Human Umbilical Cord Blood Cells Ameliorate Motor Deficits in Rabbits in a Cerebral Palsy Model

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Key Words

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Abstract

Cerebral palsy (CP) has a significant impact on both patients and society, but therapy is limited. Human umbilical cord blood cells (HUCBC), containing various stem and progenitor cells, have been used to treat various brain genetic conditions. In small animal experiments, HUCBC have improved outcomes after hypoxic-ischemic (HI) injury. Clinical trials using HUCBC are underway, testing feasibility, safety and efficacy for neonatal injury as well as CP. We tested HUCBC therapy in a validated rabbit model of CP after acute changes secondary to HI injury had subsided. Following uterine ischemia at 70% gestation, we infused HUCBC into newborn rabbit kits with either mild or severe neurobehavioral changes. Infusion of high-dose HUCBC (5×10^6 cells) dramatically altered the natural history of the injury, alleviating the abnormal phenotype including posture, righting reflex, locomotion, tone, and dystonia. Half the high dose showed lesser but still significant improvement. The swimming test, however, showed that joint function did not restore to naïve con-

trol function in either group. Tracing HUCBC with either MRI biomarkers or PCR for human DNA found little penetration of HUCBC in the newborn brain in the immediate newborn period, suggesting that the beneficial effects were not due to cellular integration or direct proliferative effects but rather to paracrine signaling. This is the first study to show that HUCBC improve motor performance in a dose-dependent manner, perhaps by improving compensatory repair processes.

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Introduction

Cerebral palsy (CP) has a very high index of disease burden, resulting in life-long neurological consequences to the patient, caretakers and social institutions. One of the major pathogenetic causes of CP is hypoxia-ischemia (HI) in the antenatal period [1, 2]. HI at the maternal, placental or fetal level often results in neonatal encephalopathy or newborn HI encephalopathy (HIE). At the present time, the only treatment available for HIE is hypothermia initiated within 6 h of birth. Hypothermia offers only 11% reduction in risk of death or disability – from 58 to 47% [3]. Further, there are considerable un-

resolved safety concerns around cooling preterm newborns [4]. Part of the reason for the modest effect in term babies is that the precise timing of the initial insult is often unclear. Promising add-on treatments such as erythropoietin and xenon are now in phase 2 clinical trials. After the child manifests CP there are no available curative therapies.

There have been excellent reviews exploring the potential use of progenitor or stem cells as a therapy for HI [5, 6]. It is postulated that they might replace lost neurons, protect endogenous host cells and promote their growth and differentiation, as well as modulate the host immune response, all of which may decrease disability after HI. Human umbilical cord blood cells (HUCBC) have been shown to be beneficial in numerous preclinical studies using models of newborn rodent HI [7–10] – except one, which used a lower dose than the others and which showed no benefit [11].

So far, >35,000 allogeneic transplants using cryopreserved and publicly banked HUCBC have been performed worldwide over the past 20 years. At Duke University alone, >1,500 have been performed, of which >300 were performed in infants and children, using autologous cells [12]. Currently, in allogeneic transplantation, the minimal effective dose after myeloablative therapy to reestablish hematopoiesis is 25 million nucleated cells/kg of recipient body weight. Doses of up to 800 million cells/kg have been given safely to infants in the first month of life. Minimally effective doses of cord blood cells in the autologous setting have not been established and are likely to vary depending on the clinical indication. We have recently reported the safety and feasibility of collection, preparation and infusion of fresh autologous HUCBC for use in infants with HIE. Cell doses of $1\text{--}5 \times 10^7$ cells/kg/dose were given intravenously for up to 4 doses [13].

We tested the use of HUCBC in the model of CP we have developed. We infused intravenous HUCBC postnatally into our newborn rabbit kits following antenatal HI at 70% gestation [14] and showed significant improvement in motor function. The initial dose we used for rabbits was comparable to the standard dose of HUCBC transfusions for humans on a per kilogram basis, 5×10^6 cells/single dose.

Materials and Methods

The Institutional Animal Care and Use Committee of NorthShore University HealthSystem Research Institute approved all experimental procedures with animals.

Collection of HUCBC

Fresh HUCBC donated to the Carolinas Cord Blood Bank (CCBB) by mothers delivering normal, term infants but not qualifying for banking for transplantation was obtained from the CCBB after processing. Cord blood was collected aseptically into cord blood collection bags (Pall Medsep, Covina, Calif., USA) containing 35 ml of citrate phosphate dextrose anticoagulant and obtained from deliveries of mothers [15]. HUCBC were transported at room temperature in validated shippers to the CCBB processing laboratory at Duke University. The collected cells were reduced in volume and RBC count after 20–30 min incubation with 6% hetastarch using the Sepax 1 automated processing system (Biosafe, Geneva, Switzerland). Pre- and postprocessing total nucleated cell counts, postprocessing CD34+ cell content, colony-forming units, sterility, and viability were assessed. HUCBC were concentrated into appropriate volumes for injection and then shipped overnight at 4°C to NorthShore University HealthSystem where they were injected the next day to kits. After arrival at NorthShore, an aliquot was taken for flow cytometry for a cell count before infusion into newborn kits.

Antenatal HI

The surgical procedure has been previously described in detail [14, 16]. In vivo global HI of fetuses was induced by sustained 40-min uterine ischemia at 22 days of gestation (70% term, embryonic day 22, E22) in timed pregnant New Zealand white rabbits (Myrtle's Rabbits, Thompson Station, Tenn., USA). Briefly, the dams were anesthetized with intravenous fentanyl (75 µg/kg/h) and droperidol (3.75 mg/kg/h) followed by spinal anesthesia using 0.75% bupivacaine. A balloon catheter was introduced into the left femoral artery and advanced into the descending aorta to above the uterine arteries. Body core temperature was monitored with a rectal temperature probe. The balloon was inflated for 40 min, causing uterine ischemia and subsequent global fetal HI. At the end of the HI period, the balloon was deflated, resulting in uterine reperfusion and a reperfusion-reoxygenation period for the fetal brains. The catheter was removed, the femoral artery was repaired and the dam was allowed to recover. The dams next underwent C-section at E31 just before term delivery (term 31.5 days). C-section was done to ensure that the HUCBC were given at the proper time for all newborn kits.

Abbreviated Neurobehavioral Examination

After birth each newborn kit was subjected to an abbreviated neurobehavioral examination investigating locomotor function and muscle tone based on the previous published protocols [16]. The newborn kits were classified as 'severe' if they had hypertonia, postural changes or obvious locomotor deficits or 'mild' if they had just mild deficit(s). The kits that were not affected were not included in the study. The division into mild and severe was performed for purposes of avoiding bias and avoiding chance imbalance in populations in treated group with HUCBC.

Infusion of HUCBC

After assignment to mild and severe groups, infusion of HUCBC, saline or media was randomly performed. This was performed in two phases. In the first phase, each of the severe and mild groups was independently randomized to receive a 1.0 ml-volume containing one of the following: (1) 5×10^6 HUCBC cells, (2) media that was used to transport HUCBC or (3) saline. The solutions

were given by intravenous injection via the external jugular or anterior abdominal vein using a 26-gauge butterfly needle or Abbot cath-T intravenous catheter (26 gauge $\times$ 3/4 inch; Abbott Laboratories, Chicago, Ill., USA). The solutions were infused at $\sim$ 4 h after birth on E31 (postnatal day 0, P0). The duration of infusion was 1–2 min. For convenience sake, the HUCBC treatment was labeled as high dose.

In the second phase, following analysis of results of phase one, each of the severe and mild groups was again independently randomized to receive either of the following: (1) 2.5×10^6 HUCBC cells or (2) saline (1-ml volume). The solutions were infused at $\sim$ 4 h after birth on E31 (P0). The duration of infusion was 5 min and delivery was by a PHD 2000 programmable pump (Harvard Apparatus, Holliston, Mass., USA). This HUCBC treatment was labeled as low dose.

Detailed Neurobehavioral Examination

At E32 (P1), P5 and P11, we performed a neurobehavioral battery of tests on the newborn kits, which assessed both motor and sensory function, as both are often affected in CP. Primary end points were as follows: (1) tone: modified Ashworth scale was used for scoring; (2) posture: evaluations of standing posture and trunk; (3) locomotion: ability to walk and jump; (4) righting: trunk and limb movement, and (5) dystonia score: assessing involuntary movements by adapting a clinical classification [17]. All tests were videotaped and scored by two investigators masked to group identity. P11 was chosen as a significant developmental milestone since it corresponds with eye opening and a substantial increase in mobility and motor abilities to near-adult levels.

Joint function and limb movement were assessed by a forced swimming test [18], which allows the detection of lack of coordination between upper and lower joints and mild motor deficits and assesses recovery with two-joint analysis. The maximum and minimum angles were calculated to define maximal extension and flexion known to be affected in CP and rabbits [18]. The mean and median angles, a measure of the mid joint motion, reflected differences between extensor and flexor tone that could change the midpoint of joint motion.

MRI Estimation of Brain Entry

As proof of principle, we assessed whether HUCBC enters the newborn kit brain and whether we could detect it noninvasively by MRI. Dams at E22 were subjected to 40 min of uterine ischemia. We added another E29 HI protocol to maximize the chances of HUCBC entering a freshly injured brain, with the differences from the E22 protocol being that at E29 the dams were subjected to 32 min of uterine ischemia, and C-section was performed at E30. The duration of ischemia at E29 was decreased to make the potency of the insult equivalent to that at E22 (as advanced gestation fetuses were more susceptible to length of insult) [19].

Cell Labeling for MRI Tracking

HUCBC cells were labeled with two compounds: Gadofluorine M (Shering, Germany), which provided positive image contrast (signal enhancement on T1-weighted images) and Feridex (11.2 mg Fe/ml; Berlex Imaging, Wayne, N.J., USA), which provided negative image contrast (signal drop on T2-weighted images) [20]. In detail, 100 μ l of 250 mM Gadofluorine stock was dissolved in a 1-ml final volume of media and incubated at 37°C/5% CO₂ for 45 min. Cells were washed and incubated in six-well plates – $5 \times$

10^7 cells in 5 ml culture medium with 25 mM Gadofluorine or 25 mM Feridex for 24 h at 37°C. Cells were washed 3 times by sedimentation (5 min, 210 g, 25°C). Unlabeled cells were prepared by incubation without labels with the same cell concentrations as above. Cells were resuspended in 1 ml of media in 1.5-ml tubes. Incubation time and label concentration for labeling were optimized in a separate set of experiments.

In vivo MRI

The dams underwent C-section at E30 and the newborn kits were infused intravenously with 5×10^6 HUCBC labeled with Gadofluorine, Feridex or unlabeled cells. The rabbit kits were imaged at 24 h ($n = 3$ per group) or 72 h ($n = 2$ per group) after HUCBC infusion.

The rabbit kits were sedated with an intramuscular injection of a mixture of ketamine (35 mg/kg), xylazine (5 mg/kg) and acepromazine (1.0 mg/kg). The animals were placed supine in a cradle heated with a water blanket at 35°C and imaged in a 4.7-tesla Bruker Biospec system (Bruker, Billerica, Mass., USA) with a 30-mm single-loop receiver-only coil. A 70-mm volume coil was used for excitation. High-resolution T2-weighted (TR/TE/NEX 5200/31/5) and T1-weighted images (TR/TE/NEX 500/9/12) were obtained in axial and coronal planes using RARE sequence (matrix 256×256 , in-plane resolution 0.08×0.11 mm, 1 mm slice thickness). FLASH T2*-weighted images (TR/TE/NEX 1000/10/5) were also obtained with the same geometry as above.

Evaluating Minimum Visibility Threshold

Gel phantoms were prepared by suspending labeled cells in 2% agarose in serial dilutions up to 1:256 from an initial concentration of $1.2\text{--}2 \times 10^6$ cells/ml. Phantoms were imaged in a 4.7-tesla Bruker Biospec system with a 30-mm single-loop receiver-only coil with the same imaging sequences and resolution as above for in vivo imaging. Signal-to-noise and contrast-to-noise (against gel phantom without cells) values were obtained for each cell concentration in T1-weighted images for Gadofluorine-labeled cells and T2-weighted images for Feridex-labeled cells.

High-Field MRI

The E32 rabbit kits (with HUCBC injection 24 h after 32 min of HI at E29) that were used in the in vivo imaging experiments were transcardially perfused with PBS followed by 4% paraformaldehyde solution. Samples were postfixed in the fixative solution for 1 week and rehydrated in PBS 24 h before imaging. A gel phantom was prepared by suspension of Feridex-labeled HUCBC in 2% agarose with a final concentration 3.65×10^3 cells/ml and 0.5 ml in a centrifuge tube. Fixed brain specimens and gel phantoms were imaged in a 14.1 vertical Bruker magnet using a 20-mm volume probe and 3D FLASH sequence (TR/TE/FA/NEX 50/23/12/6), providing T1/T2 contrast with isotropic resolution of 50 μ m and imaging time of 10 h 38 min.

Estimating for Human DNA Entry into Newborn Brain

To assess whether there was any long-term biodistribution or engraftment of HUCBC into the newborn brain, we next devised a high-sensitivity method to detect human DNA entry into the newborn brain. Rabbit brains were obtained after transcardial perfusion with saline, and total DNA was extracted. We used a PCR method using 30 cycles of PCR amplification based on a published method [21]. DNA was isolated from several rabbit kit brains at P18 using

Table 1. Results of neurobehavioral examination at P1

	Severe P1			Mild P1		
	saline (n = 5)	media (n = 5)	HUCBC (n = 7)	saline (n = 3)	media (n = 5)	HUCBC (n = 8)
Posture	1.4±0.2	1.7±0.2	1.5±0.1	2.5±0.0	2.6±0.1	2.4±0.1
Righting	1.4±0.2	1.7±0.2	1.6±0.1	2.5±0.0	2.6±0.1	2.3±0.1
Tone	3.5±0.4	2.8±0.5	3.4±0.3	1.0±0.0	0.8±0.1	0.9±0.1
Locomotion	1.2±0.4	1.7±0.2	1.6±0.2	2.5±0.0	2.5±0.0	2.3±0.1
Dystonia	1.8±0.3	1.9±0.4	2.3±0.1	1.5±0.3	0.9±0.2	0.9±0.1

Test scores were not different between the severe and mild treatment groups (ANOVA). Scores based on ordinal scoring from 0 to 4 in neurobehavioral test battery [24].

an Allprep DNA/RNA kit (Qiagen™). The DNA was PCR amplified using primers derived from a portion of the human mitochondrial cytochrome b region, which shows no homology to other animal species [21]. These primers showed no cross-reactivity to rabbit brain samples and we were able to identify as little as 40 human cells in 50 mg of rabbit brain tissue. This sensitivity was established by adding series-diluted HUCBC into 50 mg of rabbit brain tissue.

Statistical Analysis

The effect of HUCBC treatment was analyzed by repeated-measures two-way analysis of variance (ANOVA; GLM procedure in SAS statistical software). Data were presented as means ± standard error of means. The level of statistical significance, α , was set as 0.05.

Results

HUCBC Improves Motor Outcome across Severity Injury Groups

A detailed neurobehavioral examination at P1 (E32) revealed that there was initially no significant difference in motor deficit scores between the treatment and control groups in either the severe or mild groups (table 1), indicating that the groups were most likely equivalent at study entry at P0 (E31). Conversely, one could infer that there was no significant improvement at 1 day after treatment.

The differences in motor outcome measures between the saline and media groups were tested in repeated-measures ANOVA. No difference was found between the groups with respect to the interaction term ‘time × treatment’ in any outcome measure (given $\alpha = 0.05$). The saline and media groups were combined for the further analysis. The effect of HUCBC (‘treatment’) on motor outcome measures was tested using repeated-measures ANOVA with ‘time’, taking into consideration the outcomes at P1, P5 and P11 as repeated measures (n = 15), and compared

to a new control group containing both saline and media groups (n = 18). The initial severity of motor deficits, mild or severe, was added to the model as a covariate ‘severity’ (n = 17 severe, n = 16 mild). Using multivariate statistics Wilks’ Lambda, the interaction term time × treatment was found to be significantly related to locomotion, tone, posture, and righting outcome measures ($p < 0.0001$ for all outcomes), thus affirming statistically significant effects of the HUCBC on changes in motor outcome between P1, P5 and P11 time points. The interaction term time × severity was also significantly associated with the outcome measure of tone ($p = 0.0091$), indicating that the effect of the treatment was different between the mild and severe groups. Therefore, the changes in motor scores from P1 to P5 and P11 for the mild and severe groups are presented separately in figures 1 and 2. Absolute values of motor outcome variables between ages, the severity of initial injury and treatment groups are presented in online supplementary table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000374107).

High-Dose HUCBC Infusion Improves Outcome in Survivors

Following high-dose HUCBC infusion (see Methods), survivors in the severe group showed a significant improvement in motor function from P1 to P11 in all categories of neurobehavioral testing (fig. 1). There was an increase in scores of posture, locomotion and righting, with a concomitant decrease in hypertonia and dystonia scores. Survivors in the mild group also showed an improvement of motor deficits (fig. 2). The absolute change in scores was smaller in this group, but the initial impairment was also mild.

Examination of the survival curves revealed that there was a trend towards increased mortality, although not

Fig. 1. HUCBC administration improved neurobehavioral scores at days 5 and 11 in the severe group compared to saline and media. * $p < 0.05$, ** $p < 0.01$, ANOVA.

statistically significant, following high-dose HUCBC infusion relative to saline and media control groups ($p = 0.070$, χ^2 test). The initially higher mortality rate in the HUCBC group was comparable to control groups after a reduction of the infusion rate. There was no difference in weight gain in survivors.

Low-Dose HUCBC Infusion Causes No Mortality but the Effect Is Less

We then hypothesized that a lower dose of HUCBC cell infusion would result in no increase in mortality,

while retaining the capacity to improve neurobehavioral outcome. In the severe group, low-dose HUCBC or saline infusion resulted in no mortality. Again, there was significant (but milder than noted in the high-dose group) improvement in tone, posture, righting reflex, locomotion, and dystonia score from P1 to P11 ($n = 5$, repeated-measures ANOVA, $p < 0.05$ in all 5 measures; fig. 3). There was also no difference in weight gain in the survivors.

The forced swimming test [18] allows the detection of lack of coordination between the upper and lower joints

Fig. 2. HUCBC administration improved neurobehavioral scores at days 5 and 11 in the mild group. * $p < 0.05$, ** $p < 0.01$, ANOVA.

and mild motor deficits and assesses recovery with HUCBC with two-joint analysis, thus providing some insight into sites of injury. We also wanted to know whether the neurobehavioral improvement was due to a restoration to the control state or to some other compensatory state.

Functional deficits of joint movements can be discovered using graphs obtained from two-joint analysis. As

shown in figure 4, the low-dose HUCBC did not restore joint function to the control state, but joint movements in this group had a different pattern from those of the saline-treated kits, suggesting the involvement of compensatory mechanisms in the adaptation of muscle tone, range of motion and joint movement pattern in the HUCBC treated group. The range of motion and median angle did not change with HUCBC treatment (online suppl. fig. 1).

Fig. 3. Low-dose HUCBC administration improved neurobehavioral scores at days 5 and 11 (n = 5). * p < 0.05 in all 5 measures, ANOVA.

Absence of MRI Evidence of Intraparenchymal Entry of HUCBC into Newborn Brain

Cells labeled with Feridex provided larger signal difference (fig. 5a) and contrast-to-noise ratio (CNR; fig. 5b) on T2-weighted images than Gadofluorine on T1-weighted imaging. The CNR for the Feridex label was 2.05 with a signal-to-noise ratio being 129 to detect 15,600 cells/ml in the region of interest (ROI). With a resolution of $0.08 \times 0.1 \times 1$ mm in voxels used for in vivo imaging, this would theoretically provide detection of 1.23 Feridex-labeled cells in an ROI 3×3 pixel size. This calculation was done with CNR relative to agarose gel phantom, which has similar T2 properties to newborn rabbit gray matter. Detection level in a real brain requires

a much larger CNR due to the high signal inhomogeneity of brain tissue.

No clear evidence of the HUCBC penetrating brain parenchyma was found on T2-weighted images in kits infused with labeled HUCBC with slower infusion rates at 24 h (fig. 5d) and 72 h after HI and imaged 24 h later (fig. 5e), nor were hyperintensities suggestive of the presence of labeled cells found on T1-weighted images (fig. 5f). Blood vessels penetrating white and gray matter were conspicuous on T2-weighted images (fig. 5c–e, arrows) as black punctate hypointensities. Notably, the vessels were darker in images of kits infused with labeled cells than those infused with unlabeled cells (fig. 5c–e, arrows). The difference in the CNR between these two images relative

Fig. 4. Two-joint analysis of P11 kits. Saline with HI (mean, black, $n = 9$) had distinctive deficits both in forelimbs and hindlimbs compared to control (blue dashed, $n = 18$) and low-dose HUCBC-treated kits (green, $n = 4$) following E22 HI.

to gray matter was 0.38. Assuming the same CNR for labeled cells as obtained in phantom (fig. 5b) and blood volume of 6 ml in E32 rabbit kits, these numbers imply the presence of 4.11×10^3 HUCBC in the circulatory system 24 h after the infusion of 2.5×10^6 cells.

To confirm that there was no extravasation of the labeled cells, the brains from the kits that had been imaged in vivo were transcidentally perfused to remove blood and subsequently fixed and imaged overnight with high resolution using a 14.1-tesla magnet with 50 μm of isotropic resolution. Feridex-labeled single cell detection was pos-

sible at this resolution, as confirmed by imaging a gel phantom prepared from Feridex-labeled cell suspension with the same imaging parameters (fig. 6a, b). When imaged together, punctate signal voids were clearly visible in the phantom with labeled cells (fig. 6b) but not in the phantom without cells (fig. 6a). No image signatures of the labeled cells were detected in the parenchyma of the brain sample with Feridex-labeled HUCBC infusion (fig. 6d) compared to that with unlabeled cell infusion (fig. 6c). The choroid plexus was darker in the brain sample where labeled cells were infused than in the brain with

Fig. 5. Cells were labeled with T2-shortening superparamagnetic iron oxide MRI contrast (Feridex) or T1-shortening contrast Gadofluorine M. Signal change (**a**) and CNR (**b**) determined on T2-weighted images for Feridex and T1-weighted images for Gadofluorine on agarose gel phantom with serial cell dilutions on 4.7 T. Detection of intravenously infused HUCBC on MRI in vivo. Rabbit kits were imaged on a 4.7-tesla magnet 24 h after infusion of media (**c**, T2-weighted) or Feridex-labeled cells (**d**, T2-weighted) 24 or 72 h (**e**) after E29 HI. **f** T1-weighted image of rabbit kit infused with Gadofluorine-labeled cells 24 h after E29 HI. 2.5×10^6 HUCBC cells were delivered by a PHD 2000 programmable pump (Harvard Apparatus).

nonlabeled cell infusion (fig. 6c, d, arrows). This may indicate that some HUCBC may be lodged in this highly vascularized region, but no cell propagation to parenchyma was noticed.

Possible Entry of HUCBC into Newborn Brain

There are several possibilities that may explain the MRI results such as the following: (1) HUCBC did not enter the brain in appreciable numbers to be detected on MRI using our techniques, (2) loss of the MRI label with

cell division or (3) the labeling procedure changed the properties of HUCBC and they lost the ability to enter brain parenchyma and reach sites of injury. We next tested for subacute evidence of human cells in the rabbit newborn brain parenchyma by detecting human DNA in P5 kits. PCR reactions targeting specific human gene sequences were performed. In the 10 brain samples we tested, 8 cortex and 9 thalamus samples showed positive results for human DNA, with none for the saline group (fig. 6e).

Fig. 6. Detection of intravenously infused HUCBC ex vivo. High-resolution imaging with isotopic 50- μ m voxel resolution on a 14.1-tesla magnet. Agarose phantoms without (a) and with (b) Feridex-labeled cells. Rectangular ROIs placed on phantoms are shown with high magnification. Note punctate hypointensities in b, indicating labeled cells. Perfusion-fixed brain of E32 kit infused with media (c) or Feridex-labeled cells (d). Arrows point to choroid plexus on c and d. Scale bar = 1 mm. e PCR for human DNA showing most of the cortex and thalamus samples having some small amounts of human DNA.

Discussion

This is the first study to test whether HUCBC infusion after the discovery of neurobehavioral deficits following antenatal HI would ameliorate the clinical deficits cause

by HI injury. The results suggest that there may be improvements in locomotion following HUCBC (see video) over a moderate period of time, considering that rabbit days are shorter than human days in the rate of development. High-dose HUCBC may provide a larger degree of amelioration of neurobehavioral deficits, although this was not definitely proven as the experiments were designed to compare different doses. There was a definite improvement over the saline-treated group in both phases of the study, first with the high dose and later with the low dose.

In contrast to usual drug development, there has been a paucity of animal studies in this field. The translational implications of the present study reside in a narrow time period following an antenatal HI event. The antenatal insult in this study was 9 days before delivery and the infusion of HUCBC. An approximate corollary in humans would be if HI occurred at 24–28 weeks of gestation based on oligodendrocyte development in rabbits [22] and HUCBC were given at term birth. If one looks at the evolution of injury after HI, broadly there are three time epochs. The first is the ‘acute’ epoch, where cell injury occurs with the onset of primary and secondary energy failure [23]. Cytotoxic edema, cell death and apoptosis result from severe HI in the first few days. Hypothermia as a rescue therapy is given at this time to prevent further deterioration [24].

The second time epoch can be called the ‘chronic’ epoch, which spans the next few days to months and involves a period of cell repair and recovery. It may also involve secondary inflammatory processes – some of them damaging. This is the time epoch that the HUCBC were given in the present study. Mention must be made of two adjunct therapies with hypothermia that are now in clinical trials – erythropoietin and xenon. The elucidation of the mechanism of action of HUCBC runs into the same problems that have faced the mechanism of action of erythropoietin – a circulating hormone. Erythropoietin does not cross the blood-brain barrier and is also a blood-borne growth factor. Erythropoietin as a drug therapy is now being tested in clinical trials despite not knowing its exact mechanism of action. For both term and preterm infants, erythropoietin involves the modification of ‘acute’ injury initially in most animal studies [25] and extends into the ‘chronic’ epoch in some animal and human studies [26] and the proposed ongoing clinical trials. Erythropoietin is being tested in newborn babies and needs to be given in repeated doses [27, 28]. Xenon is another adjunct therapy with hypothermia now being tested for term HIE in clinical trials [29]. The cost of xenon is somewhat prohibitive at the present time.

The third time epoch is the 'late' epoch, which starts with the definitive diagnosis of CP at about 12–24 months of age. Damaging processes have become quiescent, and development adaptations result in the final clinical phenotype. Autologous HUCBC is presently in clinical trials for this time period for CP patients [30, 31]. The two actively enrolling studies are at Duke University and Georgia Regents University (see clinicaltrials.gov).

All the previous animal studies using HUCBC have been performed in the 'acute' epoch and employ the Vannucci model of unilateral carotid artery ligation followed by a period of moderate hypoxia on P7 in rats or mice. Meier et al. [8] reported reduced 'spastic paresis' when 10^7 HUCBC were given intraperitoneally 24 h after HI in P7 rats. Cells migrated to the injured side, but the cells had not differentiated 21 days after infusion. This group also showed no effect on the size of the lesion but demonstrated significantly preserved somatosensory function on the side of the lesion [32]. Notably, they found reduced hyperexcitability and restoration of the cortical maps and receptive fields in the somatosensory cortex. This strongly suggests that the benefit was mediated by improved function of the surviving brain tissue. This group also showed faster resolution of inflammation and astrocytic activation with HUCBC [33]. Similarly, Yasuhara et al. [9] found that intravenous infusion of 15,000 HUCBC, with or without cotreatment with mannitol, 7 days after HI in P7 rats was associated with significant improvement in motor asymmetry and coordination 7 and 14 days later. Only a few cells were found in the damaged area, and the authors suggest a paracrine effect as the levels of neural growth factor, glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor were increased 3 days after treatment – but only when cells were combined with mannitol to increase blood-brain barrier permeability and not with cells alone. In contrast, the same group reported that intrahippocampal injection of 200,000 marrow-derived multipotent progenitor cells (Athersys, Inc., Cleveland, Ohio, USA) did not improve rotarod testing and only improved motor asymmetry 14 days later [34]. Consistent with these findings, Pimentel-Coelho et al. [7] found that intraperitoneal injection of HUCBC in P7 rats 3 h after HI was associated with only trivial numbers of HUCBC in the lesion in contrast with slight improvements in sensorimotor reflexes 4 days after HI but not different at 2 and 7 days. The improved outcomes were associated with reduced caspase-3 cleavage around the lesion site and an overall reduction in activated microglia and macrophages consistent with antiapoptotic and

anti-inflammatory actions. In contrast, de Paula et al. [11] initially reported 8 deaths and no improvement in infarct volume or in spatial memory 3 weeks after 1×10^7 mononuclear cells were given intravenously 24 h after HI in P7 rats. Subsequently, the same group reported dose-response findings on infarction by showing reduced infarction volume with 10^7 and 10^8 HUCBC but not 10^6 HUCBC. Improvement in the Morris water maze for spatial memory was only found with a dose of 10^8 HUCBC [35].

The various stem cells present in HUCBC include hematopoietic stem cells (CD34), unrestricted somatic stem cells, umbilical cord stem cells), and very small embryonic-like stem cells, all of which contribute to the high proliferative and self-renewing capacity. HUCBC have been shown to differentiate *in vitro* under the right conditions to neurons [36], oligodendrocytes [37, 38] and astrocytes [39]. They also contain precursors of mesenchymal stromal cells [40]. Autologous HUCBC have the major advantage of not requiring immunosuppression [12, 41], but this is balanced by the hematogenous origin of the cells and, thus, less clear commitment to neural differentiation.

Given the example of hypothermia [25] and erythropoietin [42], where the therapy was tested in large animal models before implementation in humans, the Study Group for Therapies for CP recommended in a recent review that fresh autologous HUCBC is of sufficiently low risk to justify early safety and feasibility studies, but more testing should be done in animals larger and more complex than rodents [5]. Compared to rodents, rabbits are perinatal brain developers, similar to humans, especially concerning motor development. Most human perinatal HI is global in origin, similar to the present model. The timing of the antenatal insult resembles that of an insult to the extreme premature human newborn, considering the maturation of oligodendrocytes [22].

Our results showed that intravenous infusion of HUCBC may not necessarily lead to HUCBC entry into the brain tissue in appreciable numbers, since MRI demonstrated only cells in the vascular system and DNA studies showed only a very small number in the brain. With the limitation of employed MRI methods we cannot exclude the possibility of small HUCBC engraftment not detectable by MRI, as reported in some studies [43], but the amount of such engrafted cells is likely to be small. The dilution of iron labeling due to cell division is probably not a reason for the absence of signal, as this has been shown to be a factor only several days after infusion [44]. The small numbers reaching the brain are probably due

to the trapping of most HUCBC in the lungs on a first pass through the lungs [45] and additionally in the kidneys and spleen [46].

The rate of intravascular infusion of HUCBC may be a factor as too rapid an infusion may be deleterious, as reflected in the trend towards increased mortality in the beginning of the study. Mortality probably occurred from the complication of pulmonary emboli that are known to form after intravenous injection of human mesenchymal stem cells in mice [47]. It is also possible that rapid infusion caused pulmonary edema due to sudden fluid overload. When we slowed down the injection speed to 1 ml/5 min in the second phase, there was no more mortality.

Among the various possible neuroprotective mechanisms, paracrine effects [48] such as anti-inflammatory and growth factor effects [49] have been suggested as possible candidates, although the evidence is scanty. There is a suggestion that following intraperitoneal injection in the Vannucci model in rodents, HUCBC home in on the damaged cortex, and this targeting depends upon a chemokine stromal-derived factor [49]. Following intravenous injection in a similar model with concurrent cyclosporine treatment, progenitor neurons and microglia were increased in the brain, but there was not much change in brain chemokines [48]. Chemokines were shown to be increased *in vitro* in HUCBC [48], but the direct link between these chemokines originating from HUCBC and beneficial effects has not been shown and is worthy of further study.

Entry into the brain may not be totally necessary for a neuroprotective effect. The endothelial cells in the neurovascular cells can play a major role in protecting neurons [50]. The speculation is that the HUCBC possibly modify the neurovascular cells to secrete more stimulatory and neurotrophic factors. Although human DNA PCR results did not exactly correlate with neurobehavioral improvement, the presence of human DNA would suggest the possibility that even a few HUCBC may also directly stimulate endogenous proliferation of stem cells or progenitor cells.

A significant improvement in motor scores with HUCBC treatment was observed as the primary outcome of the study. The findings of the swimming test have to be taken into context as it was designed to discover mild locomotor deficits in the absence of hypertonia (as a test mitigating the effect of gravity). We also used the swimming test to give us a clue about the regional nature of the injury in the brain – whether it was affecting the forelimb or hindlimb joints. Thus, for ex-

ample, the swimming test can tell us whether an intervention caused the animal to adopt a motor strategy similar to control animals or something different. An intervention such as HUCBC that does not achieve control curves is not necessarily deemed unsuccessful. It is known that human patients with CP adapt their internal locomotor models [51] in order to be able to ambulate even with quite severe restrictions in range of motion and hypertonia. The key issue is whether an intervention can restore the ability to walk, which was the case with HUCBC for CP rabbits. A decrease in hypertonia and an increase in range of motion, which were observed in our study, would certainly improve motor abilities in CP rabbits. Furthermore, a decrease in hypertonia would greatly facilitate rehabilitation therapy and the ability for motor learning. Also, the video clearly shows the rabbits at P5 and P11 walking even with postural deficits. This is observed in humans to some extent, where there can be severe postural changes in the peripheral joints but the ability to walk is still retained or restored after an insult.

The study raises several translational research questions. HUCBC from each delivery may be different in potency. Screening for the effectiveness of HUCBC could be first tested in rabbits before use in humans. There are three mitigating factors that allow us to give xenogeneic HUCBC in rabbit newborns. First, the experiments were meant to be proof-of-concept studies justifying the use of autologous HUCBC in human newborn infants. Second, the newborn immunological response is known to be not as well developed as in adults [52, 53]. This is also true for rabbits and rodents [54]. Third, all the previous studies were done in newborn rodents without much response. Although we did not specifically study the immunological response, we would expect an absence of a major immunological reaction.

At the moment, clinical trials are only using autologous HUCBC. There is presently an ongoing clinical trial of autologous HUCBC and hypothermia for term HIE, and preliminary studies show that it is safe and may be promising [13]. Allogeneic cells have been tested in one randomized trial, along with erythropoietin, and this study had promising results [41]. Ultimately, a future topic clinically would be the feasibility of allogeneic infusions because it would expand access to therapy with HUCBC in individuals with CP. However, before we reach that stage, fundamental issues must be addressed such as which of the following mechanism of actions is used by HUCBC: (1) paracrine signaling, (2) growth factors, (3) stimulation of endogenous neuro-

genesis or (4) whether engraftment or tissue integration is needed. The clinical protocols for the use of allogeneic cells in human infants with HIE would need careful study and optimization.

In conclusion, our study adds supportive evidence for the use of HUCBC in neonatal HI brain injury. Further studies testing various dosing and timing strategies, infusions before, during or after initiation of hypothermia and further investigating mechanisms are needed.

References

- Badawi N, Kurinczuk JJ, Keogh JM, Alessandri LM, O'Sullivan F, Burton PR, Pemberton PJ, Stanley FJ: Antepartum risk factors for newborn encephalopathy: The Western Australian case-control study. *BMJ* 1998;317:1549–1553.
- Badawi N, Kurinczuk JJ, Keogh JM, Alessandri LM, O'Sullivan F, Burton PR, Pemberton PJ, Stanley FJ: Intrapartum risk factors for newborn encephalopathy: the Western Australian case-control study. *BMJ* 1998;317:1554–1558.
- Edwards AD, Brocklehurst P, Gunn AJ, Halliday H, Juszczak E, Levene M, Strohm B, Thoresen M, Whitelaw A, Azzopardi D: Neurological outcomes at 18 months of age after moderate hypothermia for perinatal hypoxic ischaemic encephalopathy: synthesis and meta-analysis of trial data. *BMJ* 2010;340:c363.
- Gunn AJ, Bennet L: Brain cooling for preterm infants. *Clin Perinatol* 2008;35:735–748, vii.
- Bennet L, Tan S, Van den Heuvel J, Derrick M, Groenendaal F, van Bel F, Juul S, Back SA, Northington F, Robertson NJ, Mallard C, Gunn AJ: Cell therapy for neonatal hypoxia-ischemia and cerebral palsy. *Ann Neurol* 2012;71:589–600.
- Liao Y, Cotten M, Tan S, Kurtzberg J, Cairo MS: Rescuing the neonatal brain from hypoxic injury with autologous cord blood. *Bone Marrow Transplant* 2013;48:890–900.
- Pimentel-Coelho PM, Magalhaes ES, Lopes LM, deAzevedo LC, Santiago MF, Mendez-Otero R: Human cord blood transplantation in a neonatal rat model of hypoxic-ischemic brain damage: functional outcome related to neuroprotection in the striatum. *Stem Cells Dev* 2010;19:351–358.
- Meier C, Middelanis J, Wasielewski B, Neuhoft S, Roth-Haerer A, Gantert M, Dinse HR, Dermietzel R, Jensen A: Spastic paresis after perinatal brain damage in rats is reduced by human cord blood mononuclear cells. *Pediatr Res* 2006;59:244–249.
- Yasuhara T, Hara K, Maki M, Xu L, Yu G, Ali MM, Masuda T, Yu SJ, Bae EK, Hayashi T, Matsukawa N, Kaneko Y, Kuzmin-Nichols N, Ellovitch S, Cruz EL, Klasko SK, Sanberg CD, Sanberg PR, Borlongan CV: Mannitol facilitates neurotrophic factor up-regulation and behavioural recovery in neonatal hypoxic-ischaemic rats with human umbilical cord blood grafts. *J Cell Mol Med* 2010;14:914–921.
- Chen J, Sanberg PR, Li Y, Wang L, Lu M, Willing AE, Sanchez-Ramos J, Chopp M: Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. *Stroke* 2001;32:2682–2688.
- de Paula S, Vitola AS, Greggio S, de PD, Mello PB, Lubianca JM, Xavier LL, Fiori HH, Dacosta JC: 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. *Pediatr Res* 2009;65:631–635.
- Sun J, Allison J, McLaughlin C, Sledge L, Waters-Pick B, Wease S, Kurtzberg J: Differences in quality between privately and publicly banked umbilical cord blood units: a pilot study of autologous cord blood infusion in children with acquired neurologic disorders. *Transfusion* 2010;50:1980–1987.
- Cotten CM, Murtha AP, Goldberg RN, Grotte CA, Smith PB, Goldstein RF, Fisher KA, Gustafson KE, Waters-Pick B, Swamy GK, Rattray B, Tan S, Kurtzberg J: Feasibility of autologous cord blood cells for infants with hypoxic-ischemic encephalopathy. *J Pediatr* 2014;164:973–979.
- Derrick M, Drobyshevsky A, Ji X, Tan S: A model of cerebral palsy from fetal hypoxia-ischemia. *Stroke* 2007;38:731–735.
- Kurtzberg J, Lyerly AD, Sugarman J: Untying the Gordian knot: Policies, practices, and ethical issues related to banking of umbilical cord blood. *J Clin Invest* 2005;115:2592–2597.
- Derrick M, Luo NL, Bregman JC, Jilling T, Ji X, Fisher K, Gladson CL, Beardsley DJ, Murdoch G, Back SA, Tan S: Preterm fetal hypoxia-ischemia causes hypertonia and motor deficits in the neonatal rabbit: a model for human cerebral palsy? *J Neurosci* 2004;24:24–34.
- Comella CL, Leurgans S, Wu J, Stebbins GT, Chmura T: Rating scales for dystonia: a multicenter assessment. *Mov Disord* 2003;18:303–312.
- Derrick M, Drobyshevsky A, Ji X, Chen L, Yang Y, Ji H, Silverman RB, Tan S: Hypoxia-ischemia causes persistent movement deficits in a perinatal rabbit model of cerebral palsy: assessed by a new swim test. *Int J Dev Neurosci* 2009;27:549–557.
- Drobyshevsky A, Derrick M, Luo K, Zhang LQ, Wu YN, Takada SH, Yu L, Tan S: Near-term fetal hypoxia-ischemia in rabbits: MRI can predict muscle tone abnormalities and deep brain injury. *Stroke* 2012;43:2757–2763.
- Henning TD, Saborowski O, Golovko D, Boddington S, Bauer JS, Fu Y, Meier R, Pietsch H, Sennino B, McDonald DM, Daldrop-Link HE: Cell labeling with the positive MR contrast agent Gadofluorine M. *Eur Radiol* 2007;17:1226–1234.
- Matsuda H, Seo Y, Kakizaki E, Kozawa S, Muraoka E, Yukawa N: Identification of DNA of human origin based on amplification of human-specific mitochondrial cytochrome b region. *Forensic Sci Int* 2005;152:109–114.
- Buser JR, Segovia KN, Dean JM, Nelson K, Beardsley D, Gong X, Luo NL, Ren J, Wan Y, Riddle A, McClure MM, Ji X, Derrick M, Hohimer AR, Back SA, Tan S: Timing of appearance of late oligodendrocyte progenitors coincides with enhanced susceptibility of preterm rabbit cerebral white matter to hypoxia-ischemia. *J Cereb Blood Flow Metab* 2010;30:1053–1065.
- Lorek A, Takei Y, Cady EB, Wyatt JS, Penrice J, Edwards AD, Peebles D, Wylezinska M, Owen-Reece H, Kirkbride V: Delayed ('secondary') cerebral energy failure after acute hypoxia-ischemia in the newborn piglet: continuous 48-hour studies by phosphorus magnetic resonance spectroscopy. *Pediatr Res* 1994;36:699–706.
- Gunn AJ, Gunn TR, de Haan HH, Williams CE, Gluckman PD: Dramatic neuronal rescue with prolonged selective head cooling after ischemia in fetal lambs. *J Clin Invest* 1997;99:248–256.
- Mazur M, Miller RH, Robinson S: Postnatal erythropoietin treatment mitigates neural cell loss after systemic prenatal hypoxic-ischemic injury. *J Neurosurg Pediatr* 2010;6:206–221.
- Neubauer AP, Voss W, Wachtendorf M, Jungmann T: Erythropoietin improves neurodevelopmental outcome of extremely preterm infants. *Ann Neurol* 2010;67:657–666.

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- 27 Wu YW, Bauer LA, Ballard RA, Ferriero DM, Glidden DV, Mayock DE, Chang T, Durand DJ, Song D, Bonifacio SL, Gonzalez FF, Glass HC, Juul SE: Erythropoietin for neuroprotection in neonatal encephalopathy: safety and pharmacokinetics. *Pediatrics* 2012;130:683–691.
- 28 Benders MJ, van der Aa NE, Roks M, van Straaten HL, Isgum I, Viergever MA, Groenendaal F, de Vries LS, van Bel F: Feasibility and safety of erythropoietin for neuroprotection after perinatal arterial ischemic stroke. *J Pediatr* 2014;164:481–486.e1–2.
- 29 Dingley J, Tooley J, Liu X, Scull-Brown E, Elstad M, Chakkarapani E, Sabir H, Thoresen M: Xenon ventilation during therapeutic hypothermia in neonatal encephalopathy: a feasibility study. *Pediatrics* 2014;135:809–818.
- 30 Bae SH, Lee HS, Kang MS, Strupp BJ, Chopp M, Moon J: The levels of pro-inflammatory factors are significantly decreased in cerebral palsy patients following an allogeneic umbilical cord blood cell transplant. *Int J Stem Cells* 2012;5:31–38.
- 31 Min K, Song J, Kang JY, Ko J, Ryu JS, Kang MS, Jang SJ, Kim SH, Oh D, Kim MK, Kim SS, Kim M: Umbilical cord blood therapy potentiated with erythropoietin for children with cerebral palsy: a double-blind, randomized, placebo-controlled trial. *Stem Cells* 2013;31:581–591.
- 32 Geissler M, Dinse HR, Neuhoﬀ S, Kreikemeier K, Meier C: Human umbilical cord blood cells restore brain damage induced changes in rat somatosensory cortex. *PloS One* 2011;6:e20194.
- 33 Wasielewski B, Jensen A, Roth-Harer A, Dermietzel R, Meier C: Neuroglial activation and Cx43 expression are reduced upon transplantation of human umbilical cord blood cells after perinatal hypoxic-ischemic injury. *Brain Res* 2012;1487:39–53.
- 34 Yasuhara T, Matsukawa N, Yu G, Xu L, Mays RW, Kovach J, Deans RJ, Hess DC, Carroll JE, Borlongan CV: 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;15:231–238.
- 35 de Paula S, Greggio S, Marinowicz DR, Machado DC, Dacosta JC: The dose-response effect of acute intravenous transplantation of human umbilical cord blood cells on brain damage and spatial memory deficits in neonatal hypoxia-ischemia. *Neuroscience* 2012;210:431–441.
- 36 Sanchez-Ramos JR, Song S, Kamath SG, Zigo-va T, Willing A, Cardozo-Pelaez F, Stedeford T, Chopp M, Sanberg PR: Expression of neural markers in human umbilical cord blood. *Exp Neurol* 2001;171:109–115.
- 37 Tracy E, Aldrink J, Panosian J, Beam D, Thacker J, Reese M, Kurtzberg J: Isolation of oligodendrocyte-like cells from human umbilical cord blood. *Cytotherapy* 2008;10:518–525.
- 38 Tracy ET, Zhang CY, Gentry T, Shoulars KW, Kurtzberg J: Isolation and expansion of oligodendrocyte progenitor cells from cryopreserved human umbilical cord blood. *Cytotherapy* 2011;13:722–729.
- 39 Buzanska L, Machaj EK, Zablocka B, Pojda Z, Domanska-Janik K: Human cord blood-derived cells attain neuronal and glial features in vitro. *J Cell Sci* 2002;115:2131–2138.
- 40 Natarajan G, Pappas A, Shankaran S, Laptok AR, Walsh M, McDonald SA, Ehrenkranz RA, Tyson JE, Goldberg RN, Bara R, Higgins RD, Das A, Munoz B: Effect of inborn vs. outborn delivery on neurodevelopmental outcomes in infants with hypoxic-ischemic encephalopathy: secondary analyses of the NICHD whole-body cooling trial. *Pediatr Res* 2012;72:414–419.
- 41 Escobar ML, Poe MD, Provenzale JM, Richards KC, Allison J, Wood S, Wenger DA, Pietryga D, Wall D, Champagne M, Morse R, Krivit W, Kurtzberg J: Transplantation of umbilical-cord blood in babies with infantile Krabbe's disease. *N Engl J Med* 2005;352:2069–2081.
- 42 Juul SE, Aylward E, Richards T, McPherson RJ, Kuratani J, Burbacher TM: Prenatal cord clamping in newborn *Macaca nemestrina*: a model of perinatal asphyxia. *Dev Neurosci* 2007;29:311–320.
- 43 Tajiri N, Acosta SA, Shahaduzzaman M, Ishikawa H, Shinozuka K, Pabon M, Hernandez-Ontiveros D, Kim DW, Metcalf C, Staples M, Dailey T, Vasconcellos J, Franyuti G, Gould L, Patel N, Cooper D, Kaneko Y, Borlongan CV, Bickford PC: Intravenous transplants of human adipose-derived stem cell protect the brain from traumatic brain injury-induced neurodegeneration and motor and cognitive impairments: cell graft biodistribution and soluble factors in young and aged rats. *J Neurosci* 2014;34:313–326.
- 44 Magnitsky S, Watson DJ, Walton RM, Pickup S, Bulte JWM, Wolfe JH, Poptani H: In vivo and ex vivo MRI detection of localized and disseminated neural stem cell grafts in the mouse brain. *Neuroimage* 2005;26:744–754.
- 45 Fischer UM, Harting MT, Jimenez F, Monzon-Posadas WO, Xue H, Savitz SI, Laine GA, Cox CS Jr: Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. *Stem Cells Dev* 2009;18:683–692.
- 46 Borlongan CV, Hadman M, Davis Sanberg C, Sanberg PR: Central nervous system entry of peripherally injected umbilical cord blood cells is not required for neuroprotection in stroke. *Stroke* 2004;35:2385–2389.
- 47 Lee RH, Pulin AA, Seo MJ, Kota DJ, Ylostalo J, Larson BL, Semprun-Prieto L, Delafontaine P, Prockop DJ: Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. *Cell Stem Cell* 2009;5:54–63.
- 48 Bae SH, Kong TH, Lee HS, Kim KS, Hong KS, Chopp M, Kang MS, Moon J: Long-lasting paracrine effects of human cord blood cells on damaged neocortex in an animal model of cerebral palsy. *Cell Transplant* 2012;21:2497–2515.
- 49 Rosenkranz K, Meier C: Umbilical cord blood cell transplantation after brain ischemia – from recovery of function to cellular mechanisms. *Ann Anat* 2011;193:371–379.
- 50 Guo S, Kim WJ, Lok J, Lee SR, Besancon E, Luo BH, Stins MF, Wang X, Dedhar S, Lo EH: Neuroprotection via matrix-trophic coupling between cerebral endothelial cells and neurons. *Proc Natl Acad Sci U S A* 2008;105:7582–7587.
- 51 Iosa M, Zoccolillo L, Montesi M, Morelli D, Paolucci S, Fusco A: The brain's sense of walking: a study on the intertwine between locomotor imagery and internal locomotor models in healthy adults, typically developing children and children with cerebral palsy. *Front Hum Neurosci* 2014;8:859.
- 52 Ygberg S, Nilsson A: The developing immune system – from foetus to toddler. *Acta Paediatr* 2012;101:120–127.
- 53 Nikonova MF, Maiskii IN, Pokrovskaya TA: Comparative study of the immune response of newborn and adult animals to the administration of tumor extracts (in Russian). *Biull Eksp Biol Med* 1975;79:85–88.
- 54 Smith RT, Bridges RA: Immunological unresponsiveness in rabbits produced by neonatal injection of defined antigens. *J Exp Med* 1958;108:227–250.