, vascuolgenis, and angiogenesis have been used to demonstrate this alternative pathway of brain repair after cell therapy in neonatal HI injury (73–75). These results taken together indicate the need to reveal the mechanism of action of the grafted cells by either direct visualization of the cells in the brain suggesting neuroregeneration or analyze the brains for an increase in graft-stimulated secreted factors that can enhance host endogenous repair processes. Direct visualization of the grafted cells is also important to reveal any untoward tumor and ectopic tissue formation in both central and peripheral organs of the transplant recipient.

Experimental Design

The experimental design of the laboratory studies should closely approximate the envisioned clinical trials to maintain the translational potential of cell therapy. One primary goal is to envision the clinical product in contemplating with the experimental design for the laboratory studies. Here, optimization of the cell dose, delivery route, and timing of administration correspond to the three most important factors that need to be determined in the laboratory. Although a bolus injection of cells seem to be the current transplant paradigm for adult stroke and adopted in neonatal brain injury, there is reason to believe that multiple transplants may prove more beneficial in further retarding and also to completely reverse the disease-induced neurobehavioral deficits. In theory, there are two stages in which treatment can be developed for neonatal brain injury, the neuroprotective stage (within 24 h of the insult) and the neurorestoration stage (beyond 24 h after the insult) (76,77). These time points may require different cell types to fulfill the therapeutic intent. With these considerations in mind, the experimental design of laboratory studies should now incorporate repeated dosing regimen of donor cells. In view of multiple cell injections, lower cell dose may be possible thereby circumventing possibility of microembolism with high cell dose. Moreover, with this repeated cell dosing, the route of administration is likely *via* peripheral vasculature rather than direct intracerebral transplantation.

Mechanisms of Action

As noted above, functional outcome assays and evaluation of the status of grafted cells are important criteria in translational cell therapy for neonatal brain injury. These two criteria overlap in terms of their overarching focus on mechanisms of action underlying functional recovery produced by the grafted cells. Cell signaling and growth pathways along with neurorestorative processes such as neurogenesis, angiogenesis, synaptogensis, immunomedulation, trophic factor secretion, and cell replacement are effective targets for treatments for cell therapy (5–7,59). The two major postulated mechanisms of action for cell based treatment of neurological disorders include cell replacement and bystander effects. Imaging techniques such as *in vivo* functional MRI (fMRI) can be used to reveal both graft survival and endogenous repair mechanisms, as previously demonstrated in adult stroke models (78–81). These observations can be extended to neonatal brain injury as has been recently proposed (82–84).

Safety Outcome Measures

Operating under the Hippocratic Oath of "to do no harm to the patients," cell therapy should be scrutinized not only for their efficacy but equally for their safety. As noted above, phenotypic characterization of the donor cells is a prerequisite before transplantation to delete any tumor-forming cells. After transplantation, the survival, migration, and differentiation of the grafted cells should also be monitored, if possible with minimally invasive visualization techniques such as MRI. To that end, the experimental design should carefully address all safety issues. When moving forward to the clinic, cell therapy studies should have a method of identifying tumor or ectopic tissue formation, cell fate and status, and adverse behavioral effects. Moreover, solicitation of advice from the FDA must be initiated early on during the design of pivotal preclinical studies to get guidance on both efficacy and safety outcome measures.

Relevance of Baby STEPS to Adult Stroke's STEPS Guidelines

Many of the neonatal brain injury guidelines being proposed here have been derived from the original recommendations set forth by the STEPS consortium. Here, we identified

STEPS guidelines for adult stroke that can be extended to neonatal brain injury. We discussed above the importance of animal modeling, characterization of donor cells, careful considerations for the experimental design, the choice of functional and safety outcomes, and the need to incorporate mechanism-based investigations at the preclinical stage, which we have borrowed from STEPS but highlighted caveats for these translational criteria to be applied in Baby STEPS.

There are, however, many key differences between adult stroke and neonatal brain injury, supporting the need to establish Baby STEPS separate from STEPS. Clearly, the age of the targeted population differs between adult stroke and neonatal brain injury, with the latter likely to be more responsive to cell therapy due largely to increased brain plasticity accompanying the neonatal brain.

A major area of research need for cell-based therapies in neonates is the development of objective test that can accurately predict long-term neurologic deficits shortly after injury. These tests will likely include biomarkers, physical examination findings, amplitude-integrated electroencephalography (aEEG), imaging studies, and cerebral oximetry. Ideally, a scoring system using all these tests to accurately predict long-term deficits such as CP should be developed. Predicting long-term outcomes is a daunting task, because these tests must predict the attainment of baseline function many years into the future with brain plasticity serving as a confounding variable. This situation is markedly different from an adult who suffers brain injury and loses function shortly after injury and can be readily tested for this loss of function.

On the basis of these critical laboratory and clinical variables, distinguishing neonatal brain injury from adult stroke, we recognize the need for establishing the Baby STEPS. Although we are focused on cell therapy, we envision that these Baby STEPS guidelines will also apply to other experimental neuroprotective and neurorestorative treatments for neonatal brain injury (85–88) and complement other existing pediatric stroke recommendations for research and treatment interventions (89–96). We plan to set up a consortium that will include the NIH, the FDA, and multiple clinicians and scientists from numerous disciplines to better amplify the therapeutic potential of cell transplantation in neonatal brain injury. This consortium will enhance the execution of experimental designs that maximize the efficacy and safety of cell therapy in neonatal brain injury as we translate this treatment into clinical application. In addition to the NIH and the FDA, we would like to include biotech companies that will be able to offer important resources especially their expertise and infrastructure for providing clinical grade cells processed under strict GMP and GLP; these companies (while not an inclusive list) will involve Celgene Cellular Therapeutics, Cord Blood Registry, and Cryo-Cell. Although the many guidelines enumerated here seem daunting and appear to suggest additional administrative hurdles before clinical trial initiation, our goal is to expedite the transition of cell therapy from the laboratory to the clinic. With the involvement of the FDA regulatory board, the scientific vision support of NIH, and the participation of stem cell-based companies allowing access to their established cell manufacturing protocols, this academic-regulatory-industry consortium should advance cell therapy for neonatal brain injury.

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