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Intra-arterial transplantation of human umbilical cord blood mononuclear cells in neonatal hypoxic–ischemic rats

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ABSTRACT

Based on preclinical findings, cellular therapy has become a promising therapeutic approach for neonatal hypoxia–ischemia (HI). However, before translation into the clinical setting, new and effective routes of cell delivery must be determined. Intra-arterial (IA) delivery is an attractive route of cellular administration but has never been used in neonatal HI rats. Aims: In this study, we investigated the feasibility of IA transplantation of human umbilical cord blood (HUCB) mononuclear cells for the treatment of long-term behavior dysfunction and brain lesion after neonatal HI. Main methods: Seven-day-old rats were subjected to a HI model and the animals received HUCB mononuclear cells into the left common carotid artery 24 h after HI insult. Key findings: At 9 weeks post-HI, intra-arterially transplanted HUCB mononuclear cells significantly improved learning and long-term spatial memory impairments when evaluated by the Morris water maze paradigm. There was no effect of neonatal HI insult or IA procedure on body weight and on motor coordination and balance when evaluated by the accelerating rotarod test. Cellular transplantation by the IA route did not restore neonatal HI-induced brain damage according to stereological volume assessment. Furthermore, HUCB mononuclear cells were tracked in the injured brain and peripheral organs of HI transplanted-rats by nested polymerase chain reaction analysis at different time points. Significance: Our findings contribute to the translational knowledge of cell based-therapy in neonatal HI and demonstrate for the first time that IA transplantation into rat pups is a feasible route for cellular delivery and prevents long-term cognitive deficits induced by experimental neonatal HI.

Introduction

Neonatal hypoxia ischemia (HI) remains an important cause of mortality and is associated with a high incidence of life-long disabilities. Unfortunately, the clinical treatment of HI is quite limited, and no available intervention can effectively hinder the long-term sequelae of HI insult (Johnston et al., 2011). Presently, only hypothermia has some beneficial effects in moderate-affected HI children born at term and treated in a narrow therapeutic window (Shankaran, 2012). In the preclinical context, there is increasing evidence that cell based-therapy exhibits a neuroprotective effect against HI brain injury and subsequent deleterious outcomes (Liao et al., 2013; Pimentel-Coelho et al., 2012). According to the Baby STEPS (Baby Stem cell Therapeutics as an Emerging Paradigm in Stroke) consortium, the design of preclinical studies should closely approximate clinical trials to favor the translational potential of cell therapy in neonatal HI (Borlongan and Weiss, 2011). Additionally, the experimental design should consider and determine the cell type, dose, timing of administration and the delivery route. Despite these recommendations, intraparenchymal injection is the most commonly used technique for cell delivery into the rodent HI brain (de Paula et al., 2010). Intracerebroventricular administration precludes systemic dissemination but fails due to the limited cell numbers that can be injected. The same trade-off can be observed with the intracerebral (IC) route, where cells can be delivered directly to the target, but they are nonuniformly distributed which may cause additional brain injury. As an alternative to the aforementioned methods, we and others have used intravenous (IV) delivery to transplant cells into neonatal HI animals (de Paula et al., 2009, 2012; Yasuhara et al., 2010). However, the IV route does not yield a large number of cells reaching the brain, and the majority of transplanted cells are trapped within the filtering organs such as the lungs, liver, spleen and kidneys (Fischer et al., 2009; Lappalainen et al., 2008). Non-conventional cell delivery methods, such as intraperitoneal (Rosenkranz et al., 2012), intracardiac (Lee et al., 2010) and intranasal (Donega et al., 2013)

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delivery, were also applied in the HI animal model. Therefore, it is reasonable to investigate alternative ways to deliver stem cells for the treatment of neonatal HI.

The intra-arterial (IA) route has the advantage of selectively targeting a larger number of cells to an injured brain area, bypassing the filter of the peripheral organs, and permits a multiple treatment paradigm (Misra et al., 2012). Additionally, it was demonstrated that cell administration via IA delivery could spread cells uniformly throughout the ischemic brain (Li et al., 2010; Walczak et al., 2008). Although the IA transplantation of cells has numerous precedents in clinical trials (Barbosa da Fonseca et al., 2010; Friedrich et al., 2011) and in animal models of adult HI (Andres et al., 2011; Guzman et al., 2008; Pendharkar et al., 2010), stroke (Brenneman et al., 2010; Chua et al., 2011; Chung et al., 2009; Gutierrez-Fernandez et al., 2011; Kamiya et al., 2008; Lappalainen et al., 2008; Li et al., 2001, 2010; Mitkari et al., 2012; Ohta et al., 2006; Vasconcelos-dos-Santos et al., 2012; Walczak et al., 2008; Zhang et al., 2012) and traumatic brain injury (Lundberg et al., 2012; Osanai et al., 2012), it has never been tested in neonatal HI rodents. Here we investigate for the first time the use of the IA route for the transplantation of human umbilical cord blood (HUCB) mononuclear cells in a neonatal rat HI model. The HUCB mononuclear cell fraction was chosen for this study since it is easily collected with minimal ex vivo processing. Parameters such as long-term behavior impairment, body and cerebral weight, brain damage and cell migration were evaluated.

Materials and methods

Neonatal hypoxia–ischemia model and experimental groups

All analyses were performed in a blinded set-up, and all experimental procedures were performed with the approval of the Animal Care and Ethics Committee of PUCRS, Rio Grande do Sul, Brazil (CEUA 09/00105). A schedule of the surgical procedure, the treatment and tests of the animals is shown in Table 1. Neonatal HI model was established as described before (de Paula et al., 2012; Greggio et al., 2011; Rice et al., 1981). Briefly, the right common carotid artery of 7-day-old Wistar rat was permanently doubly ligated. After 3-hour recovery, the pups were subjected to a humidified mixture of 8% O_2 and 92% N_2 at 37 °C for another 2 h. The animals were randomly assigned into five experimental groups: sham-operated rats (sham, n = 10), rats subjected to the HI model (HI, n = 11), HI rats IA administered with vehicle (VEH, n = 9) and HI rats IA transplanted with 1×10^6 (HI+10^6, n = 10) or 1×10^7 (HI+10^7, n = 10) HUCB mononuclear cells. The rat pups from each litter were randomly divided among the groups to avoid "litter effects" on the study outcome. Besides, only male rat pups were used in our study in order to avoid gender effects upon the histological and behavioral outcomes. Following the hypoxic exposure, all of the pups were returned to their dams for recovery. The sham-operated animals were anesthetized with halothane, and the right common carotid artery was exposed but did not receive ligation or hypoxia.

HUCB mononuclear cell preparation and IA transplantation

After obtaining informed consent, HUCB cells were collected ex-utero from healthy volunteers immediately after full-term delivery using sterile syringes containing 5000 UI of heparin. For the separation of mononuclear cells, the obtained material was diluted in RPMI-1640 medium (1:1) (Gibco, Grand Island, NY, USA). The cells were resuspended and fractionated on a density gradient generated by centrifugation, over a Ficoll-Paque solution with a density of 1.077 g/L (Histopaque 1077, Sigma Aldrich, St. Louis, MO, USA), at 400×*g* for 30 min at 25 °C. The mononuclear fraction over the Ficoll-Paque layer was collected and washed twice with Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco, Grand Island, NY, USA). The cell density was determined with a Neubauer-counting chamber, and the number of viable cells was determined using the Trypan Blue 0.4% exclusion method. For the detection of surface antigens, HUCB mononuclear cells were incubated with fluorescein isothiocyanate- (FITC) or phycoerythrin- (PE) conjugated monoclonal antibody against CD45 (hematopoietic precursor cells), CD105 (bone marrow precursor cells), CD34 (hematopoietic and endothelial precursor cells), and CD117 (hematopoietic precursor cells) (Becton Dickinson Biosciences, San Jose, CA). Labeled cells were collected and analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ). The immunophenotypic analysis of mononuclear fraction derived from HUCB revealed that 2.4% of the cells expressed CD34, 23.14% expressed CD45, 50.41% expressed CD105, and 71.25% expressed CD117. Taken together, the data showed that the isolated cord blood cells exhibited a mixture of different cellular types, consistent with the literature (de Paula et al., 2009, 2012). Twenty-four hours after HI insult, animals received HUCB mononuclear cells (1×10^6 or 1×10^7 cells resuspended in 50 μl of PBS) or vehicle delivered into the left (contralateral) common carotid artery using an ultrafine 34 gauge microneedle (outer-Ø 0.20 mm, inner-Ø 0.10 mm; Nodegraf, Tokyo, Japan). For this procedure, animals were anesthetized again, the previous neck suture was carefully opened, and the left carotid artery was isolated from adjacent tissue to facilitate the IA injection. Thereafter, the skin was once again closed with a suture, and the animals were returned to their dams for recovery.

Table 1
Timeline of experimental procedures.

PND 7	Neonatal rats subjected to the hypoxia–ischemia model
PND 8	Intra-arterial administration of HUCB mononuclear cells or vehicle
PND 65–69	Training for spatial version of the Morris water maze (MWM) learning task
PND 70	Probe test for the MWM and rotarod habituation
PND 71	Accelerated rotarod test
PND 72	Cerebral hemispheric volume assessment

Spatial version of the Morris water maze learning task

The animals were tested in the spatial version of the Morris water maze (MWM) learning task beginning at PND 65. The MWM was undertaken to investigate the impact of IA cellular transplantation upon neonatal HI-induced spatial memory impairment as described previously (de Paula et al., 2012; Greggio et al., 2011; Venturin et al., 2011). Briefly, the spaced training protocol was performed for 5 successive days. On each day, the rats received 8 consecutive training trials during which the hidden platform was kept in a constant location. A different starting location was used for each trial, which consisted of a swim followed by a 30-s platform sit. Any rat that did not find the platform within 60 s was guided to it by the experimenter. Memory retention was evaluated in a 60-s probe trial performed in the absence of the escape platform 24 h after the last training session.

Accelerated rotarod performance

At PND 71, the rotarod test was performed to measure motor coordination and balance in the rats. An apparatus equipped with a sensor that detected the fall and automatically stopped the timer was used (EFF 411, Insight, SP, Brazil). One day prior to the test, all animals were habituated to the apparatus using a protocol of three trials of 3 min each at 16-rpm speed. One day after the habituation, the speed of the rotarod was set to gradually increase from 4 to 37 rpm over 6 min across ten phases (1–2: 16 rpm; 3–4: 20 rpm; 5–6: 25 rpm; 7–8: 28 rpm; 9–10: 37 rpm). Upon reaching the maximum speed, the animals were kept in place for an additional minute. The test session consisted of five trials separated by 15-minute intervals (Daadi et al., 2010).

Stereological volume assessment of the cerebral hemispheres

After the accelerated rotarod test (PND 72), the animals were weighed, deeply anesthetized with thiopental sodium (0.1 ml/100 g, i.p.) and then perfused transcardially with saline followed by 4% paraformaldehyde at 4 °C. The brains were histologically processed and the hemispheric volume was assessed by using the Cavalieri principle associated with the counting point method as described elsewhere (Alles et al., 2010; de Paula et al., 2012).

Nested polymerase chain reaction (PCR) analysis

An additional group of HI+10^7 rats was euthanized and samples from the right and left cerebral hemispheres, lungs, liver and spleen were collected at 1, 3, 6, 12 and 24 h, and 7 and 30 days after IA transplantation of HUCB mononuclear cells (n = 2 male rats for each time point). PCR analysis was performed to identify the presence of the administered HUCB mononuclear cells in the samples of transplanted animals using complementary primers to the human β-actin gene sequence. DNA isolation and nested-PCR analysis were performed as described before (de Paula et al., 2012).

Statistical analysis

The statistical analysis was performed using PrismGraph 5.0 software (Graph-Pad Software, San Diego, CA). Comparisons between the experimental groups were made by using one-way ANOVA followed by Dunnett's post hoc test or by using two-way ANOVA followed by Bonferroni's post hoc test. A statistical significance level of $\alpha = 0.05$ and $p < 0.05$ was applied to all tests.

Results

IA transplantation of 1 × 10^7 HUCB mononuclear cells rescues long-term spatial memory impairments in rats previously subjected to neonatal HI

To assess the effects of IA transplantation of HUCB mononuclear cells on long-term spatial memory deficits, we employed the MWM paradigm. The mean escape latencies to the hidden platform decreased as training progressed, and the experimental groups showed a distinct performance over time ($F_{(4,225)} = 2.824$, $p = 0.0003$, Fig. 1A). As shown in Table 2, two-way ANOVA followed by Bonferroni's post hoc test revealed a significant difference between the HI and sham groups throughout the entire 5-day training session. The HI group animals IA administered with either vehicle or 1 × 10^6 HUCB mononuclear cells had similar learning performances when compared with the HI only group and were significantly different from the sham rats. Conversely, the animals subjected to neonatal HI injury and transplanted with 1 × 10^7 HUCB mononuclear cells learned differently from the sham rats but performed better than the HI, Veh and HI+10^6 groups. Since a dysfunction in the learning performance among the groups in the training session was verified, we also expected to find some impairment in the probe tests of the HI animals as a consequence of it. The probe test was performed 24 h after the last training session in the absence of the escape platform. One-way ANOVA followed by Dunnett's post hoc test showed that the escape latency to swim over the previous position of the escape platform was longer in the HI (42.45 ± 6.73 s, $p < 0.01$), HI+10^6 (38.66 ± 6.67 s, $p < 0.05$) and Veh (45.24 ± 5.36 s, $p < 0.01$) groups when compared with the sham group (14.96 ± 2.91 s, $p < 0.01$ versus HI group) (Fig. 1B). Only the HI+10^7 group had a tendency to a shorter time required to localize the platform position (27.53 ± 6.93 s, $p > 0.05$ versus sham and HI groups). In addition, the HI and Veh animals spent less time swimming in the target quadrant that previously contained the escape platform (28.88 ± 5.35% and 29.55 ± 4.77%, respectively, $p < 0.01$) compared with the sham-operated rats (57.22 ± 4.18%) (Fig. 1C). Neither HI group transplanted with 1 × 10^6 or 1 × 10^7 HUCB mononuclear cells had any significant difference when compared with the sham group for the time spent in the target quadrant (41.57 ± 4.86% and 48.38 ± 7.03%, respectively). However, only the HI+10^7 group had a significantly statistical difference when compared to HI animals ($p < 0.05$), demonstrating cognitive impairment recovery. With respect to the number of crossings over the former target position, all groups displayed significantly lower values than the sham animals (6.10 ± 0.78) (Fig. 1D). However, this difference was more evident in the HI (1.54 ± 0.45, $p < 0.001$), Veh (1.77 ± 0.66, $p < 0.001$) and HI+10^6 (2.60 ± 0.87, $p < 0.01$) animals than in the HI+10^7 group (3.30 ± 0.65, $p < 0.05$). No statistically significant differences were observed for swimming speed among the experimental groups, demonstrating that the results in the MWM probe test were not influenced by any HI-induced motor impairment (Fig. 1E).

IA transplantation of HUCB mononuclear cells or vehicle does not induce impairments in rotarod performance and body weight in adult rats previously subjected to neonatal HI

In the present study, the rotarod test was performed to measure motor coordination and balance in rats. Using an accelerating rotarod protocol, there were no statistically significant differences between the HI group and the sham rats in the mean time spent on the treadmill (Fig. 2A) and mean phase in which the fall occurred (Fig. 2B). One-way ANOVA followed by Dunnett's test showed that the HUCB-treated and Veh animals performed equally in both variables analyzed in the rotarod test when compared with the sham group. These results are in agreement with the normal motor function observed in the evaluation

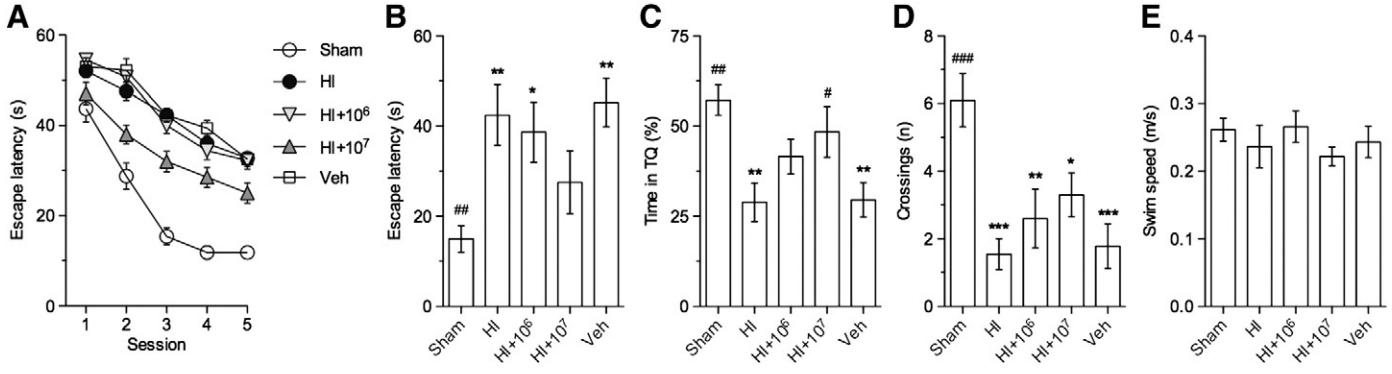

Fig. 1. IA transplantation of 1 × 10^7 HUCB mononuclear cells reduced the injury-induced spatial memory impairment in hypoxic–ischemic rats. (A) The mean escape latencies to the hidden platform were obtained from a 5-day training session. (B) Mean escape latency of swimming over the previous platform location. (C) Percentage of time searching the quadrant in which the platform had been submerged during training. (D) Number of crossings over the former target position. (E) Mean swimming speed of the rats during the probe test. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus sham and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ versus HI in Dunnett's post hoc test after one-way ANOVA.

Table 2
Morris water maze acquisition performance.

Group	Training session				
	1	2	3	4	5
Sham	43.7 ± 2.9**	28.8 ± 2.9***	15.4 ± 1.9***	11.8 ± 1.5***	11.8 ± 1.1***
HI	52.1 ± 1.5††	47.6 ± 2.0†††§§	42.3 ± 1.4†††§§§	36.0 ± 0.9†††§	32.8 ± 1.2†††§
HI+10^6	54.5 ± 1.0†††§	50.7 ± 0.9†††§§§	40.0 ± 1.8†††§	34.4 ± 2.0†††	32.2 ± 1.9†††§
HI+10^7	47.1 ± 2.5	38.0 ± 2.1†††***	32.0 ± 2.3†††***	28.5 ± 2.2†††*	25.0 ± 2.3†††*
Veh	53.0 ± 1.9††	52.2 ± 2.6†††§§§	42.3 ± 1.5†††§§	39.4 ± 1.7†††§§§	32.5 ± 1.6†††§

Values represent the mean ± S.E.M. (values in seconds). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. HI group; $^{\dagger\dagger}p < 0.01$ and $^{\dagger\dagger\dagger}p < 0.001$ vs. sham group; $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$ vs. HI+10^7 group in Bonferroni post hoc test after two-way ANOVA.

of the swimming speed of the rats in the MWM test. After the rotarod test, the animals were weighed and no significant differences were observed among the experimental groups, as sham (273.2 ± 42.6 g), HI (267.2 ± 21.5 g), HI+10^6 (250.8 ± 32.3 g), HI+10^7 (241.7 ± 34.49 g) and Veh (257.2 ± 26.0 g). Additionally, the mortality rate was the same between the control and the transplanted animals (~15%).

IA transplanted HUCB mononuclear cells are not able to prevent brain injury in adult rats previously subjected to neonatal HI

We next examined whether IA transplantation of HUCB mononuclear cells could block the long-term brain morphological alterations by using the Cavalieri principle and the counting point method. Morphological examination of cresyl violet-stained sections of rat brains from the sham group revealed no histological abnormalities or volume differences between the cerebral hemispheres (542.4 ± 10.17 versus 535.6 ± 9.13 mm^3) (Fig. 3A and E). Conversely, absolute hemispheric volume analysis showed significant atrophy of the hemisphere ipsilateral to the carotid occlusion (right side) when compared with the contralateral hemisphere (551.1 ± 10.27 versus 385.4 ± 57.49 mm^3, $p < 0.05$) (Fig. 3A and F) 9 weeks after the HI injury. Similarly, the HI+10^6 (Fig. 3A and G) and Veh (Fig. 3A and I) groups also had a significantly reduced right hemisphere volume when compared with the left hemisphere (510.2 ± 18.43 versus 352.0 ± 66.95 mm^3, $p < 0.05$; and 570.7 ± 20.73 versus 396.1 ± 65.10 mm^3, $p < 0.05$; respectively). However, no statistically significant difference was observed between the right and left hemispheres in HI rats IA transplanted with 1×10^7 HUCB mononuclear cells (505.2 ± 13.47 versus 362.6 ± 67.25 mm^3) (Fig. 3A and H). In a different analysis approach, we examined cerebral atrophy in terms of percentage of brain tissue loss (Fig. 3B). One-way ANOVA followed by Bonferroni's post hoc test revealed no significant difference between the sham (1.22 ± 0.32%) and HI (30.21± 10.47%), HI+10^6 (33.99 ± 11.57%), HI+10^7 (29.73 ± 12.48%) or Veh (32.02 ± 10.29%) groups. Based on visual and histological investigation, the percent of porencephalic cysts (~40%) was the same among transplanted and non-treated HI animals. Corroborating this evidence, one-way ANOVA followed by Dunnett's post hoc test demonstrated no significant difference in brain weight among the groups, but only when compared with sham animals ($p < 0.05$) (Fig. 3C).

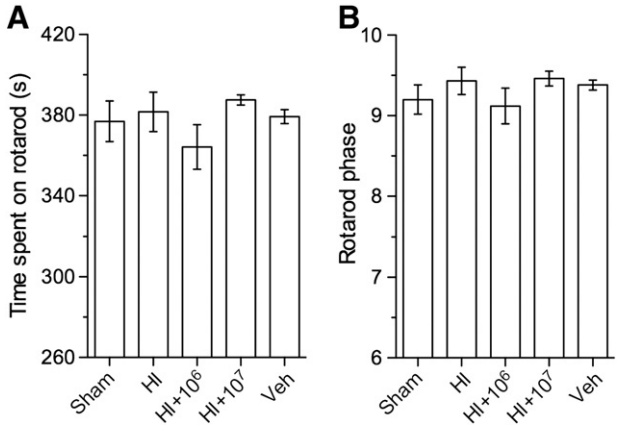

Fig. 2. Effect of IA transplantation of HUCB mononuclear cells or vehicle on rotarod performance and body weight of adult rats previously subjected to neonatal HI. There were no statistically significant differences between the HI groups and the sham rats on the mean time spent on the treadmill (A) and mean phase in which the fall occurred. Data were analyzed by one-way ANOVA followed by the Dunnett's post hoc test.

Human β-actin gene expression was detected in samples obtained from HI rats IA transplanted with HUCB mononuclear cells

We employed nested PCR analysis to elucidate whether the observed beneficial cognitive effects were associated with the migration of cells to the injured brain and to monitor their spreading in the rodent body. The expression of the band corresponding to the human β-actin gene was initially detected (1 h post-transplantation) in the left cerebral hemisphere, liver and lungs of HI rats that received cellular administration into the left carotid artery. At 3 h after cellular transplantation, human DNA was detected in the liver and lungs and not detected in the left hemisphere, but it was detected in the right injured brain hemisphere. However, at 6 h post-transplantation, the human β-actin gene was only amplified in the right hemisphere. We could not detect any expression for the human gene in the brain or systemic organs at 24 h after IA transplantation of HUCB mononuclear cells. At later analysis time points, we could detect human DNA in the left cerebral hemisphere and lungs (7 and 30 days post-transplantation), and in the right hemisphere (30 days post-transplantation). It was not possible to detect the expression of human β-actin gene in the spleen of IA transplanted HI rats at any time point analyzed.

Discussion

Here we demonstrate for the first time that IA transplantation is a feasible delivery route for cellular therapy in neonatal rats subjected to a HI model. The IA delivered cells prevented cognitive impairments 9 weeks after neonatal HI insult but did affect recovery from brain damage. Additionally we further showed that HI insult or IA cell transplantation had no long-term impact on body weight or motor function in rodents. HUCB mononuclear cells were rapidly identified in the ischemic brain after IA transplantation and also in several peripheral organs. Altogether the data indicate that the IA approach is a potential delivery method for cell based-therapy for neonatal HI.

The HUCB mononuclear cell fraction was chosen for this study because these cells are one of the most convenient and rich sources of therapeutic cells in pediatrics and can be used in the case of neonates affected by HI insult. Cord blood is easily collected after delivery and can be promptly used because it requires minimal ex vivo processing, or it can be cryopreserved for a longer period in cord blood banks (Liao et al., 2013). We have recently demonstrated that acute IV administration of HUCB mononuclear cells exerts a dose-dependent effect on

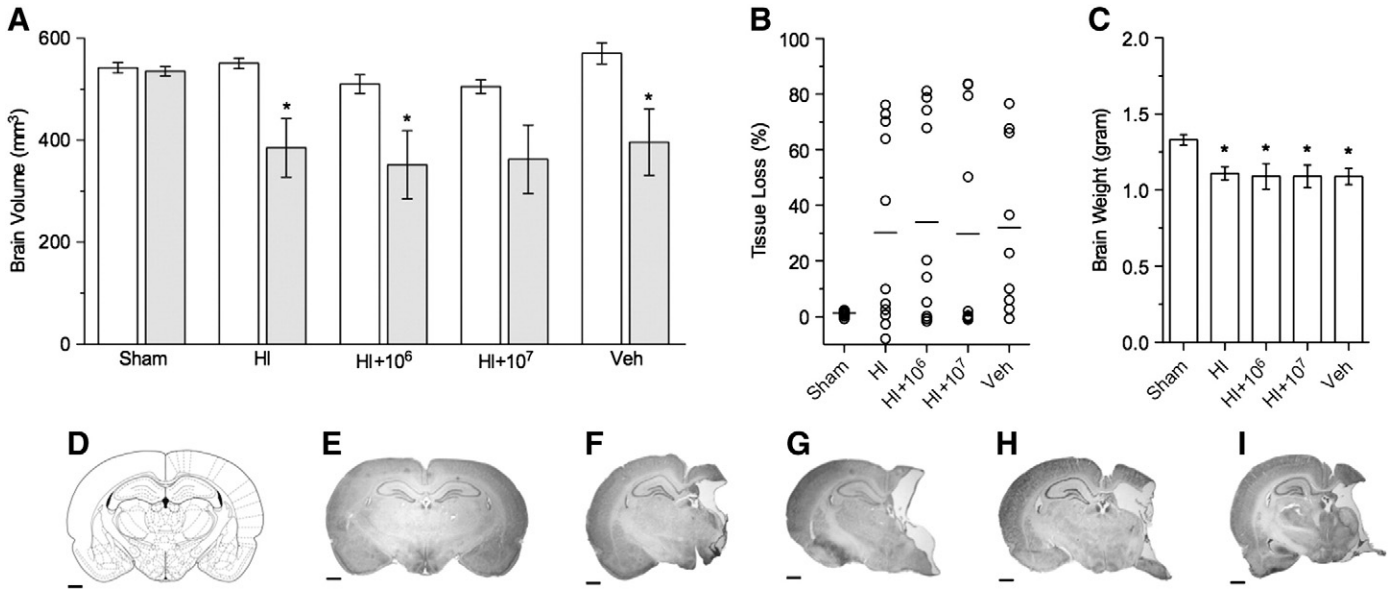

Fig. 3. IA transplantation of HUCB mononuclear cells is not able to prevent long-term brain injury in adult rats subjected to neonatal HI. (A) The estimated volumes of the contralateral (white columns) and ipsilateral (gray columns) hemispheres. (B) Percentage of brain tissue loss. $^{*}p < 0.05$ vs. left hemisphere volume in Bonferroni's post hoc test after one-way ANOVA. (C) Brain weight measurements. $^{*}p < 0.05$ vs. sham in Dunnett's post hoc test after one-way ANOVA. (D) A schematic drawing from the Paxinos and Watson's atlas (interaural 5.70 mm; bregma −3.30 mm). (E–I) Digitized images of the rat brains that corresponded to the coronal sections from the sham (E), HI (F), HI+10^6 (G), HI+10^7 (H) and Veh (I) groups. Calibration bars = 1 mm.

long-term behavior and morphological outcomes in HI-injured rats (de Paula et al., 2012). Therefore, HUCB mononuclear cells represent a promising candidate for neonatal HI therapy. Nevertheless, the ideal route for stem cell delivery is a key factor that needs to be addressed in the preclinical context to help in the design of future clinical trials for neonatal HI treatment (Borlongan and Weiss, 2011).

IA delivery route has never been used for cellular transplantation in immature rats following neonatal HI insult. The main advantage of IA transplantation is the possibility of directing a larger number of cells to a specific injured brain area by a less invasive procedure than the intraparenchymal injection. Moreover, this approach circumvents cellular entrapment by internal organs and allows for repetitive transplant regimens (Misra et al., 2012). In the present study, the cell doses and volumes were based on previously published studies (Gutierrez-Fernandez et al., 2011; Kamiya et al., 2008; Lappalainen et al., 2008; Walczak et al., 2008). We adapted the microneedle technique for cellular transplantation in neonatal rats. This technique uses the high rate of blood flow in the common carotid artery to dilute and carry the injected single-cell suspension (Chua et al., 2011). In contrast to studies that had shown that intra-carotid delivery of cells induces increased mortality in stroke animals (Li et al., 2010; Walczak et al., 2008), we found no differences in the mortality rate, body weight, motor function and left brain hemisphere integrity among the experimental groups, indicating the safety of microneedle-based injection method. The IA transplantation was performed in the left common carotid artery, contralateral to the injured brain hemisphere, because we used Rice's model of neonatal HI in which the right common carotid artery is permanently occluded and does not allow reperfusion (Rice et al., 1981). Some studies suggest that transplanted cells migrate preferentially to the ischemic hemisphere (Lundberg et al., 2012) and that chemoattraction may recruit therapeutic cells through the interhemispheric vasculature, followed by transendothelial diapedesis and subsequent intraparenchymal migration to the HI brain lesion (Andres et al., 2011; Pendharkar et al., 2010).

In this study, the IA transplantation of HUCB mononuclear cells dose-dependently reduced cognitive deficits induced by neonatal HI brain injury. We have previously demonstrated the same beneficial effect on cognitive function using the IV route for HUCB mononuclear cell delivery (de Paula et al., 2012). According to our studies, the cellular dose capable of promoting cognitive recovery in HI rats is lower with IA transplantation than with IV (1×10^7 versus 1×10^8 cells, respectively) transplantation. It was also demonstrated that 1×10^4 embryonic stem cell-derived cells improve the learning ability and memory of HI mice at 2 and 8 months after intracerebroventricular transplantation using the MWM paradigm (Ma et al., 2007). Additionally, because the Veh and HI rats had the same performance on the training session and the probe tests of MWM, we can assume that the IA administration procedure is safe and does not exacerbate the cognitive dysfunction found in neonatal rats.

According to accelerating rotarod test, there was no significant impact of the neonatal HI model, IA administration of HUCB mononuclear cells or vehicle on balance and motor coordination in adult rats. The finding of normal locomotor activity in adult rats subject to neonatal HI insult may be due to the spontaneous recovery of sensorimotor deficits because of plasticity of the immature brain (Balduini et al., 2000; de Paula et al., 2012; Lubics et al., 2005; Rojas et al., 2012). There is evidence for a positive effect of cell-based therapy on neonatal HI-induced sensorimotor impairment using the IC (Daadi et al., 2010) and IV (Yasuhara et al., 2010) routes. With regard to IA transplantation, stem cells have been shown to recover sensorimotor deficits in experimental models of stroke (Kamiya et al., 2008; Ohta et al., 2006) and traumatic brain injury (Osanai et al., 2012). A positive correlation was demonstrated between the number of IA transplanted cells found in the brain and the degree of motor recovery in the adult HI model (Guzman et al., 2008). Conversely, two studies using the neonatal HI model failed to demonstrate motor recovery in the rotarod test by using IC (Yasuhara et al., 2006) and intra-cardiac (Lee et al., 2010) delivery methods. In our study, intra-carotid transplantation was unable to reduce brain damage independently of cognitive recovery in the HI rats. It is possible that IA transplantation of HUCB mononuclear cells enhances brain plasticity rather than neuroprotection. Similarly, others have also shown functional recovery without brain injury reduction through IC (Daadi et al., 2010) and IA (Gutierrez-Fernandez et al., 2011; Guzman et al., 2008; Zhang et al., 2012) cellular transplantation. Interestingly, the greater brain engraftment of IA transplanted cells may not necessarily be associated with higher brain lesion recovery in

the stroke model (Gutierrez-Fernandez et al., 2011; Li et al., 2010; Zhang et al., 2012). Intra-cardiac injection of mesenchymal stem cells neither restores brain infarction nor improves the performance on the rotarod test in neonatal HI (Lee et al., 2010). In contrast, IA delivery of therapeutic cells favored both histological and cognitive outcomes in adult HI (Andres et al., 2011) and stroke (Brenneman et al., 2010; Kamiya et al., 2008; Ohta et al., 2006) models. Interestingly, the transplantation of 1×10^7 HUCB mononuclear cells decreased infarction volume in animals receiving IV treatment (de Paula et al., 2012). Conversely, the same dosage of HUCB mononuclear cells was not effective against HI brain injury through IA cellular transplantation. Furthermore, no further brain damage was identified in the ipsilateral hemisphere due to HUCB transplantation, indicating the safety of the IA delivery method.

We verified cellular migration to the injured brain hemisphere as early as 3 and 6 h post-transplantation and also at 7 and 30 days later. However, human cells were not detectable at 24 h after their IA administration into the neonatal rat brain. This evidence is debatable because different sets of animals were used for each time point analyzed. Furthermore, interindividual variability of the IA transplant efficiency and degree of HI brain damage might have affected cellular migration to the target injured brain (Li et al., 2010). We also detected human cells in the liver and lungs at 1 and 3 h post-IA transplantation, which is consistent with preclinical and clinical studies (Barbosa da Fonseca et al., 2010; Vasconcelos-dos-Santos et al., 2012). PCR analyses are commonly used to investigate cell migration in preclinical studies (de Paula et al., 2009, 2012; Venturin et al., 2011). However, the drawback of the PCR technique lies in its inability to permit longitudinal studies in the same animals. It has been shown in animal models of ischemic stroke (Gutierrez-Fernandez et al., 2011; Kamiya et al., 2008; Lappalainen et al., 2008; Li et al., 2010; Pendharkar et al., 2010; Walczak et al., 2008; Zhang et al., 2012) and traumatic brain injury (Lundberg et al., 2012) that IA delivery results in a wider distribution and larger number of grafted cells in the injured brain than by IV injection. In contrast, a recent study showed that IA and IV delivery routes provide similar brain homing of bone marrow mononuclear cells in focal cerebral ischemia (Vasconcelos-dos-Santos et al., 2012). Notwithstanding, it was demonstrated that IA transplantation of stem cells after transient ischemia results in a high variability of cell homing in the rat brain (Brenneman et al., 2010; Lappalainen et al., 2008; Li et al., 2001; Walczak et al., 2008). An explanation for this variability could be the ratio of the cell diameter to the capillary size, which may affect the biodistribution of transplanted cells into cerebral tissue (Lappalainen et al., 2008; Walczak et al., 2008). Therefore, it is likely that IA injected cells become initially entrapped in the cerebrovasculature due to cellular adhesion and size and are then gradually recruited by chemotactic signals to the ischemic tissue (Mitkari et al., 2012; Osanai et al., 2012; Vasconcelos-dos-Santos et al., 2012).

The present study was not designed to investigate the mechanisms of action by which HUCB mononuclear cells exert their neuroprotective effects on HI neonatal rats. In contrast to the initial concept, studies indicate that engraftment and direct differentiation of the transplanted cells are not the key mechanisms of cell-based therapies. Currently, it has been postulated that stem cells exert therapeutic effects mediated by blood vessel regeneration, greater survival of intrinsic cells, synaptic plasticity enhancement, anti-inflammatory action and immunomodulation, most likely via neurotrophic factors and cytokine secretion (Liao et al., 2013; Pimentel-Coelho et al., 2012). Some studies suggest that only the presence of transplanted HUCB cells within the systemic circulation is sufficient for functional recovery and brain repair (Borlongan et al., 2004; Yasuhara et al., 2010). Conversely, others point to the importance of stem cell engraftment into the brain for behavioral improvement (Guzman et al., 2008). It could therefore be hypothesized that the IA delivery route would allow the convergence of systemic effects and the focal action of HUCB mononuclear cells in the brain of HI animals.

The femoral artery catheterization is not usual in the neonatal intensive care units but it is eventually used for cardiac catheterization. In the case of asphyxiated newborns, the procedure would be done in the first hours after birth through the umbilical artery which is performed when attempts to obtain peripheral access are unsuccessful. The placement of umbilical catheters has relatively low risk and is an essential technique for the treatment of many newborns in unstable condition (Anderson et al., 2008). Since the umbilical artery is usually available for two or three days, the cellular transplantation would be performed before that period. In the practice, we believe that the catheter could be introduced until the carotid artery under echography monitoring for the cellular transplantation, and after that the catheter would be placed in its previous position (Berenstein et al., 1997). In another study from our research group, asphyxiated piglets subjected to umbilical catheterization for cellular transplantation had HUCB cells detected in their brains. However, the HUCB cells were not detected when intravenous injection was performed (data not published).

To our knowledge, this is the first study to demonstrate the feasibility and protective effects of IA transplantation of HUCB mononuclear cells in an experimental model of neonatal HI. Major factors such as the aspects of the human cerebral vasculature and hemodynamics, the dosage and infusion regimen, the adjuvant interventions and the cell type and treatment timing will need to be addressed before translating the IA approach for the delivery of stem cells into clinical applications. It would be suitable to compare multiple routes of cell delivery using the same neonatal HI model paradigm, assessments of brain lesions and behavioral outcomes and cellular tracking by small animal imaging, as performed elsewhere in experimental cerebral ischemia (Zhang et al., 2012). Therefore, we propose that the IA route might provide an additional alternative for cellular transplantation for pediatric patients with HI injury.

Conclusions

In this study, we show that IA transplantation is a feasible delivery route for HUCB mononuclear cells in neonatal rats subjected to a HI model. The IA delivered 1 x 107 cells hindered cognitive impairments 9 weeks after neonatal HI insult but did not protect against brain damage. We further demonstrated that IA cellular transplantation is safe since there was no long-term impact on body weight or motor function in rodents.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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