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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).