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Hemispheric Brain Injury and Behavioral Deficits Induced by Severe Neonatal Hypoxia-Ischemia in Rats Are Not Attenuated by Intravenous Administration of Human Umbilical Cord Blood Cells

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ABSTRACT: Neonatal hypoxia-ischemia (HI) is an important cause of mortality and morbidity in infants. Human umbilical cord blood (HUCB) is a potential source of cellular therapy in perinatology. We investigated the effects of HUCB cells on spatial memory, motor performance, and brain morphologic changes in neonate rats submitted to HI. Seven-day-old rats underwent right carotid artery occlusion followed by exposure to 8% O_2 inhalation for 2 h. Twenty-four hours after HI, rats received either saline solution or HUCB cells i.v. After 3 wk, rats were assessed using a Morris Water Maze and four motor tests. Subsequently, rats were killed for histologic, immunohistochemical, and polymerase chain reaction (PCR) analyses. HI rats showed significant spatial memory deficits and a volumetric decrease in the hemisphere ipsilateral to arterial occlusion. These deficits and decreases were not significantly attenuated by the injection of HUCB cells. Moreover, immunofluorescence and PCR analysis revealed few HUCB cells located in rat brain. Intravenous administration of HUCB cells requires optimization to achieve improved therapeutic outcomes in neonatal hypoxic-ischemic injury. **(*Pediatr Res* 65: 631–635, 2009)**

Neonatal hypoxia-ischemia (HI) is a major cause of mortality and morbidity in infants and occurs in approximately 2–4 per 1000 full-term births. Between 20 and 50% of asphyxiated newborns with hypoxic-ischemic encephalopathy die within the neonatal period, and up to 25% of the survivors will exhibit neurodevelopment morbidity, such as cerebral palsy, mental retardation, and epilepsy. The most widely used and accepted animal model of neonatal HI is the Levine method as modified by Rice *et al*. (1), which represents a useful tool to study long-term effects of neuroprotective strategies in behavioral changes, especially in learning and memory tasks (2). Although promising neuroprotective strategies have been studied in animal models and clinical trials, current management techniques have reached only limited success (3).

Human umbilical cord blood (HUCB), is rich in adult stem cells and seems to be a potential source for transplantation, especially for perinatal neuronal repair. Studies have shown behavioral and neurologic recovery in stroke (4–7) and HI-insulted animals (8,9) that received i.v. injection of HUCB, indicating that cells migrate toward ischemic regions and cross the blood brain barrier (BBB), especially in acute periods postischemia (10). The i.v. route is less invasive and a safer access to clinical applications when compared with intracerebral delivery. However, very few transplanted cells are found in the brain when delivered intravascularly. Therefore, evidence suggests that these cells increase endogenous mechanisms of brain repair by trophic factor secretion rather than by replacing the damaged tissue (11,12).

The aim of this study was to assess the effects of HUCB cells on spatial memory, motor performance, and brain morphologic changes in 30-d-old rats after neonatal HI on postnatal d 7. In addition, we tested whether the injected HUCB cells migrate to the injured brain 24 h, and 1 and 3 wk after HI insult.

METHODS

Experimental groups. The animals were randomly divided into three experimental groups: A) sham-operated animals ($n = 9$); B) rats infused with saline solution 24 h after HI injury (HI + saline; $n = 10$); and C) rats infused with HUCB cells 24 h after HI injury (HI + HUCB; $n = 15$). After 3 wk, rats were assessed in Morris Water Maze (MWM) and four motor tests. All evaluations were performed by blinded investigators.

Neonatal hypoxia-ischemia model. We used the Levine rat model, modified by Rice *et al*. (1) for neonatal rats. On postnatal d 7, Wistar rats were briefly anesthetized with halothane, and the right common carotid artery was identified, isolated from the vagus nerve, and permanently occluded with 7.0 surgical silk sutures. The entire surgical procedure was completed within 15 min.

After surgery, animals were put back into their cages and allowed to recover for 2–4 h in the company of their mothers. Rats were then placed for 2 h in a hypoxia chamber, with constant flow of humidified 8% oxygen balanced with nitrogen. The environmental temperature was maintained at 37–38°C.

The sham-operated animals were anesthetized with halothane and exposure of the right common carotid artery without ligation and hypoxia. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institutes of Health (USA).

Human umbilical cord blood cell separation procedure and administration. Cord blood collections were obtained *ex utero* using sterile syringes containing anticoagulant from full-term births of healthy donors. All samples ($n = 5$) were collected after obtaining written informed consent forms.

Blood samples were processed within a range of 24 h after collection. HUCB was diluted with RPMI medium 1640 (GIBCO, Langley, OK) and this suspension was fractionated on Histopaque density (Sigma Chemical-Aldrich) at 400 g for 30 min at room temperature. The mononuclear fraction was collected and rinsed twice with phosphate-buffered saline (PBS) contain-

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Abbreviations: BBB, blood brain barrier; **HI,** hypoxia-ischemia; **HUCB,** human umbilical cord blood; **MWM,** Morris Water Maze

ing 1% of Liquemine (Roche, Swiss). The cell viability was evaluated using the Trypan Blue Stain 0.4% (GIBCO, Langley, OK) exclusion method. CD 34+ cells represented, on average, 0.05% of cord blood mononuclear cells after flow cytometric analysis.

Twenty-four hours after HI, randomly selected rats received i.v. either HUCB cells or saline solution. Using a micropipette with ultrafine tip (diameter <5 μm) connected to an insulin syringe, we injected 1×10^7 mononuclear cells (6,8) suspended in 100 μL total fluid volume into the external jugular vein.

Morris water maze. The spatial memory performance was evaluated 3 wk after HI lesion using an MWM (13,14). The water maze consists of a black circular pool conceptually divided in four equal imaginary quadrants. Two centimeters beneath the surface of the water and hidden from the rat's view was a black circular platform. The water maze was located in a well-lit white room with several spatial cues.

Training on spatial version of the MWM was carried out over 5 consecutive days. On each day, rats received four training trials in which the hidden platform was kept in a constant location. The mean latency to find the platform was measured for individual animals on each day.

A different starting location was used in each trial, which consisted of a swim followed by a 30 s platform sit. Rats that did not find the platform within 60 s were guided to it by the experimenter. To assess long-term memory, 24 h after the final trial, the platform was removed from the maze and the parameter measured was time spent in the target quadrant.

Open-field activity. Rats were placed in a 40×30 cm wood box and left to explore the arena freely for a 5-min period. The floor was divided into 12 equal squares by black lines. The number of line crossings, number of rearings, and locomotion time were measured (15).

Cylinder test. Forelimb use was analyzed by videotaping movements of each rat during vertical exploration in a transparent cylinder (15×30 cm). Each animal was placed in the cylinder and observed for 5 min. The forelimb use asymmetry score was calculated as the percentage of nonimpaired forelimb use subtracted from the percentage of impaired forelimb use (16).

Grid walking test. The rats were placed on a stainless steel grid floor elevated 1 m above the floor. For 1-min observation period, the total number of steps was counted as well as the number of foot fault errors (15).

Tapered/ledged beam walking test. The beam-walking apparatus consisted of a tapered wooden beam with ledges on each side to permit foot faults without falling. The end of the beam was connected to a black box. A bright light was placed above the start point to motivate the rats to traverse the beam. The score for each trial was calculated as follows: [(vertical slips $\times$ 0.5 + horizontal slips)/(steps + vertical slips + horizontal slips)] $\times$ 100 (17).

Morphologic study. The animals were deeply anesthetized with thiopental sodium (0.1 mL/100 g, i.p.) and perfused transcardially with saline followed with 4% paraformaldehyde. The brains were removed from the skull and stored in the same solution for 24 h. Coronal sections of the brain (50 μm) were cut using a cryostat (Shandon, United Kingdom), with 250 μm intervals, and stained with cresyl violet using the Nissl method. Digitized images of cross-sectional areas were obtained with a video camera installed in an Olympus BX40 microscope, interfaced by a software (Image Pro-Plus 6.1, Media Cybernetics) run on a personal computer. Images of the hemispheres were displayed onto a high-resolution video monitor and its boundaries were outlined for area measurements in accordance with the Paxinos and Watson atlas (18).

The Cavalieri method was used to estimate the hemispheric volumes (mm^3) by summation of areas multiplied by the distance between sections. The cross-sectional area of the hemisphere was obtained outlining edges of each hemisphere. Volume estimation was performed in about 10 sections for each rat (19,20).

Immunofluorescent staining. Rats from the HUCB cell group were killed 24 h ($n = 2$), and 1 ($n = 2$) and 3 ($n = 2$) wk after neonatal HI injury. Brains were removed from the skulls and frozen in liquid nitrogen (-70°C). Coronal sections (15 μm) were obtained using a cryostat at -20°C and postfixed in acetone. To identify grafted human cells, sections were incubated with mouse antihuman nuclear monoclonal antibody (MAb) (MAb-1281; dilution 1:100; Chemicon International) overnight at 4°C. After washing in 0.1 M PBS, FITC (dilution 1:100; Novocastra Laboratories) was added and incubated at room temperature in the absence of light for 1 h. To specifically stain the nuclei with blue fluorescence, 4′,6-diamino-2-phenylindole (DAPI, Santa Cruz Biotechnology) was used. To detect human cells in rat brain, double-stained sections were examined to identify those MAb-1281 positive cells that colabeled with DAPI, as previously described (6).

Reactivity of these antibodies with human cells had been confirmed in positive samples and negative control sections were processed as per the experimental tissue, but the primary or secondary antibodies were omitted. Slides were examined qualitatively in a Nikon Eclipse E800 fluorescence microscope coupled to a Pro-Series High Performance CCD camera and Image Pro Plus Software 6.1 (Media Cybernetics, Bethesda, MD).

Polymerase chain reaction analysis. DNA was obtained from the brain and other organs 24 h, and 1 and 3 wk after HUCB cell injection using Trizol Reagent (Carlsbad, CA). To evaluate HUCB cells migration, polymerase chain reaction (PCR) analysis for human β-actin gene was performed using primers (direct 5′-CCTCATGAAGATCCTCAC-3′, and reverse 5′-TGGAGAAGAGCTACAAGC-3′), which results in a 161 bp amplicon. Amplicons were analyzed after electrophoresis on 2% agarose gel and visualized with ethidium bromide staining.

Statistical analysis. The sample size (nine animals per group) was calculated to provide more than 80% power to detect a reduction in behavioral outcomes of 40% between the groups.

Variables with normal distribution were presented as means $\pm$ SEM. Comparisons between the groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test, and behavioral performance on the training day was analyzed using a two-way ANOVA followed by the Bonferroni test. Variables with abnormal distribution were presented as median (minimum and maximum), and the Kruskal-Wallis test was used after Dunn's comparison. Data were considered significantly different if $p < 0.05$.

RESULTS

Eight rats died during different steps of the experiment (19%). These animals were not considered in the final sample size.

To determine whether i.v. administration of HUCB cells can improve cognitive deficits, 30-d-old rats were trained in the spatial version of the MWM. Except on d 1, two-way ANOVA revealed significant differences between the experimental groups during the training period. As shown in Figure 1*A*, the mean latency of the second to fifth day was shorter in sham-operated rats when compared with the saline and HUCB cell groups, indicating that HI impaired memory performance in the injured animals. During this test, rats that had received i.v. HUCB cells 24 h after HI did not present a statistically significant shorter latency to find the platform on the d 2 to d 4.

Analysis of the probe trials (Fig. 1*B*) using one-way ANOVA followed by the Tukey test also showed that the sham-operated group spent significantly more time in the target quadrant when compared with HI animals treated with HUCB cells ($p = 0.008$) and saline ($p < 0.001$). No statistically significant difference was found between the transplanted HI rats and the saline group in terms of the latency to swim over the previous location of the escape platform ($p = 0.25$).

To analyze the motor performance of the experimental groups, we performed four motor tests. Table 1 shows that open-field activity, cylinder, grid walking, and ledged beam walking tests did not reveal significant differences between the HUCB, saline, and sham-operated groups.

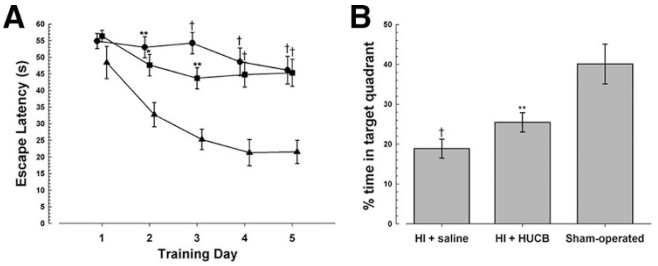

Figure 1. MWM performed 3 wk after HI injury. (*A*) Mean escape latency during the 5 d of training for sham-operated (▲; $n = 9$), HI + saline (●; $n = 10$) and HI + HUCB (■; $n = 15$) rats. (*B*) Mean percentage of time spent in the target quadrant during a 60-s probe test in the absence of the escape platform carried out 24 h after the fifth training day for rats that received saline or HUCB cells as in *A*. *Post hoc* test shows HI + saline *vs.* sham: $p < 0.001$; HI + HUCB *vs.* sham: $p = 0.008$; HI + saline *vs.* HI + HUCB: $p = 0.25$. $*p < 0.05$; $**p < 0.01$; $\dagger p < 0.001$ *vs.* sham-operated rats.

Table 1. *Motor performance in three groups within the study*

Test/group	HI + HUCB	HI + saline	Sham-operated	*p*
Locomotion time (s): mean ± SEM	107.09 ± 5.61	99.75 ± 5.39	109.13 ± 4.76	0.44
No. rearing: mean ± SEM	31.64 ± 3.08	35.75 ± 2.01	29.38 ± 2.01	0.21
No. crossings: mean ± SEM	115.55 ± 6.55	113.17 ± 5.50	124.63 ± 4.47	0.40
Cylinder test (%): median (minimum–maximum)	12.90 (−2.7 to 69.23)	13.17 (−7.14 to 53.85)	2.94 (−1.37 to 11.32)	0.06
Grid walking (%): median (minimum–maximum)	16.67 (4 to 21.43)	11.24 (7.14 to 21.43)	5.72 (0 to 33.33)	0.07
Ledged beam walking (%): means ± SEM	10.94 ± 1.01	8.62 ± 0.59	10.98 ± 1.47	0.15

Variables with normal distribution are presented as means ± SEM and are compared using one-way ANOVA. Otherwise, variables are presented as median (minimum and maximum) and are compared using the Kruskal-Wallis test.

We also examined whether HI resulted in morphologic deficits and if i.v. administration of HUCB cells attenuated neuronal loss. The results of the morphologic assessment in animals treated only with saline solution showed that neonatal HI lesion caused a significant decrease in the volume of the hemisphere ipsilateral to carotid occlusion (right) when compared with the contralateral hemisphere ($p < 0.001$) 3 wk after injury (Fig. 2*A*). The group that received HUCB cells also presented significantly reduced volume in the right hemisphere when compared with the left hemisphere ($p < 0.01$). Figure 2*B* shows that there is no statistically significant difference between the HI + saline and HI animals treated with HUCB cells in terms of the relative difference between left and right hemispheric volume (hemisphere volume ratio = left hemisphere volume/right hemisphere volume) ($p = 0.095$).

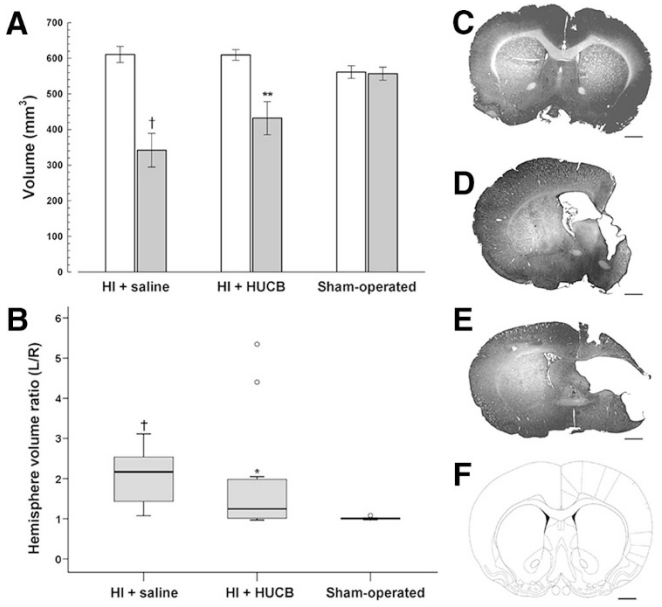

Figure 2. (*A*) The estimated volumes of brain hemispheres, using the Cavalieri method in sham-operated ($n = 9$), HI + saline ($n = 10$) and HI + HUCB ($n = 15$) rats 3 wk after HI injury. There is a significant decrease in the right hemisphere volume (*gray columns*) in HI + saline ($p < 0.001$) and HI + HUCB groups ($p < 0.01$), when compared with the left side (*white columns*). Data expressed as means ± SEM. ANOVA was used followed by a Tukey test (*$p < 0.05$; **$p < 0.01$; †$p < 0.001$) (*B*) Hemispheric volume ratio determined by division of the left hemisphere by the right hemisphere, showing a tendency to reduction in HUCB cell group when compared with saline animals. Data are expressed as median (minimum and maximum). Circles represent outliers. Post hoc analysis shows HI + saline *vs.* sham: $p < 0.001$; HI + HUCB *vs.* sham: $p = 0.02$; HI + saline *vs.* HI + HUCB: $p = 0.095$. (*C–F*) We can observe digitized images of coronal sections of the rat brains stained using the Nissl procedure. (*C*) Sham-operated. (*D*) HI + saline. (*E*) HI + HUCB. (*F*) Schematic drawings obtained from Paxinos and Watson's atlas (interaural 9.70 mm; bregma 0.70 mm). Calibration bars = 1 mm.

Qualitative analysis of HUCB cell migration detected few MAb-1281 immunoreactive cells in either the ipsilateral or contralateral hemispheres 24 h, and 1 and 3 wk after HI injury (Fig. 3*A–I*). To confirm the distribution of HUCB cells after i.v. administration, PCR analysis using human β-actin was performed (Fig. 3*J*). The expression of human β-actin was detected in the ipsilateral and contralateral hemispheres 24 h, and 1 and 3-wk postinjury.

DISCUSSION

This study showed that neonatal HI brain injury induced significant long-term spatial memory deficits and extensive brain hemisphere atrophy. In those animals that received i.v. administration of HUCB cells 24 h after HI induction, the degree of behavioral and morphologic impairment was not significantly reduced. Importantly, the administration of human cells did not worsen the outcomes in HI rats.

As with other investigations, our study showed that, in spite of severe brain damage, motor function tests were insufficiently sensitive to detect neuromotor alterations in all HI rats (2). In contrast to human neonates, pups that underwent HI injury did not show obvious postural or locomotor abnormalities due to a higher degree of plasticity in immature rat brain (21). However, studies have demonstrated that injured brain regions required for memory and learning processing result in significantly decreased spatial memory (2). Our results showed that the MWM test was sensitive to brain damage in neonatal HI rats.

Several groups have reported that HUCB cells delivered either intracerebrally or i.v. dramatically enhance functional recovery after ischemic injury in adult rats. The mechanisms behind such reported neuroprotection are not known, but may include cytokines and trophic factors produced by HUCB cells (6,7,22–24). However, some authors do not confirm these results (25).

Although there are few reported studies on the use of cellular therapy in neonatal brain damage models, there is a wide range of methods used (8,9,26,27). Ma *et al.* used a HI mouse model similar to ours when studying the effect of stem cell transplantation. They showed that the transplanted cells significantly improved the learning and memory deficits 8 mo posttransplantation. However, this study used mice embryonic stem cells injected directly into the lesion site (26).

The route of delivery may contribute to contrasting data. To date, no study had used the acute transplant (24 h after insult)

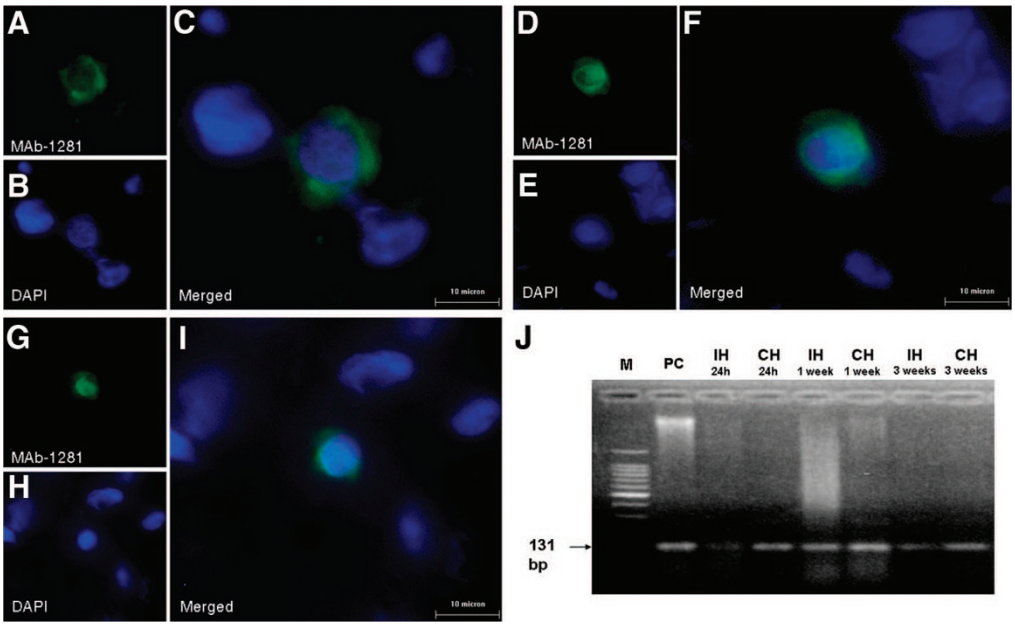

Figure 3. (*A–I*) Digitized images of rat brain showing the immunoreaction to human nuclei antibody (MAb-1281). Use of immunofluorescence FITC (*green*) shows few MAb-1281 immunoreactive cells in rat brain at (*A*) 24 h, and (*D*) 1 and (*G*) 3 wk after HI injury. (*B*, *E*, *H*) Nuclei visualized by DAPI staining (*blue*). (*C*, *F*, *I*) Colocalization of immunofluorescent labels DAPI and MAb-1281 was observed. (*J*) Representative PCR analysis results for human β-actin of rats after 24-h, and 1- and 3-wk postinjury. Positive control (PC) = human DNA extracted from human hippocampus. IH, ipsilateral hemisphere; CH, contralateral hemisphere.

of adult stem cells into neonatal rat by i.v. delivery. Intravenous administration has the advantage of being less invasive but raises the problem of cell homing to organs and target site (11). Yasuhara *et al.* (9) demonstrated that both intracerebral and i.v. injection of multipotent adult progenitor cells resulted in behavioral improvement in HI rats although the percentage of graft survival was small. However, Borlongan *et al.* (28) showed that in contrast with intracerebral transplantation, i.v. delivery of bone marrow stem cells produced only limited functional effects in stroke rats. In our study, the nonsignificant functional recovery could also be explained by the small number of MAb1281-positive cells found in rat brain.

We chose to administer the cells 24 h after injury to target acute ischemic processes, such as the inflammation and BBB opening. The optimal time for transplantation after injury is also a critical factor in initiating the repair process. Acute delivery of cells will be critical once the ischemic brain elicits a strong inflammatory response about 24 h postinsult (10,22,28). Also, the BBB may be more accessible at 24–72 h postischemia and combined opening of the BBB and the high expression of chemokines could have facilitated the present mononuclear cell graft migration toward and survival within the ischemic area (29). However, Borlongan *et al.* reported that BBB opening produced by middle cerebral artery occlusion was not permissive enough to allow CNS entry of graft-derived trophic factors. In that study, improved behavioral functions were observed only in rats treated with cord blood stem cells associated with mannitol (4). Previously, we evaluated the BBB permeability in five rats 24 h after HI. Mild leakage was observed in these animals after trypan blue injection (data not shown).

Our morphologic analyses showed that neonatal HI resulted in an extensive infarcted area and that i.v. injection of HUCB cells was unable to significantly reduce the severity of the morphologic damage. We used a dose of 1×10^7 HUCB cells in accordance with previous studies (8). However, few reports about stem cell therapy in brain damage have studied specific dose range (6).

Our study has some limitations. Seven-day-old rats are considered to be similar to human newborns with regard to brain maturation, and the animal is highly suitable for long-term behavioral evaluations. However, Levine rat model results in variable degrees of damage due to individual differences in brain susceptibility among animals (2,30). We hypothesized that the large extension of the hemispheric brain lesion obtained in our study could have contributed to the fact that we did not observe repair after the infusion of 1×10^7 HUCB cells. It is possible that rats with smaller brain injury will have a different response to i.v. injection of HUCB cells.

Only a low number of HUCB cells were detected in rat brain 24 h, and 1 and 3 wk after neonatal HI, as reported in other investigations (4,6,8,22). Despite controversies, immunosuppressive treatment may be a key determinant for homing of HUCB cells i.v. infused into rats (31). Several investigations suggest that T cell-mediated immune reaction plays a significant role in graft rejection and that interspecies incompatibility contributes significantly to phagocytosis of xenogeneic cells (32). Although xenoreactivity to human mesenchymal stem cells transplanted into infarcted rat myocardium has been demonstrated (33), these findings contrast with the results of Saito *et al.* (34). Indeed, xenotransplantation of human bone marrow stem cells ameliorates neurologic deficits after grafting into ischemic brain of rats (35). Also, the results obtained by Chen *et al.* (22) with HUCB cells in rats with stroke suggest an immunologic tolerance to human cells. Although we do not have evidence of host immune response to the transplanted cells, the administration of human cells could have played a role in the negative results obtained in this study.

In addition, the unilateral carotid artery permanent occlusion results in reduction of cerebral blood flow within the various structures of the hemisphere ipsilateral to the vascular occlusion (30). We speculated that low perfusion in distal field

regions may hamper migration of HUCB cells to injured structures, which could also explain our immunohistochemical, behavioral, and morphologic results.

In conclusion, this study suggests that, according to our experimental design, HI neonatal rats with severe brain damage that received i.v. administration of HUCB cells showed nonsignificant improvement to functional and morphologic outcomes. Aspects such as dose, timing, peripheral route of HUCB cell delivery, immunosuppression, and use of associated therapies need to be investigated in depth before cellular therapy is clinically applied to optimize neuroprotection and, consequently, neurobehavioral outcomes.

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