J. Cell. Mol. Med. Vol 14, No 4, 2010 pp. 914-921

# Mannitol facilitates neurotrophic factor up-regulation and behavioural recovery in neonatal hypoxic-ischaemic rats with human umbilical cord blood grafts

T. Yasuhara [a], K. Hara [a], M. Maki [a], L. Xu [a], G. Yu [a], M. M. Ali [a], T. Masuda [a], S. J. Yu [a], E. K. Bae [a], T. Hayashi [a], N. Matsukawa [a], Y. Kaneko [a], N. Kuzmin-Nichols [b], S. Ellovitch [c], E. L. Cruz [c], S. K. Klasko [d], C. D. Sanberg [b], P. R. Sanberg [d], C. V. Borlongan [a, *]

[a] *Department of Neurology, Medical College of Georgia, Augusta, GA, USA*
[b] *Saneron CCEL Therapeutics, Tampa, FL, USA*
[c] *Cryopraxis Criobiologia, Rio de Janeiro, Brazil*
[d] *Center of Excellence for Aging and Brain Repair, Department of Neurosurgery, University of South Florida College of Medicine, Tampa, FL, USA*

*Received: October 12, 2008; Accepted: January 5, 2009*

## Abstract

We recently demonstrated that blood–brain barrier permeabilization using mannitol enhances the therapeutic efficacy of systemically administered human umbilical cord blood (HUCB) by facilitating the entry of neurotrophic factors from the periphery into the adult stroke brain. Here, we examined whether the same blood–brain barrier manipulation approach increases the therapeutic effects of intravenously delivered HUCB in a neonatal hypoxic-ischaemic (HI) injury model. Seven-day-old Sprague–Dawley rats were subjected to unilateral HI injury and then at day 7 after the insult, animals intravenously received vehicle alone, mannitol alone, HUCB cells (15k mononuclear fraction) alone or a combination of mannitol and HUCB cells. Behavioural tests at post-transplantation days 7 and 14 showed that HI animals that received HUCB cells alone or when combined with mannitol were significantly less impaired in motor asymmetry and motor coordination compared with those that received vehicle alone or mannitol alone. Brain tissues from a separate animal cohort from the four treatment conditions were processed for enzyme-linked immunosorbent assay at day 3 post-transplantation, and revealed elevated levels of GDNF, NGF and BDNF in those that received HUCB cells alone or when combined with mannitol compared with those that received vehicle or mannitol alone, with the combined HUCB cells and mannitol exhibiting the most robust neurotropic factor up-regulation. Histological assays revealed only sporadic detection of HUCB cells, suggesting that the trophic factor–mediated mechanism, rather than cell replacement *per se*, principally contributed to the behavioural improvement. These findings extend the utility of blood–brain barrier permeabilization in facilitating cell therapy for treating neonatal HI injury.

**Keywords:** stem cells • transplantation • cerebral palsy • neurotrophic factor • blood–brain barrier

## Introduction

Cell therapy remains a controversial approach for treating human disorders [1–3]. Laboratory and clinical data are divided on trans-differentiation of cells from non-neuronal origin, with studies reporting failure of hematopoietic bone marrow cells to differentiate into brain cells [4, 5], whereas equally compelling evidence demonstrates that peripherally transplanted adult human bone marrow cells in patients with hematologic malignancies entered the brain and generated neurons [6, 7]. Clinical trials of cell therapy for neurological disorders have been performed in Parkinson's disease, Huntington's disease and stroke [8–10]. Two major lines of mechanistic investigations on cell therapy have focussed on the concepts that either transplanted cells will replace the damaged host cells or the grafts will serve as neurotrophic factor–secreting vehicles, both pathways implicated to afford neuroprotection and/or neurorestoration. Because the absence of surviving grafts

*Correspondence to: Cesar V. BORLONGAN, Ph.D.,
Department of Neurosurgery, MDC 78,
University of South Florida College Medicine, Tampa, FL 33612, USA.
Tel.: 813-974-3988
Fax: 813-974-3078
E-mail: cborlong@health.usf.edu

doi:10.1111/j.1582-4934.2008.00671.x

**Table 1** Timeline of experimental procedures

| | |
|---|---|
| Day 0 | Seven-day-old rats exposed to hypoxic-ischaemic injury |
| Day 0 | Transplantation of HUCB, manniol, vehicle or combination |
| Day 3 | Separate cohort of animals processed for ELISA |
| Day 7 and 14 | Behavioural tests |
| Day 14 | Histological assays |

correlates with continued display of neurological deficits in transplant recipients, in both animal models of CNS disorders and patients [11, 12], a scientific bias exists for dismissing transplantation results as 'placebo effects' when graft survival could not be unequivocally demonstrated in the event of functional recovery. To this end, we previously showed that transplant-mediated functional recovery could occur in stroke without CNS entry of grafted cells, provided that neuroprotective molecules secreted by these cells crossed the blood–brain barrier (BBB) and reached the injured brain site [13]. The aim of the present study was drawn from our previous report [13] in adult stroke animals showing mannitol facilitation of trophic factor effects produced by intravenously administered HUCB. Here, we now hypothesized that mannitol treatment in neonatal HI injured animals would similarly enhance the therapeutic benefits of HUCB grafts by permeating the BBB, thereby improving the access of trophic factors secreted by the transplants from the periphery to the brain. In the present study, we tested whether the same BBB manipulation approach potentiates the therapeutic effects of intravenously delivered human umbilical cord blood (HUCB) in a neonatal hypoxic-ischaemic (HI) injury model. The rat neonatal HI model mimics the brain injury, such as cerebral palsy, occurring in infants. No treatment is available for this disorder; thus demonstrating efficacy with cell therapy in HI model would potentially benefit a large number of infants.

# Materials and methods

## Animals

We examined the behavioural effects and histological status of early intravenous delivery of HUCB cells into neonatal rats (Table 1). NIH and IACUC guidelines for use of animals in research were followed. Seven-day-old Sprague–Dawley rats were initially subjected to unilateral HI injury [14]. Briefly, rat pups underwent permanent ligation of the right common carotid artery under anaesthesia with 2% isoflurane. A midline cervical incision was made to expose the right common carotid artery, which was ligated by a single suture. The incision was closed with interrupted 6-0 silk sutures. The pups were then placed with the dams for 2 hrs before placement in an 8% oxygen chamber partially immersed in a water bath at 37°C for 2.5 hrs. The animals were then placed on a temperature-controlled blanket until they recovered from anaesthesia. At day 7 after HI injury, animals ($n = 8$ per treatment group) received injections of vehicle alone, mannitol alone, HUCB cells (15k mononuclear fraction) alone or a combination of mannitol and HUCB *via* the jugular vein. For transplantation and mannitol treatment, anaesthetized (equithesin, 300 mg/kg IP) animals received intravenous (jugular vein) injection of HUCB (from Saneron CCEL Therapeutics, Inc, Tampa, FL, in 0.2 ml PBS) or vehicle (PBS, same volume) over 2 min. Immediately thereafter, using the same intravenous line, animals received either 1.1 mol/l mannitol (maintained at 4°C) or vehicle (PBS, also maintained at 4°C) at a volume of 0.01 ml/g body weight over 5 min.

## Behavioural testing

Behavioural tests were conducted at post-transplantation days 7 and 14 to reveal HI-induced deficits in motor asymmetry and motor coordination using the elevated body swing test (EBST) and the Rotarod, respectively. The EBST provided a motor asymmetry parameter and involved handling the animal by its tail and recording the direction of the biased body swings [15]. The EBST consisted of 20 trials with the number of swings ipsilateral and contralateral to the ischaemic hemisphere recorded and expressed in percentage to determine the biased swing activity. The Rotarod treadmill (Accuscan, Inc., Columbus, OH) generated data by averaging the scores (total time spent on treadmill divided by five trials) for each animal on days 7 and 14. Each animal was placed in a neutral position on a 3-cm-diameter cylinder, then the rod rotated with the speed accelerated linearly from 0 rpm to 24 rpm within 60 sec., and the time spent on the Rotarod was recorded automatically. The maximum score given to an animal was fixed to 60.

## Immunohistochemistry

HUCB cell graft survival was examined using a human-specific antibody. Animals were anaesthetized with xylazine (13 mg/kg i.p.) and ketamine (44 mg/kg i.p.) then perfused with saline (150 ml) *via* a cardiac catheter. The brain was removed and stored in 4% paraformaldehyde with 25% sucrose until cryostat sectioning. Based on our previous study showing hippocampal damage following HI injury, we focussed our histological analyses within the hippocampal region. Brain sections were cut at 20 μm cryostat thickness and processed for immunohistochemistry. Free-floating sections were incubated overnight at 4°C with an anti-human nuclei (HuNu) antibody (mouse monoclonal IgG, 1:300, Chemicon, Billerica, MA, USA) with 10% normal horse serum (Vector, Burlingame, CA, USA) and 0.2% TritonX (Fischer Scientific, Pittsburg, PA, USA). After several rinses in PBS, sections were incubated for 1 hr in bisBenzimideH 33342 trihydrochloride (Hoechst33342, 1:1000, Sigma, St Louis, MO, USA). Alternate sections were processed for MAP2 (1:500, Abcam, Cambridge, MA, USA) to reveal dendritic density in the CA1 region. The sections were washed three times in PBS and mounted on Superfrost® Plus glass slides (Erie Scientific, Portsmouth, NH, USA) and embedded with mounting medium (Biomeda, Foster City, CA, USA). The fluorescent images were captured by Zeiss Axio Imager (Thornwood, NY, USA). Control studies included exclusion of primary antibody substituted with 10% normal horse serum in PBS. No immunoreactivity was observed in these controls. For estimation of preservation of dendrites in the CA1, two randomly selected visual fields in each coronal level, using three levels, were photographically captured (Zeiss Axio Imager). For analyses of the density of MAP2-positive density

in the CA1, two areas of the HI-injured CA1 and the two corresponding areas in the control, non–HI-injured CA1 were analyzed using Scion Image software (Scion, Frederick, MD). Binary images were created using a distinct threshold, and then the positive areas were calculated and summed up. To further control the estimation of the MAP2 fibre density, the sections were counterstained with the cell nuclear marker DAPI, thereby allowing calculation of the number of dendrites divided by cell numbers in the identical area to show an average dendrite per cell. The percent ratio of the value in the HI-injured CA1 to the intact side was used for statistical analyses.

### Enzyme-linked immunosorbent assay (ELISA)

Parallel groups of animals (an additional $n = 5$ per treatment group) from the four treatment conditions were euthanized at day 3 post-transplantation for ELISA of known CNS growth factors, including GDNF, NGF and BDNF, which have been previously shown to exert neurotrophic effects in ischaemic injury. We followed the ELISA method described elsewhere [16] with minor modifications. Anaesthetized animals were decapitated, their brains removed quickly and the hemisphere ipsilateral to the stroke side was dissected and stored at 270°C until analysis. Brains were homogenized using a Teflon homogenizer (setting 7, 20 strokes) in a lysis buffer (137 mM NaCl, 20 mM Tris, pH 8.0, 1% NP-40, 10% glycerol, 1 mM phenylmethyl-sulfonyl-fluoride [PMSF], 10 mg/ml aprotinin, 2 mg/ml leupeptin, 1 mM sodium vanadate). The homogenates were centrifuged at 12,000*g* for 20 min. The pellets were discarded and the supernatant was acidified to enhance the detection of neurotrophic factors. Samples were then neutralized to pH 7.4 and then adjusted with buffer to contain the same amount of protein per ml (2 mg/ml). Protein concentrations were measured by using the BCA kit (Pierse, Rockford, IL). The samples were assayed for GDNF, NGF and BDNF by ELISA, using trophic factor antibodies obtained from R&D Systems (Minneapolis, MN). A THERMOmax 96-well microplate reader (Molecular Devices Corp., Sunnyvale, CA) was used to measure the optical densities.

### Statistical analysis

Behavioural scores and trophic factor expression levels were initially analyzed using ANOVA, followed by post hoc Bonferroni t-tests for pair-wise comparisons between treatment groups. The level of significance was set at $<0.05$.

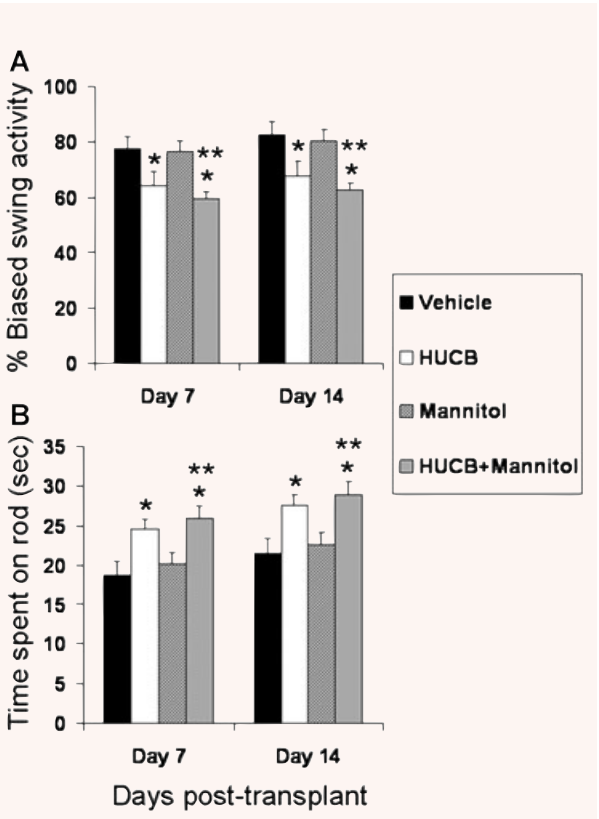
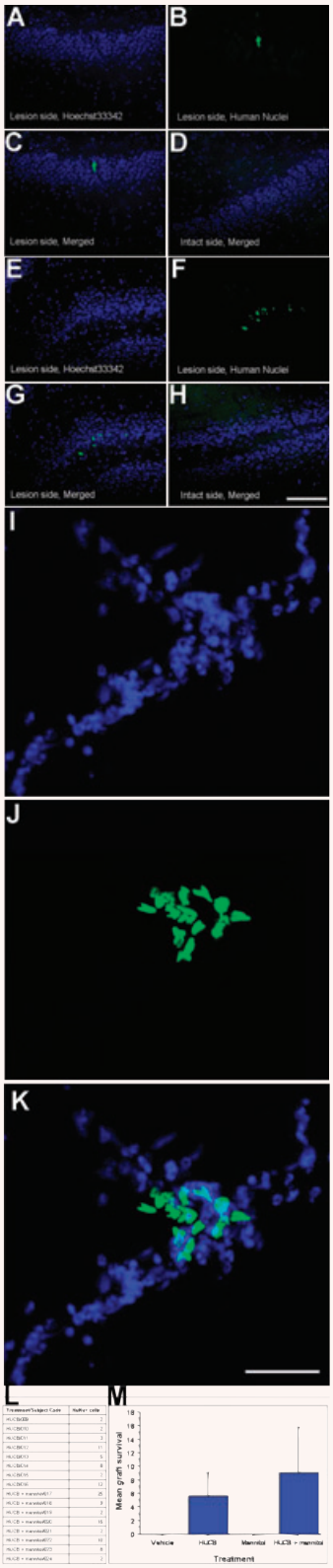



**Fig. 1** HUCB grafts ameliorate HI-induced behavioural deficits. EBST (**A**), Rotarod (**B**). Significant behavioural recovery of locomotor tasks in transplanted HI-injured animals ($P < 0.05$ *versus* vehicle or mannitol alone) was detected at both post-transplant testing days. Both groups of HI animals that received HUCB cells alone or when combined with mannitol were significantly less impaired in EBST and Rotarod test compared with those that received vehicle alone or mannitol alone at both post-transplant testing days 7 and 14, but the HI animals that received combined HUCB cells and mannitol displayed significantly better improvement than those that received HUCB cells alone ($P$s $< 0.01$ *versus* *vehicle/mannitol or **HUCB alone). Data are shown as mean values + S.E.

## Results

### Mannitol enhances behavioural recovery of HI-injured animals transplanted with HUCB

Behavioural tests were conducted at post-transplantation days 7 and 14 (Fig. 1). Repeated measures of ANOVA revealed significant treatment effects (EBST: $F_{3,28} = 64.63$, $P < 0.0001$; Rotarod: $F_{3,28} = 88.68$, $P < 0.0001$) and pair-wise t-test comparisons revealed that HI animals that received HUCB cells alone or when combined with mannitol were significantly less impaired in motor asymmetry and motor coordination as revealed by the EBST ($P < 0.01$) and the Rotarod test ($P < 0.01$), respectively, compared with those that received vehicle alone or mannitol alone at both post-transplant testing days (EBST, day 7: $F_{3,28} = 44.70$, $P < 0.0001$; EBST, day 14: $F_{3,28} = 69.24$, $P < 0.0001$; Rotarod, day 7: $F_{3,28} = 39.93$, $P < 0.0001$; Rotarod, day 14: $F_{3,28} = 52.23$, $P < 0.0001$). Moreover, the HI animals that received combined HUCB cells and mannitol (22% reduction and 28% increment in EBST and Rotarod) displayed significantly better improvement than those that received HUCB cells alone (16% and 19%) at both post-transplant testing days (EBST: $P < 0.01$ and $P < 0.001$ for days 7 and 14; Rotarod: $P$s $< 0.01$ for days 7 and 14).



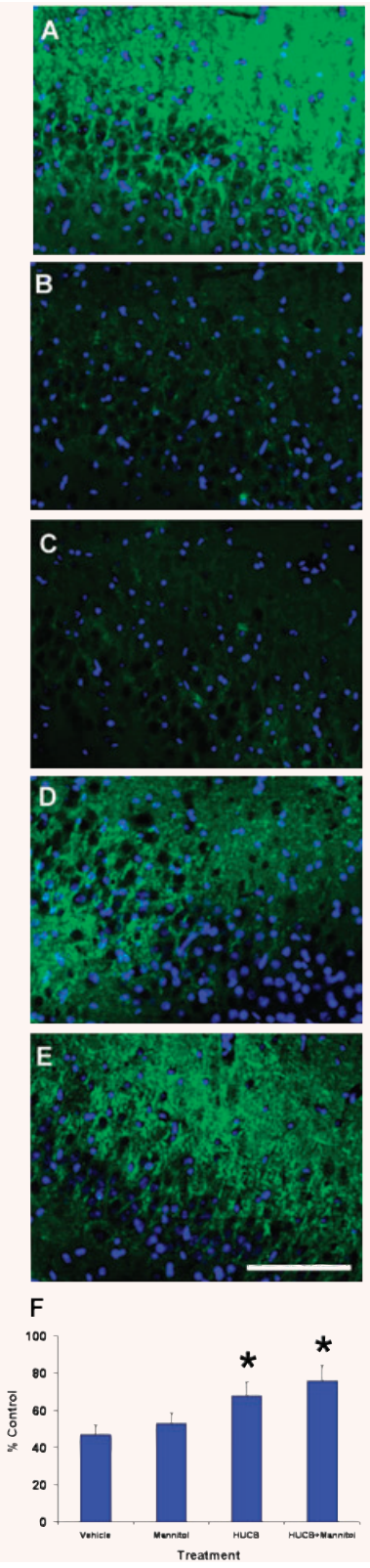

## Few HUCB cells survive in the brains of HI-injured animals

Histological assays of graft survival at day 14 post-transplantation revealed only sporadic surviving HUCB cells detected in the HI-injured hippocampus (*i.e.* dentate gyrus) (Fig. 2). Although surviving HUCB cells were observed in all transplanted animals with or without mannitol treatment, we only found about 2–25 HUCB cells per brain. There was no significant difference in HUCB graft survival between transplanted animals that were treated or did not receive mannitol treatment ($P > 0.1$). No HUCB cell was recognized in vehicle and mannitol-only treatment groups. Further histological analyses to reveal neurotrophic effects of HUCB grafts on host endogenous cells revealed that hippocampal CA1 dendritic density (Fig. 3) was partially normalized by HUCB alone or HUCB and mannitol combination ($F_{3,28} = 54.28$, $P < 0.0001$; post hoc t-tests $P$s $< 0.01$ *versus* vehicle or mannitol).

## Mannitol up-regulates CNS growth factors in HI-injured animals transplanted with HUCB

Parallel groups of animals from the three treatment conditions ($n = 5$ per treatment group) were euthanized at day 3 post-transplantation for ELISA (Fig. 4). ANOVA revealed significant treatment effects on CNS levels of all three growth factors examined (GDNF: $F_{3,16} = 207.44$, $P < 0.0001$; NGF: $F_{3,16} = 391.96$, $P < 0.0001$; BDNF: $F_{3,16} = 167.85$, $P < 0.0001$). Pair-wise t-test comparisons consistently showed elevated levels of GDNF, NGF and BDNF in the brains of HI animals that received HUCB cells alone or when combined with mannitol compared with those that received vehicle alone or mannitol alone ($P$s $< 0.0001$ for all three neurotrophic factor). In addition, each neurotrophic factor up-regulation was markedly higher in HI animals that received the combined HUCB cells and mannitol (threefold) compared with those that received HUCB cells alone (twofold) (GDNF: $P < 0.0001$; NGF: $P < 0.0001$; BDNF: $P < 0.0004$).

**Fig. 2** Sporadic HUCB grafts survive in HI brains. Representative images of microscopically detected HUCB cells alone (**A–D**) and HUCB cells + mannitol (**E–H**; adjacent section with high magnification images shown in I–K). Very few HuNu-positive HUCB cells (green) were detected in the HI-injured hippocampal dentate gyrus, which co-labelled with the nuclei marker Hoechst (blue). These data indicate that only a handful of intravenously transplanted HUCB cells reached the ischaemic hippocampus. Bar = 60 μm. Individual and mean cell counts of HuNu positive cells are shown in panels **L** and **M**, respectively.




## Discussion

The present study extends the utility of mannitol in potentiating the therapeutic efficacy of cell therapy in adult stroke to neonatal HI injury animal model. The permeation of the BBB *via* mannitol enhanced the behavioural recovery achieved by intravenously transplanted HUCB cells in HI-injured animals. In addition, such mannitol treatment when combined with HUCB transplantation further increased the CNS levels of at least three neurotrophic factors, namely, GDNF, NGF and BDNF. Although mannitol treatment did not increase HUCB graft survival, the combined BBB permeation and HUCB transplantation significantly elevated trophic factor levels in the HI brain, which could have mediated the robust functional improvement. These results support our concept that the therapeutic outcome produced by cell therapy can be recognized not solely based on graft survival in the injured brain but also through improved entry of neuroprotective and/or neurorestorative molecules from the periphery into the CNS. We previously reported that ELISA revealed partially increased (15% above control non-stroke animals) levels of trophic factors from circulating blood in stroke animals that received intravenous HUCB grafts plus mannitol [13]. In contrast, no detectable elevations in trophic factors in circulating blood were obtained from stroke animals injected either with intravenous HUCB grafts alone, mannitol alone or vehicle alone compared with controls. These observations further strengthen the notion that significant elevation of trophic factors in the brain, instead of the peripheral blood circulation, likely mediated the functional recovery of HI-injured animals that received the combination treatment of HUCB and mannitol.

Cerebral palsy accounts for a number of neurological disorders that occur in infancy or early childhood, with majority due to genetic syndromes [17, 18] and others caused by brain infections [19], such as bacterial meningitis or viral encephalitis, or head injury from perinatal brain damage [20], vehicular accident [21] or child abuse and neglect [22]. There is no cure for cerebral palsy, but physical therapy [23] along with other rehabilitation treatments (*e.g.* occupational therapy, speech therapy), as well as drugs for seizure and muscle spasm regulation, and pain medication may improve the child's daily activities [24–26]. The brain damage produced by the rat neonatal HI model recapitulates some of the CNS malformations seen in cerebral palsy infants. We previously reported consistent hippocampal cell loss following HI

**Fig. 3** HUCB grafts partially normalize hippocampal dendritic density. Transplantation of HUCB alone or in combination with mannitol led to partial normalization of hippocampal dendritic density in HI-injured animals. Data are presented as percentage + S.E. of control (**A**). (**B**) Vehicle, (**C**) Mannitol, (**D**) HUCB alone and (**E**) HUCB + mannitol. Quantitative analyses are shown in (**F**). **P*s $< 0.01$ *versus* vehicle or mannitol. Bar = 60 μm.

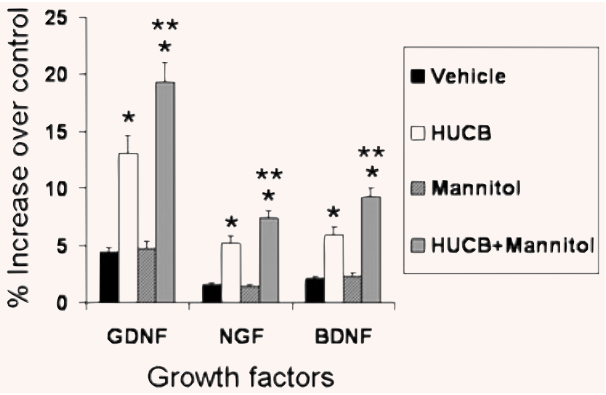



**Fig. 4** HUCB grafts promote neurotrophic factor up-regulation in HI brains. ELISA revealed that both groups of HI animals that received HUCB cells alone or when combined with mannitol significantly increased GDNF, NGF and BDNF brain levels at day 3 after HI compared with animals treated with vehicle alone or mannitol alone, but the HI animals that received combined HUCB cells and mannitol displayed significantly better improvement than those that received HUCB cells alone ($P$s $< 0.0001$ *versus* *vehicle/mannitol or **HUCB alone). Data are shown as mean values + S.E.

insult in the neonatal rat [27], thereby allowing investigations into the potential of cell therapy for cerebral palsy.

Intravenous delivery of HUCB alone promoted behavioural recovery in HI-injured animals, but the functional improvement was more pronounced when HUCB transplantation was combined with mannitol, which is used clinically for hyperosmolar therapy. The amelioration of behavioural abnormalities represents improvement in both motor asymmetry and motor coordination as revealed by EBST and Rotarod test, respectively, which persisted in the two test periods of days 7 and 14 post-transplantation. The combination of HUCB transplantation and mannitol treatment enhanced the behavioural recovery by about 40% in EBST and 50% in Rotarod compared with HUCB transplantation alone. Long-term monitoring of these motor tasks to reveal stable functional outcome and to determine the need for booster HUCB transplantation and mannitol treatment should further provide guidance on the optimal therapeutic regimen for HI. In addition, in view of the established involvement of the hippocampus in learning and memory, the assessment of cognitive performance in HI-injured animals undergoing this treatment should be pursued in subsequent studies.

The present HUCB cell dose of 15k is dramatically low compared with previously reported therapeutically active doses for HUCB. In our previous study in adult stroke [13], we found that 200,000 HUCB cells (already considered a low dose) when combined with mannitol mimics those seen in other reports using much higher doses (>500,000 cells without mannitol) of IV HUCB in stroke [28, 29]. A plausible explanation for the ability of a much lower cell dose of HUCB to exert therapeutic benefits in HI is likely due to the neonatal brain being more plastic than the adult brain, thereby allowing the young host microenvironment to respond more appropriately to therapeutic intervention. Although our initial concept for BBB permeation was to allow for the donor cells to be injected acutely after brain injury, we recognized that the diagnosis of cerebral palsy normally takes place after days or a few weeks after birth. Accordingly, we adjusted the timing of cell transplantation and mannitol, to augment the clinical application of this treatment for cerebral palsy. Here, we show that even with a 1-week delay post-injury, mannitol was able to potentiate the therapeutic effects of HUCB, suggesting that BBB permeabilization in neonates is critical for optimizing the functional outcome of cell therapy when the chosen route of cell administration is from the periphery. With the mannitol enhancement of HUCB-induced behavioural recovery, such minimally invasive intravenous cell delivery offers a more practical and a less traumatic approach than directly injecting cells into the brain, especially when the transplant recipient is an infant.

In agreement with our study in adult stroke [13], the present results indicate that spontaneous BBB opening produced by HI injury was not permissive enough to allow CNS entry of endogenous or graft-derived trophic factors, suggesting the need for exogenous BBB manipulation. We concur that BBB may not be fully developed in neonatal rats, but even such immature BBB appears capable of blocking the entry of peripherally secreted growth factors as seen in our neonatal animals that received HUCB alone without mannitol treatment. In contrast, mannitol treatment in neonatal animals that received HUCB resulted in elevated CNS levels of growth factors, suggesting that even an immature BBB needs to be permeabilized in order to achieve enhanced entry of peripherally secreted growth factors by HUCB. A perturbed BBB permeability produced by mannitol facilitates mobilization of graft-derived trophic factors to be present in the brain to exert therapeutic benefits. The lack of significant increments in brain trophic factor levels in HI-injured animals that received vehicle alone or mannitol alone suggests that HUCB grafts instead of the host tissues *per se* were likely the source of elevated CNS levels of trophic factors. In concert with preclinical evidence demonstrating the neuroprotective and neuroregenerative effects of GDNF in ischaemic injury [30, 31], cell-based CNS therapies designed to deliver GDNF, in combination with a cocktail of known and yet to be determined trophic factors secreted by stem cells such as HUCB, may further enhance the therapeutic benefits produced by exogenous delivery of a single or a few specific trophic factors. Although host-derived growth factors have been shown to be up-regulated after ischaemic injury, this endogenous compensatory mechanism is not sufficient to afford neuroprotection and neuroregeneration. Accordingly, we postulate that the observed elevation of CNS growth factors following mannitol treatment is likely derived from the peripherally transplanted HUCB. Our previous study [13] showed that the use of neutralizing trophic factor antibodies by exposing HUCB cells to antibodies against GDNF, NGF and BDNF before transplantation prevented elevations in trophic factor levels, and completely blocked the behavioural and histological beneficial effects produced by combined HUCB and mannitol treatment. These observations implicate the pivotal role of mannitol permeabilization of BBB in promoting the trophic factor effects of

peripherally administered HUCB grafts to be manifested in ischaemic brain injury.

The visualization of a few surviving HUCB cells in the brains of HI-injured brains is unexpected because we did not detect any grafted HUCB cells in transplanted adult stroke animals [13]. As noted earlier, it is likely the plastic neonatal brain creates a more conducive host microenvironment for the HUCB cells to survive. This favourable neonatal host brain, in tandem with mannitol treatment, might have facilitated the migration of the HUCB cells from the periphery since chemokine up-regulation, such as stromal cell–derived factor 1, accompanies neonatal HI injury [32]. We have previously shown that cultured [33, 34] or grafted HUCB [35] cells express phenotypic neuronal markers, which could have contributed to the observed functional recovery. Indeed, differentiation of grafted cells into neural lineage, including neurons and astrocytes, has been implicated in reorganization of the HI-injured neonatal brain [36]. However, the very low number of HUCB cells that crossed the BBB even with mannitol treatment supports the notion that the primary mechanism underlying the functional recovery was likely mediated by neurotrophic factor secretion rather than HUCB graft survival in the brain *per se*. Additional immunohistochemical assays (not shown) using neuronal (MAP2), glial (GFAP) and oligodendrocytic (O4) markers reveal that none of the few cells reaching the brain expressed any of these phenotypic labelling, suggesting that HUCB cells did not differentiate into a neuronal lineage. These observations further strengthen our claim that graft survival and/or neuronal commitment only partially contributed to the therapeutic benefits, and that CNS elevation of neurotrophic factors either directly by HUCB cells or indirectly whereby HUCB cells stimulated the spared endogenous cells to up-regulate known, as well as yet to be determined, growth factors largely played the key role in affording protection from and/or repair of the HI-injured brain regions. In the end, the primary mechanistic pathway we invoke for the observed therapeutic benefits is that mannitol facilitates the up-regulation of growth factors by HUCB cells, host cells or both in the CNS where these molecules abrogated HI injury. The present cell transplant–mediated trophic factor neuroprotection and/or neuroregeneration parallels our previous reports showing that the cell dose administered, rather than the subsequent number of surviving cells, appears to correspond to the therapeutic threshold to recognize functional recovery [13, 27, 37, 38]. This concept of 'bystander effects' (*i.e.* growth factor secretion) overriding the 'cell replacement' (*i.e.* graft survival) has recently received direct scientific support [39, 40]. Of note, we observed here that HUCB grafts alone or in combination with mannitol partially normalized dendritic density in hippocampal CA1 region, suggesting neurotrophic factor effects.

These results, taken together, advance the strategy of BBB permeation *via* mannitol treatment as an adjunct to intravenous transplantation of HUCB for treating neonatal HI injury, as demonstrated previously in adult stroke [13]. The present therapeutic benefits of combined mannitol and HUCB were achieved without immunosuppression, which is often accompanied by deleterious side effects, thereby further paving the way for autologous, as well as allograft transplantation.

## Conflict of Interests

PRS and CVB are Founder and Consultant for Saneron CCEL Therapeutics, Inc. Both are inventors on a patent application related to this research.

## References


1. **Blau H, Brazelton T, Keshet G, *et al.*** Something in the eye of the beholder. *Science*. 2002; 298: 361–2.
2. **Mezey E, Nagy A, Szalayova I, *et al.*** Comment on "Failure of bone marrow cells to transdifferentiate into neural cells *in vivo*". *Science*. 2003; 299: 1184.
3. **Theise ND, Krause DS, Sharkis S.** Comment on "Little evidence for developmental plasticity of adult hematopoietic stem cells". *Science*. 2003; 299: 1317.
4. **Wagers AJ, Sherwood RI, Christensen JL, *et al.*** Little evidence for developmental plasticity of adult hematopoietic stem cells. *Science*. 2002; 297: 2256–9.
5. **Castro RF, Jackson KA, Goodell MA, *et al.*** Failure of bone marrow cells to transdifferentiate into neural cells *in vivo*. *Science*. 2002; 297: 1299.
6. **Mezey E, Key S, Vogelsang G, *et al.*** Transplanted bone marrow generates new neurons in human brains. *Proc Natl Acad Sci USA*. 2003; 100: 1364–9.
7. **Weimann JM, Charlton CA, Brazelton TR, *et al.*** Contribution of transplanted bone marrow cells to Purkinje neurons in human adult brains. *Proc Natl Acad Sci USA*. 2003; 100: 2088–93.
8. **Redmond DE Jr.** Cellular replacement therapy for Parkinson's disease–where we are today? *Neuroscientist*. 2002; 8: 457–88.
9. **Dunnett SB, Bjorklund A, Lindvall O.** Cell therapy in Parkinson's disease – stop or go? *Nat Rev Neurosci*. 2001; 2: 365–9.
10. **Borlongan CV, Sanberg PR, Freeman TB.** Neural transplantation for neurodegenerative disorders. *Lancet*. 1999; 353: S129–30.
11. **Borlongan CV, Tajima Y, Trojanowski JQ, *et al.*** Transplantation of cryopreserved human embryonal carcinoma-derived neurons (NT2N cells) promotes functional recovery in ischemic rats. *Exp Neurol*. 1998; 149: 310–21.
12. **Lindvall O, Brundin P, Widner H, *et al.*** Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. *Science*. 1990; 247: 574–77.
13. **Borlongan CV, Hadman M, Sanberg CD, *et al.*** Central nervous system entry of peripherally injected umbilical cord blood cells is not required for neuroprotection in stroke. *Stroke*. 2004; 35: 2385–9.
14. **Rice JE 3rd, Vannucci RC, Brierley JB.** The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Ann Neurol*. 1981; 9: 131–41.
15. **Borlongan CV, Sanberg PR.** Elevated body swing test: a new behavioral parameter for rats with 6-hydroxydopamine-induced hemiparkinsonism. *J Neurosci*. 1995; 15: 5372–8.

16. **Okragly AJ, Haak-Frendscho M.** An acid-treatment method for the enhanced detection of GDNF in biological samples. *Exp Neurol*. 1997; 145: 592–6.
17. **Dabney KW, Lipton GE, Miller F.** Cerebral palsy. *Curr Opin Pediatr*. 1997; 9: 81–8.
18. **Lequin MH, Barkovich AJ.** Current concepts of cerebral malformation syndromes. *Curr Opin Pediatr*. 1999; 11: 492–6.
19. **Hermansen MC, Hermansen MG.** Perinatal infections and cerebral palsy. *Clin Perinatol*. 2006; 33: 315–33.
20. **Berger R, Garnier Y.** Pathophysiology of perinatal brain damage. *Brain Res Rev*. 1999; 30: 107–34.
21. **Lipson AH, Gillerot Y, Tannenberg AE, *et al.*** Two cases of maternal antenatal splenic rupture and hypotension associated with Moebius syndrome and cerebral palsy in offspring. Further evidence for a utero placental vascular aetiology for the Moebius syndrome and some cases of cerebral palsy. *Eur J Pediatr*. 1996; 155: 800–4.
22. **Spencer N, Devereux E, Wallace A, *et al.*** Disabling conditions and registration for child abuse and neglect: a population-based study. *Pediatrics*. 2005; 116: 609–13.
23. **Damiano DL.** Activity, activity, activity: rethinking our physical therapy approach to cerebral palsy. *Phys Ther*. 2006; 86: 1534–40.
24. **Buonaguro V, Scelsa B, Curci D, *et al.*** Epilepsy and intrathecal baclofen therapy in children with cerebral palsy. *Pediatr Neurol*. 2005; 33: 110–3.
25. **Lubsch L, Habersang R, Haase M, *et al.*** Oral baclofen and clonidine for treatment of spasticity in children. *J Child Neurol*. 2006; 21: 1090–2.
26. **Van Schaeybroeck P, Nuttin B, Lagae L, *et al.*** Intrathecal baclofen for intractable cerebral spasticity: a prospective placebo-controlled, double-blind study. *Neurosurgery*. 2000; 46: 603–9.
27. **Yasuhara T, Matsukawa N, Yu G, *et al.*** Behavioral and histological characterization of intrahippocampal grafts of human bone marrow-derived multipotent progenitor cells in neonatal rats with hypoxic-ischemic injury. *Cell Transplant*. 2006; 15: 231–8.
28. **Chen J, Sanberg PR, Li Y, *et al.*** Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. *Stroke*. 2001; 32: 2682–8.
29. **Willing AE, Lixian J, Milliken M, *et al.*** Intravenous versus intrastriatal cord blood administration in a rodent model of stroke. *J Neurosci Res*. 2003; 73: 296–307.
30. **Harvey BK, Chang CF, Chiang YH, *et al.*** HSV amplicon delivery of glial cell line-derived neurotrophic factor is neuroprotective against ischemic injury. *Exp Neurol*. 2003; 183: 47–55.
31. **Wang Y, Lin SZ, Chiou AL, *et al.*** Glial cell line-derived neurotrophic factor protects against ischemia-induced injury in the cerebral cortex. *J Neurosci*. 1997; 17: 4341–8.
32. **Miller J, Bartley JH, Wimbourne HJC, *et al.*** The neuroblast and angioblast chemotaxic factor SDF-1 (CXCL12) expression is briefly upregulated by reactive astrocytes in brain following neonatal hypoxic-ischemic injury. *BMC Neurosci*. 2005; 6: 63.
33. **Chen N, Hudson JE, Walczak P, *et al.*** Human umbilical cord blood progenitors: the potential of these hematopoietic cells to become neural. *Stem Cells*. 2005; 23: 1560–70.
34. **Sanchez-Ramos JR, Song S, Kamath SG, *et al.*** Expression of neural markers in human umbilical cord blood. *Exp Neurol*. 2001; 171: 109–15.
35. **Zigova T, Song S, Willing AE, *et al.*** Human umbilical cord blood cells express neural antigens after transplantation into the developing rat brain. *Cell Transplant*. 2002; 11: 265–74.
36. **Zheng T, Rossignol C, Leibovici A, *et al.*** Transplantation of multipotent astrocytic stem cells into a rat model of neonatal hypoxic-ischemic encephalopathy. *Brain Res*. 2006; 1112: 99–105.
37. **Vendrame M, Cassady J, Newcomb J, *et al.*** Infusion of human umbilical cord blood cells in a rat model of stroke dose-dependently rescues behavioral deficits and reduces infarct volume. *Stroke*. 2004; 35: 2390–5.
38. **Yasuhara T, Hara K, Maki M, *et al.*** Intravenous grafts recapitulate the neurorestoration afforded by intracerebrally delivered multipotent adult progenitor cells in neonatal hypoxic-ischemic rats. *J Cereb Blood Flow Metab.* 2008; 28: 1804–10.
39. **Yasuhara T, Matsukawa N, Hara K, *et al.*** Transplantation of human neural stem cells exerts neuroprotection in a rat model of Parkinson's disease. *J Neurosci*. 2006; 26: 12497–511.
40. **Redmond DE Jr, Bjugstad KB, Teng YD, *et al.*** Behavioral improvement in a primate Parkinson's model is associated with multiple homeostatic effects of human neural stem cells. *Proc Natl Acad Sci USA*. 2007; 104: 12175–80.