Term vs. preterm cord blood cells for the prevention of preterm brain injury

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BACKGROUND: White matter brain injury in preterm infants can induce neurodevelopmental deficits. Umbilical cord blood (UCB) cells demonstrate neuroprotective properties, but it is unknown whether cells obtained from preterm cord blood (PCB) vs. term cord blood (TCB) have similar efficacy. This study compared the ability of TCB vs. PCB cells to reduce white matter injury in preterm fetal sheep.

METHODS: Hypoxia–ischemia (HI) was induced in fetal sheep (0.7 gestation) by 25 min umbilical cord occlusion. Allogeneic UCB cells from term or preterm sheep, or saline, were administered to the fetus at 12 h after HI. The fetal brain was collected at 10-day post HI for assessment of white matter neuropathology.

RESULTS: HI ($n=7$) induced cell death and microglial activation and reduced total oligodendrocytes and CNPase +myelin protein in the periventricular white matter and internal capsule when compared with control ($n=10$). Administration of TCB or PCB cells normalized white matter density and reduced cell death and microgliosis ($P<0.05$). PCB prevented upregulation of plasma tumor necrosis factor (TNF)- α , whereas TCB increased anti-inflammatory interleukin (IL)-10 ($P<0.05$). TCB, but not PCB, reduced circulating oxidative stress.

CONCLUSIONS: TCB and PCB cells reduced preterm HI-induced white matter injury, primarily via anti-inflammatory actions. The secondary mechanisms of neuroprotection appear different following TCB vs. PCB administration.

subsequent clinical trials are now underway for term infant or childhood neuropathologies (6). Recent studies also show that UCB treatment is effective in HI-induced preterm brain injury (7,8). In ovine studies, we have also shown that early administration of autologous or allogeneic UCB cells ameliorate gray and white matter brain damage in term and preterm sheep (9,10). However, in all studies to date that have examined the neuroprotective effects of UCB, the cells were obtained from term pregnancies (2–10). This raises the important question—do UCB cells from preterm birth also demonstrate protective benefits, particularly for the preterm brain? This is critical, given that infants born preterm demonstrate the highest incidence of cerebral palsy (11), and could benefit from stem-cell therapy after birth. A recent meta-analysis demonstrates that stem-cell therapies show excellent promise for treating cerebral palsy (12), but it is also likely that treating cerebral palsy as soon as possible after birth will hold the greatest neurotherapeutic benefit (9,13).

It is known that the neuroprotective actions of UCB are mediated by cell composition within the mononuclear cell (MNC) fraction. However, the cellular composition of term and preterm human UCB is quite different (14–19). The MNC fraction of UCB contains three major types of stem/progenitor cells: hematopoietic, endothelial, and mesenchymal stem/progenitor cells (HSCs, EPCs, and MSCs, respectively), along with lymphocytes and monocytes (6). It is unclear how each of these cell types contributes to neuroprotection; however, it is certain that the relative proportion and developmental profile of these stem/progenitor cells change within the fetal circulation as pregnancy progresses (14–20). We hypothesized that the neuroprotective effect of preterm cord blood (PCB) differs from term cord blood (TCB), given that cellular composition in UCB varies with gestation. Therefore, the present study compared the efficacy of allogeneic PCB vs. TCB cells in an established fetal sheep model of preterm WMI induced by acute severe hypoxia–ischemia (HI).

Very preterm birth, before 32 weeks of gestation, has significant adverse outcomes for the developing brain, leading to lifelong neurodevelopmental deficits (1). White matter injury (WMI) is the predominant form of brain injury present in about half of infants born very preterm; 25–50% of these infants will develop cognitive or behavioral deficits and up to 15% will develop cerebral palsy (1). Currently, there are no therapies for reducing WMI in preterm infants.

Preclinical animal studies demonstrate significant neuroprotective benefits of human umbilical cord blood (UCB) cells in rodent models of term HI brain injury (2–6), and

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METHODS

Animals and Surgery

The animal studies were approved by the Monash University Animal Ethics Committee (MMCA/2013/17). Surgery was performed on pregnant Merino-Border Leicester ewes at 97.5 ± 0.2 -day gestation (term = 147 days). Under general anesthesia, the fetus was exteriorized for insertion of fetal polyvinyl catheters (0.8 mm inner diameter and 1.2 mm outer diameter, Dural Plastics, Australia) into a fetal femoral artery and vein. An inflatable balloon occluder (16HD, *In Vivo* Medical) was placed around the umbilical cord, and an amniotic catheter was inserted. Catheters were exteriorized through the maternal flank. A maternal jugular vein catheter was implanted for antibiotic administration (500 mg engemycin, 1 g ampicillin) on the day of surgery and for 3 days after. Fetal catheters were maintained by an infusion of heparinized saline and, ~5 days after surgery, recording of fetal heart rate and mean arterial pressure was commenced (Power Lab, ADInstruments, Castle Hill, Australia).

Experimental Protocol

At 102.3 ± 0.2 -day gestation, the animals were randomized into one of the following four groups: (i) control (sham-occlusion+saline, $n = 10$); (ii) HI (HI+saline, $n = 7$); (iii) HI+TCB (HI+50 million TCB cells at 12 h, $n = 6$); and (iv) HI+PCB (HI+50 million PCB cells at 12 h, $n = 6$). All groups comprised single fetuses, with the exception of the control and HI groups (with twins); there was no difference in sex distribution (Table 1). In our previous study (9), we observed no differences between sham-control animals with and without term UCB treatment (control+saline vs. control+TCB), and, therefore, we did not include additional control+TCB or control+PCB fetuses in this study. HI was induced by complete umbilical cord occlusion, in which the balloon occluder was filled with 2.0–2.5 ml sterile water for 23–25 min. The occlusion was discontinued after 25 min, or sooner if the occlusion was >23 min duration and the mean arterial pressure was decreased to <8 mm Hg. Fetal arterial blood samples were collected 24 h before, during, and 6, 12, 24, 48, 72, 120, and 240 h after HI for determining blood gas parameters (ABL 800, Radiometer, Copenhagen, Denmark). Malondialdehyde (MDA) and cytokine concentrations were also assessed in selected plasma samples, stored at -80°C until assays were performed.

Collection, Processing, and Transplantation of UCB

Ovine UCB was collected into heparinized syringes from cesarean-section delivery of term (141 days) or preterm lambs (112 days), which were separate cohorts to the animals used in this study. The blood was centrifuged at 3,100 r.p.m. for 12 min at room temperature, without brake, and the buffy coat layer was collected with the excess red blood cells removed using red blood cell lysis

buffer (Sigma-Aldrich, Melbourne, Victoria, Australia). The cells were re-suspended in fetal bovine serum with 10% dimethyl sulfoxide (Merck, Darmstadt, Germany), and cryopreserved in liquid nitrogen. TCB or PCB cells were thawed and labeled with carboxyfluorescein succinimidyl ester to facilitate cell tracking within the brain (9). Fifty million viable cells were re-suspended in 2.5 ml sterile saline and administered to the fetus (over 3 min) via the femoral vein 12 h after HI. On average, one dose of cells (~50 million) from each preterm UCB collection and one to two doses from each term UCB collection (~50–100 million cells) could be obtained. When cell number obtained was insufficient for 50 million total cells, cells were supplemented from another collection.

Tissue Collection and Processing

At 10 days after HI, the ewe and the fetus were killed with sodium pentobarbital IV. The fetal body and brain weights were recorded. The right cerebral hemisphere was cut transversely and immersion-fixed in 10% formalin for 5 days. Paraffin-embedded 10 μm coronal forebrain sections at the level of the head of the caudate nucleus (CN) were mounted on slides. Brain regions of interest were the subventricular zone (SVZ), periventricular white matter (PVWM), internal capsule (IC), and CN.

Immunohistochemistry

Immunohistochemistry on sections of the fetal brain was undertaken as we have described previously (9). Briefly, rabbit polyclonal oligodendrocyte transcription factor 2 (Olig2, 1:1,000; Millipore, Melbourne, Victoria, Australia) and mouse-anti-human 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase, 1:200; Sigma Chemical, Melbourne, Victoria, Australia) antibodies were used to identify oligodendroglial lineage cells and myelinated axons, respectively. Proliferating cells were visualized with rabbit anti-human Ki-67 antibody (1:100; Dako, Santa Clara, CA, USA). Activated microglia were identified using rabbit anti-ionized calcium-binding adaptor molecule 1 (Iba-1, 1:500; Wako, Osaka, Japan). Cell death was identified using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Promega, Melbourne, Victoria, Australia). The slides were imaged at $\times 400$ magnification under light microscopy (Olympus BX-41, Melbourne, Victoria, Australia). The number of Olig2-, Ki-67-, TUNEL-, and Iba-1-immunoreactive cells per field of view were counted. The percentage of CNPase-positive area, indicative of the combined density of immature and mature oligodendrocytes and myelin protein covering of axons, was quantified by means of ImageJ (NIH, Bethesda, MD, USA). Immunohistochemical outcomes were assessed in two sections per animal and three fields of view per region on each non-adjacent sections, with the results averaged per animal and then across animals in each group.

Double-label immunohistochemistry was used for oligodendrocyte proliferation as previously described (9). Sections were incubated with mouse anti-Olig2 (1:1,000; Millipore) and rabbit anti-Ki-67. Immunoreactivity was visualized with Alexa Fluor 594 goat anti-mouse (Red, 1/1,000; Molecular Probes) and Alexa Fluor 488 goat anti-rabbit (Green, 1/1,000; Molecular Probes) and viewed with fluorescence microscopy (Olympus BX-41).

MDA Assay

Lipid peroxidation was assessed via the thiobarbituric acid reactive substances, TBARS method of measuring MDA, in plasma at 6, 12, 24, 48, and 72 h ((ref. 9)), following the manufacturer's protocol (Cayman Chemical, Ann Arbor, MI).

Cytokine Assay

Plasma pro-inflammatory tumor necrosis factor (TNF)- α and anti-inflammatory interleukin (IL)-10 concentrations were analyzed against recombinant cytokines on day 1 and day 10 after HI using standard capture enzyme-linked immunosorbent assay for ovine-specific monoclonal antibodies, as previously described (9).

Table 1. Fetal characteristics

Variables	Control, ($n = 10$)	HI, ($n = 7$)	HI+TCB, ($n = 6$)	HI+PCB, ($n = 6$)
Female, n (%)	7 (70)	3 (43)	3 (50)	3 (50)
Twin, n (%)	6 (60)	2 (29)	0 (0)*	0 (0)*
UCO duration, min	0	$24.4 \pm 0.3^{**}$	$24.2 \pm 0.4^{**}$	$24.3 \pm 0.3^{**}$
Brain weight, g	34.5 ± 0.7	$30.5 \pm 0.9^{**}$	$29.8 \pm 1.0^{**}$	$28.2 \pm 0.9^{**}$
Body weight, kg	2.0 ± 0.1	2.4 ± 0.1	2.1 ± 0.2	2.1 ± 0.2
Brain/body weight, g/kg	17.6 ± 0.7	$13.6 \pm 0.9^{**}$	$14.4 \pm 0.9^{**}$	$14.0 \pm 0.9^{**}$

HI, hypoxia-ischemia; PCB, preterm cord blood; TCB, term cord blood; UCO, umbilical cord occlusion.

Mean (%) or mean $\pm$ SE are presented for each group. One-way ANOVA and *post hoc* Bonferroni tests were carried out on comparison between groups. * $P < 0.05$ vs. control. ** $P < 0.01$ vs. control.

Data Analysis

Animals that completed the whole experimental course were included in data analyses. All assessments were conducted on coded slides or samples, with the examiner blinded to the group. Data are presented as mean $\pm$ SE. Statistical analysis was performed with JMP (version 11, SAS, Cary, NC). Differences between three or more groups were analyzed by a two-way ANOVA (histology data) or one-way ANOVA (fetal weight, arterial blood gas, physiology, plasma cytokine, and MDA levels), followed by the Bonferroni *post hoc* test when a significant difference was found. Differences between the two groups were analyzed with either the Wilcoxon or Fisher's test. Regression analysis was performed for estimating the relationships among variables. $P < 0.05$ was considered statistically significant.

RESULTS

We have previously reported data for animals that received TCB from this cohort (9). The overall fetal survival rate for the study was 85%, with no significant differences in mortality between experimental groups. Fetal body weight was not different between groups. Brain weight and brain/body weight ratios were reduced in all HI groups (HI, HI+TCB, and HI+PCB), when compared with that in control ($P < 0.01$, Table 1).

Physiological Measures

HI caused severe hypotension, bradycardia, acidosis, and hypoxia when compared with the controls (Figure 1), $P < 0.01$. There was no difference in the duration of the HI insult, or any physiological parameter, measured immediately after HI, between the three HI groups. Following cell administration, the mean arterial pressure in HI+TCB and HI+PCB fetuses was increased for 24 h; however, this was significant only in HI+TCB fetuses ($P = 0.04$). No intergroup differences were observed.

Brain Histopathology

Ten days following fluorescent UCB administration, the cells were found within some, but not all brains (3/7 HI+TCB, 2/6 HI+PCB, Figure 2a). Where present, the cells were observed across white and gray matter, but more commonly in white matter (65% of cells observed).

White Matter Injury

Following HI, the density of Olig2+ oligodendrocytes was reduced by 24, 30, and 41% within the SVZ, PVWM, and IC regions when compared with the density in the control fetuses ($P = 0.7$, $P = 0.1$, and $P = 0.06$, respectively). Compared with HI animals, oligodendrocyte density was significantly improved toward control levels within the IC ($P = 0.02$) in HI+TCB fetuses, and in the PVWM ($P = 0.02$) in HI+PCB fetuses (Figure 3B). HI caused a reduction in CNPase+ oligodendrocytes and myelin density, decreased 22% in PVWM and 27% in IC ($P = 0.01$), when compared with that in control. Both TCB and PCB administration prevented the reduction of CNPase+ protein density (Figure 3B).

Microglial Activation

HI induced a significant increase in microglial cells within the PVWM and IC ($P < 0.05$) regions, when compared with those regions in control (Figure 4). In HI brains, the microglia demonstrated an activated morphology, with an enlarged amoeboid cell body and loss of processes (Figure 4b). In contrast, microglial cell density was similar in control, HI+TCB, and HI+PCB brains, and showed characteristic morphology of ramified resting microglia, with small cell bodies and long branching processes (Figure 4a,c,d). While both TCB and PCB reduced microglial density, TCB administration produced a more pronounced anti-inflammatory effect when compared with PCB administration, with microglial cell counts in line with control values in TCB-treated animals (Figure 4) and significant reduction in more regions when comparing HI and HI+TCB (PVWM: $P = 0.04$; IC: $P < 0.01$) groups vs. with HI and HI+PCB (PVWM: NS; IC: $P = 0.03$) groups. Regression analysis revealed a strong negative correlation between the number of activated microglia and oligodendrocytes in PVWM ($R^2 = 0.62$, $P < 0.001$) and IC ($R^2 = 0.68$, $P < 0.001$).

Cell Death and Cell Proliferation

HI induced TUNEL+ cell death within the IC ($P < 0.01$, Figure 5A,a,b), and this was prevented with TCB or PCB administration (Figure 5A,a,c,d). Regression analyses showed no significant correlation between Olig2+ and TUNEL+ cell densities in any regions examined.

Cell proliferation was increased within the SVZ in HI fetuses when compared with that in controls ($P = 0.03$; Figure 5B,f). Double-label immunohistochemistry demonstrated colocalization of Ki-67 and Olig2, indicative of oligodendrocyte proliferation within the SVZ following acute HI (Figure 2b). There was no difference in proliferating cell number between control and HI+TCB and HI+PCB fetuses (Figure 5B,e,g,h). PCB administration reduced aberrant proliferation to a greater extent than TCB in the SVZ region (HI+PCB vs. HI; $P < 0.01$).

Fetal Plasma Oxidative Stress and Cytokine Analysis

Fetal plasma MDA, an established marker of oxidative stress, was increased at 6–12 h in HI animals and remained above baseline for 48 h (Table 2a). The administration of PCB did not alter the MDA profile in response to HI. In contrast, TCB treatment at 12 h post HI demonstrated an antioxidant benefit, reducing plasma MDA levels from their 12 h peak such that MDA was not different to basal levels by 24 h; MDA was reduced in HI+TCB when compared with the MDA in HI at 48 h ($P = 0.03$). HI induced an inflammatory response shown by an increase in plasma TNF- α , as well as an endogenous anti-inflammatory response, revealed by an increase in plasma IL-10 observed at 24 h, but not sustained to 10 days (Table 2b,c, respectively). PCB administration moderated the TNF- α response at 24 h and resulted in decreased plasma TNF- α in HI+PCB when compared with the plasma TNF- α in HI+TCB ($P = 0.04$, Table 2b). In contrast,

Figure 1. Physiological measurements during HI. HI, induced by umbilical cord occlusion, is shown by the gray columns and UCB cell administration is shown by vertical lines. HI resulted in severe hypotension (a) and bradycardia (b), markedly reduced fetal arterial pH (c), PaO₂ (d), and base excess (f), and increased PaCO₂ (g) and lactate (e) levels when compared with that in control groups ($P < 0.01$). Data are presented as mean $\pm$ SEM. BE, base excess; FHR, fetal heart rate; HI, hypoxia–ischemia; MAP, mean arterial pressure; PCB, preterm cord blood; TCB, term cord blood; UCB, umbilical cord blood. * $P < 0.05$ vs. control, ** $P < 0.01$ within group comparisons vs. baseline.

Figure 2. Fluorescent microscopy. (a) A representative microphotograph of carboxyfluorescein succinimidyl ester-labeled cells detected in the periventricular white matter of the preterm brain. (b) Double-label fluorescent immunostaining of Olig2 (red) and Ki-67 (green) with merged images in the SVZ of the brain in an HI fetus. White arrow denotes an example of a cell with dual signal. Bar = 50 μ m. HI, hypoxia–ischemia; SVZ, subventricular zone.

TCB administration upregulated IL-10 at 10 days with elevated plasma IL-10 concentration in HI+TCB fetuses when compared with HI fetuses ($P = 0.04$); no difference was found between HI+PCB and HI fetuses (Table 2c).

DISCUSSION

White matter brain injury in infants born preterm is the principal neuropathology underlying neurodevelopmental deficits including cerebral palsy (1). This study is the first to

Figure 3. Effects of TCB and PCB cell on white matter cells following HI. The graphs of the number of Olig2+ oligodendrocyte (A) and density of oligodendrocyte and myelinating axons (CNPase+, B), and the representative photomicrograph images in control (a, e), HI (b, f), HI+TCB (c, g), and HI+PCB (d, h) groups. SVZ, PVWM, IC, and CN. Data are mean $\pm$ SEM and were analyzed using ANOVA followed by the Bonferroni *post hoc* test for individual brain regions. * $P < 0.05$, ** $P < 0.01$. Bar = 50 μ m. CN, caudate nucleus; HI, hypoxia-ischemia; IC, internal capsule; PCB, preterm cord blood; PVWM, periventricular white matter; SVZ, subventricular zone; TCB, term cord blood.

compare the neuroprotective effects of allogeneic preterm with TCB cells for preterm WMI. We show that administration of either PCB or TCB significantly reduced oligodendrocyte and CNPase+ myelin loss in response to HI, and to a similar extent. Interestingly, while both PCB and TCB preserved white matter structure, PCB and TCB may act differently. Both TCB and PCB mediated the neuroinflammatory response to HI; however, TCB also demonstrated a strong antioxidant ability, whereas PCB reduced cell proliferation within the brain and reduced acute systemic inflammation. These results support early administration of allogeneic PCB or TCB to reduce WMI, and further show that the systemic and cerebral anti-inflammatory actions of cord blood therapy is central to their neuroprotective benefit.

HI induced by umbilical cord occlusion for 25 min caused WMI at 10 days post insult, with oligodendrocyte and myelin reduction across the PVWM and IC, as evidenced as a decrease in Olig2+ and CNPase+ proteins. It is well described that neuroinflammation, particularly via microglial activation, is a principal cause of preterm WMI (20,21). Supporting this, we show a strong correlation between increasing activated microglia density and oligodendrocyte loss, as observed for preterm WMI induced by fetal inflammation (9). Both PCB and TCB demonstrated an excellent ability to reduce neuroinflammation, reducing the number of activated

microglia and maintaining microglial phenotype in a resting state. HI also increased cell proliferation within the SVZ, a response recognized as an aberrant production of oligodendrocytes with limited functionality (22). Both PCB and TCB significantly reduced total oligodendrocyte cell death and restored CNPase+ oligodendrocyte and myelin density. However, PCB treatment normalized SVZ cell proliferation, whereas TCB did not, indicating that PCB may be more effective at restoring the balance between cell death and proliferation.

In this study, TCB cell administration reduced circulating markers of oxidative stress following HI. *In vitro* evidence has shown that stem cells possess antioxidant capacity (23,24), with more immature and younger cells demonstrating a stronger antioxidant ability (25). The current study, the first *in vivo* assessment of PCB vs. TCB, suggests that TCB has a greater antioxidant capacity than PCB. However, we did observe a large variability and higher baseline values in MDA concentration within HI+PCB fetuses (Table 2a), which might be due to the severity of the initial injury and somewhat limits interpretation of these data. Similarly, TCB, but not PCB, induced an increase in systemic anti-inflammatory IL-10 concentration following HI (Table 2c). IL-10 is primarily produced by monocytes, and to a lesser extent by lymphocytes (26), and an increased proportion of monocytes

Figure 4. Effects of TCB and PCB cell following HI on the density of Iba-1+ activated microglia. The photomicrograph images show that Iba-1+ inflammatory cells in control brains exhibited the characteristics of ramified (resting) microglia, with small cell bodies and long branching processes (a). However, the cells in HI group show loss of microglial branching and transformation into rounded macrophages (b). The changes are to a large extent reversed following TCB and PCB cell administration at 12 h (c,d). SVZ, PVWM, IC, and CN. Data are mean $\pm$ SEM, and were analyzed using ANOVA followed by the Bonferroni *post hoc* test for individual brain regions. * $P < 0.05$, ** $P < 0.01$. Bar = 50 μ m. CN, caudate nucleus; HI, hypoxia-ischemia; IC, internal capsule; PCB, preterm cord blood; PVWM, periventricular white matter; SVZ, subventricular zone; TCB, term cord blood.

in TCB might account for this result (27). In contrast, only PCB moderated the pro-inflammatory TNF- α response following HI (Table 2c). Suppressing pro-inflammatory cytokines (e.g., TNF- α and IL-1) is one of the modes of action of MSCs (28), and increased MSCs in PCB are likely to be a principal reason for this systemic anti-inflammatory effect (15). Although the cytokine data show wide variability, results indicate that the neuroprotective actions demonstrated by PCB and TCB may be mediated, at least in part, by different secondary mechanisms.

We observed an acute response of blood pressure elevation by allogeneic UCB cell administration, albeit this was statistically significant in the TCB-treated group only. In our previous study, we examined the neuroprotective effects of autologous UCB cells in term HIE lambs and did not

observe any cardiovascular effects of the cells (10). That allogeneic UCB cell therapy causes a systemic response is not entirely surprising, considering that the administration of human (allogeneic) UCB to children with cerebral palsy induced an immune/inflammatory reaction, and children who showed the greatest response also demonstrated the most improved functional outcomes (29). We utilized allogeneic rather than autologous UCB administration for the following two reasons: (i) to avoid possible harm to preterm fetuses by withdrawing a large volume of UCB in this *in utero* model and (ii) to compare PCB with TCB in the same immunological (allogeneic) setting. Our findings also have implications for the use of autologous preterm UCB for neuroprotection. Unlike hypoxic-ischemic encephalopathy at term, the timely diagnosis of preterm WMI is extremely challenging (6). In

Figure 5. Effects of TCB cell and PCB cell on cell death and cell proliferation following HI. The graphs of the density of TUNEL+ cell death (A) and Ki-67+ proliferating cells (B), and the representative photomicrograph images in control (a,e), HI (b,f), HI+TCB (c,g), and HI+PCB (d,h) groups. Black arrows show examples of TUNEL-positive cells (b). Data are mean $\pm$ SEM, and were analyzed using ANOVA followed by the Bonferroni *post hoc* test for individual brain regions. * $P < 0.05$, ** $P < 0.01$. Bar = 50 μ m. HI, hypoxia–ischemia; PCB, preterm cord blood; TCB, term cord blood; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Table 2a. MDA (μ M)

	Control	HI	HI+TCB	HI+PCB
Base	9.7 $\pm$ 2.3	9.6 $\pm$ 2.3	8.0 $\pm$ 0.9	12.9 $\pm$ 3.4
6 h	6.5 $\pm$ 2.0	11.2 $\pm$ 2.0 ^a	12.5 $\pm$ 1.4 ^a	18.2 $\pm$ 9.6
12 h	9.0 $\pm$ 2.5	17.8 $\pm$ 2.5 ^a	15.5 $\pm$ 3.1	16.6 $\pm$ 7.5
24 h	6.7 $\pm$ 1.7	17.1 $\pm$ 1.7 ^a	10.6 $\pm$ 2.1	20.6 $\pm$ 9.5
48 h	9.4 $\pm$ 2.3	18.1 $\pm$ 2.3 ^a	10.2 $\pm$ 2.4 ^b	17.1 $\pm$ 3.0
72 h	8.3 $\pm$ 4.7	11.7 $\pm$ 4.7	9.1 $\pm$ 4.2	13.7 $\pm$ 3.3

HI, hypoxia–ischemia; MDA, malondialdehyde; PCB, preterm cord blood; TCB, term cord blood.
^a $P < 0.05$ vs. control. ^b $P < 0.05$ vs. HI.

this study we administered cells at 12 h after HI, as the therapeutic window of UCB therapy for optimal neuroprotection is up to 3 days after HI (9,13,30). The collection and administration of autologous UCB soon after birth may provide increased benefit in high-risk very preterm or extremely preterm infants. In this situation, autologous cells are readily available, free from immunological side effects, and

potentially have a cellular composition that is developmentally appropriate with a high relative proportion of stem/progenitor cells. In contrast, UCB banks predominantly collect from full-term births. The volume of available UCB for collection is correlated with birth weight (i.e., 18–23 ml/kg) (31,32), and therefore, whereas the volume of UCB for collection is low at preterm birth, the total number of cells would be sufficient for treatment of a preterm infant (62 $\pm$ 31 ml, 3.6×10^8 cells at 25–33 weeks vs. 102 $\pm$ 30 ml, 11.3×10^8 cells at term (14)). It should, however, be considered that, although volume and cell number are feasible for PCB transplantation in preterm infants (19,31), preterm birth is frequently associated with obstetric complications (e.g., chorioamnionitis and intrauterine growth restriction), which may alter the volume and cell composition of UCB (33,34), and may prohibit autologous PCB administration.

It is likely that the differential effects of PCB vs. TCB are mediated via individual cell composition. HSCs (CD34+, CD45+) give rise to multiple blood lineages and comprise the largest progenitor cell population in human UCB (~0.5% of

Table 2b. TNF- α (pg/ml)

	Control	HI	HI+TCB	HI+PCB
24 h	2.9 $\pm$ 0.5	4.0 $\pm$ 1.2	5.0 $\pm$ 0.9	1.6 $\pm$ 0.7 ^a
10d	3.1 $\pm$ 1.0	2.7 $\pm$ 2.7	2.6 $\pm$ 1.0	1.3 $\pm$ 0.8

d, day; HI, hypoxia-ischemia; PCB, preterm cord blood; TCB, term cord blood; TNF, tumor necrosis factor.

^a $P < 0.05$ vs. HI+TCB.

Table 2c. IL-10 (BU/ml)

	Control	HI	HI+TCB	HI+PCB
24 h	0.7 $\pm$ 0.7	7.0 $\pm$ 3.5	5.4 $\pm$ 4.1	2.6 $\pm$ 1.4
10d	13.5 $\pm$ 2.0	11.3 $\pm$ 1.4	16.0 $\pm$ 1.4 ^a	12.0 $\pm$ 1.4

d, day; HI, hypoxia-ischemia; IL, interleukin; PCB, preterm cord blood; TCB, term cord blood.

^a $P < 0.05$ vs. HI.

MNCs) (14). EPCs have a potent ability for neovascularization (35), accounting for 1–2% of the HSC-containing CD34+ cell fraction in UCB. MSCs (CD34–, CD45–, and plastic adherent) are multipotent stem cells, capable of differentiating into multiple lineages, and have immunomodulatory/anti-inflammatory properties and high proliferation capacity (28,36). The majority of studies investigating UCB for perinatal brain injury have administered xenogeneic (human) whole MNCs (2–6); however, UCB-CD34+ cells, UCB-MSCs, as well as monocytes and lymphocytes in UCB may all contribute to neuroprotection (37–40). The relative proportion and developmental profile of these stem/progenitor cells change as pregnancy progresses (14–19), suggesting that the neuroprotective capacity of UCB may also change over gestation. The proportion of HSCs decreases over the last trimester, whereas MNCs remain constant (14,17,19). MSCs account for only 0.002% of MNCs in term UCB, but increase with decreasing gestation, with a 10-fold increase at 28–31 weeks compared with term (16), and a >40-fold increase at 24–28 weeks' gestation (15). Furthermore, PCB has a higher proportion of immature stem/progenitor cells (14–19), more immature subsets of lymphocytes, and a decreasing monocyte population with increasing gestation (17,27). This cell composition may be critical for normal developmental processes within the preterm brain.

We acknowledge that our ability to compare the cellular composition of ovine PCB and TCB was limited because of a lack of suitable monoclonal antibodies for ovine cell surface markers. However, we are now characterizing ovine cord blood and, to date, have observed that ovine PCB contains a greater frequency of colony-forming adherent cells (MSCs and EPCs) when compared with TCB (personal observation, Jingang Li), as occurs in human UCB (15,16). This is indicative that across species, or at least in humans and sheep, there is a similar gestational profile for change in cord blood cell content. We used CNPase as an immunohistochemical marker of white matter integrity within the developing brain, where CNPase stains immature/mature oligodendrocytes and

is a structural protein in the myelin membrane, constituting ~5% of myelin protein in the adult brain (41,42). The reduction in CNPase+ staining observed in this study could be attributed to a delay in oligodendrocyte maturation and/or reduced myelination. A further limitation of the current study was that relatively small group numbers meant that we were unable to examine whether male and female fetuses respond to term and preterm UCB in a qualitatively similar manner.

In summary, UCB obtained from preterm or term pregnancy was effective at reducing preterm WMI following acute HI. Both PCB and TCB reduced the neuroinflammatory response in the preterm brain—a principal mechanism of oligodendrocyte cell loss and hypomyelination. However, the secondary mechanisms of neuroprotection appear to be different in response to PCB and TCB administration. PCB suppressed acute systemic inflammation by reducing plasma TNF- α following HI, whereas TCB induced a late increase in anti-inflammatory cytokine IL-10 and decreased systemic oxidative stress. Changes in stem/progenitor cell composition of UCB over the course of gestation are likely to influence the mode/s of neuroprotective action.

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