Cord blood stem cells to reduce preterm brain injury

Jingang Li, MD

Hudson Institute of Medical Research

&

Department of Obstetrics and Gynaecology

Monash University

Australia

Table of Contents

Summary

Preterm infants, particularly infants born under 32 weeks of gestational age, are at a high risk of neonatal complications including brain injury. Currently no effective treatment is available to prevent or treat preterm brain injury. There is increasing evidence that umbilical cord blood (UCB) may have therapeutic potential in preventing or repairing perinatal brain injury in rodent models of neonatal asphyxia. However, studies to date have focused on the efficacy and mechanisms of action of UCB stem cells for term perinatal brain injury, and not the white matter brain injury that is the principal feature of injury to the preterm brain. This set of studies aimed to examine whether administration of allogeneic UCB cells protects and/or repairs preterm brain damage.

This thesis used a fetal sheep model of brain injury induced by hypoxia-ischemia (HI) at a developmental age equivalent to approximately 28-32 weeks brain development in the human infant. HI was induced by 25 min of complete umbilical cord occlusion (UCO) to fetal sheep at 98-103 d of gestational age (=0.7 gestation), and an allogeneic source of cells derived from UCB were administered intravenously to the fetus. The fetal brains were collected at 10 d after the HI insult. The first study in this thesis specifically investigated the efficacy, optimal timing and mechanisms of action of term ovine UCB cell administration, by examining UCB cell administration to the fetus at 12 h or 5 d after HI (Chapter 2). The second study compared the effects of UCB cells derived from term or preterm UCB to examine whether there are differential effects (Chapter 3). The third study further investigated whether UCB derived mesenchymal stem/stromal cells (MSC), play a specific therapeutic role in protecting the preterm brain (Chapter 4).

Results demonstrated that 25 minutes HI induced significant white matter injury, reducing the

total number of oligodendrocytes (Olig2+) and myelin density (CNPase+), and increasing the density of activated microglia (Iba-1+) in cerebral white matter, compared to control fetuses (P<0.05). UCB cells administered at 12 h, but not 5 d after HI, significantly protected white matter architecture and suppressed cerebral inflammation. Activated microglial density showed a correlation with decreasing oligodendrocyte number (P<0.001). Additionally, UCB cells administered at 12 h induced a significant systemic increase in interleukin-10 at 10 d, and reduced circulating levels of oxidative stress (malondialdehyde) following HI (P<0.05). This study concluded that term allogeneic UCB cell administration at 12 h after HI reduces preterm white matter brain injury, principally via anti-inflammatory and antioxidant actions.

The second study demonstrated that administration of preterm UCB cells normalized white matter density, and reduced cell death and microgliosis to a similar degree to that shown for term UCB in the preterm brain. Both preterm and term UCB mediated the neuroinflammatory response to HI, which appears to be the critical mechanism of oligodendrocyte loss and white matter damage. However, the secondary mechanisms of neuroprotection were different for term and preterm UCB cells, reflecting a shift in cell composition over the course of normal gestation.

The third and final study showed that the administration of MSC (ex-vivo expanded MSC derived from preterm UCB; UCB-MSC) preserved myelination (P<0.05) and modified microglial activation in response to acute HI. UCB-MSC promoted cell proliferation (Ki-67) within the brain, and macrophage (CD163) migration from the circulation into the white matter of the brain, compared to control and HI alone fetuses (P<0.05). Assessment of multiple cytokines, chemokines and trophic factors via assay showed that the level of CXCL10 was significantly increased following UCB-MSC administration within the preterm brain (P<0.05), which likely contributes to the macrophage migration and immature oligodendrocyte proliferation within the white matter. UCB-MSC administration also reduced systemic pro-inflammatory cytokine TNFα at 3d post-HI (P<0.05). Administration of UCB-MSC preserved white matter structure following

HI, by promoting cell proliferation and macrophage migration in the preterm brain, and by decreasing systemic inflammation.

The data presented in this thesis demonstrates that whole white blood cell fraction of cells derived from term and preterm UCB, and ex-vivo expanded UCB-MSC, modified fetal brain damage after HI insult. Cells derived from UCB are an effective neuroprotective strategy, principally acting via a decrease in neuroinflammation to protect preterm brain development after HI. UCB-MSC also appears to be effective for neuroregeneration, acting via central anti-inflammatory and cerebral chemokine modulation. Combined, these results strongly suggest that cord blood cells could be used to reduce white matter brain injury in the preterm brain.

General Declaration

In accordance with Monash University Doctorate Regulation 17 Doctor of Philosophy (PhD) and Master of Philosophy (MPhil) regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes two original papers and one review paper published in peer reviewed journals and one unpublished publications that is currently under review by a journal. The core theme of the thesis is *"Cord blood stem cells to reduce preterm brain injury"*. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Hudson Institute of Medical Research under the supervision of Associate Professor Suzanne Miller, Professor Graham Jenkin, Dr Tamara Yawno, and Associate Professor Flora Wong.

In the case of Chapter 1 to 4, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status (published, in press, accepted or returned for revision)	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
1	*Could cord blood cell therapy reduce preterm brain injury?*	*Accepted*	*70%. Concept, and writing manuscript*	*1)Courtney McDonald, writing section 5%, 2)Michael Fahey, writing section 5%, 3)Graham Jenkin, editting manuscript 10%, 4) Suzanne Miller, writing section, editing manuscript 10%*	*No*

2	*Preterm white matter brain injury is prevented by early administration of umbilical cord blood cells.*	*Accepted*	*75%. Concept, data collection, data analysis, and writing manuscript*	*1)Tamara Yawno, animal surgery, data analysis, editing manuscript 5%, 2)Amy Sutherland, animal surgery 2%, 3)Jan Loose, data collection 2%, 4)Ilias Nitsos, animal surgery 2%, 5)Robert Bischof, data analysis 1%, 6)Margie Castillo-Melendez, data analysis 1%, 7)Courtney McDonald, data analysis 1%, 8)Flora Wong, concept 1%, 9)Graham Jenkin, concept, 5%, 10)Suzanne Miller, concept, editing manuscript 5%*	*No*
3	*Term versus preterm cord blood cells for the prevention of preterm brain injury.*	*Accepted*	*80%. Concept, data collection, data analysis, and writing manuscript*	*1)Tamara Yawno, animal surgery, data analysis 5%, 2) Amy Sutherland, animal surgery 2%, 3)Jan Loose, data collection 1%, 4)Ilias Nitsos, animal surgery 1%, 5)Beth Allison, animal surgery 1%, 6)Robert Bischof, data analysis 1%, 7)Courtney McDonald, data analysis 1%, 8)Graham Jenkin, concept 3%, 9)Suzanne Miller, concept, editing manuscript 5%*	*No*
4	*Umbilical cord blood derived mesenchymal stem/stromal cells protect preterm white matter brain development against hypoxia-ischemia.*	*Under Review*	*77%. Concept, data collection, data analysis, and writing manuscript*	*1)Tamara Yawno, animal surgery, data analysis 5%, 2)Amy Sutherland, animal surgery, data collection 2%, 3)Shanti Gurung, data analysis 1%, 4)Madison Paton, data analysis 1%, 5)Courtney McDonald, data analysis 1%, 6)Abhilasha Tiwari, data analysis 1%, 7)Yen Pham, data collection 1%, 8) Margie Castillo-Melendez, data analysis 1%, 9)Graham Jenkin, concept, editing manuscript 5%, 10)Suzanne Miller, concept, data analysis, editing manuscript 5%*	*Yes*

*If no co-authors, leave fields blank

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: ███████████ **Date:** **15/11/2017**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: ███████████ Date: 15/11/2017

Acknowledgements

I would like to convey my earnest appreciation to all of the people who have helped me throughout my memorable PhD years.

First and foremost to my supervisors Associate Professor Suzie Miller, Professor Graham Jenkin, Dr Tamara Yawno, and Associate Professor Flora Wong. I deeply appreciate you giving me the invaluable opportunity to take part in this exciting study and to be a part of such a fantastic research environment. Suzie, your support is excellent, and I can't tell you how much help I gained from you. You are always open for discussion, and willing to offer a chance to try my research ideas. I really cannot imagine how I would have gotten through my PhD without your support. Also a big thank you to Graham, you welcomed me to join your research group, and offered me this priceless research opportunity. You were always willing to offer help, and provided excellent feedbacks and guidance. Also a big thank you to Tamara, you always offered me a huge amount of help, with everything from animal work to analysis of data, preparation of conference presentation, and drafting papers. I cannot describe how much I value your tremendous support.

A big thank you to Ilias, Amy, Dal, and Beth for your help with sheep surgeries. And a big thanks to Jan for your tuition and help in the lab and animal house. You were always willing to help out, with the biggest kindness. A big thank you to Yen for your tremendous support, especially in administrative support and laboratory work. You always solved everything instantly, which made everything easier throughout my PhD. Big thanks to Shanti, Abhi, and Lakshmi for your kind help in teaching me cell work, which enabled me to begin and complete my final study. Thanks to Margie for your help with the immunos, and to Rob for your help with ELISAs. Thanks to my funding bodies, Inner Wheel Australia, L.E.W. Carty Charitable Fund, the

Finally to my family, my wife, Yuling, thank you for supporting me throughout all these years. Without your understanding and support, I could not even start this invaluable experience in Australia. And thanks for my two kids, who were born within these PhD years. With your lovely smile and sleeping faces, I could keep writing this thesis while you were sleeping, and complete this thesis.

I sincerely appreciate everyone involved in my PhD study.

Publications and Abstracts

Publications arising from this thesis

Li, J., McDonald, C., Fahey, M. C., Jenkin, G., & Miller, S. L. (2014). Could cord blood cell therapy reduce preterm brain injury? Frontiers in Neurology, 2014 Oct 9;5 (200) 1-17.

Li, J., Yawno, T., Sutherland, A., Loose, J., Nitsos, I., Bischof, R., Castillo-Melendez, M., McDonald, C. A., Wong, F. Y., Jenkin, G., & Miller, S. L. (2016). Preterm white matter brain injury is prevented by early administration of umbilical cord blood cells. Exp Neurol, 283(Pt A), 179-187.

Li, J., Yawno, T., Sutherland, A., Loose, J., Nitsos, I., Allison, B. J., Bischof, R., McDonald, C. A., Jenkin, G., & Miller, S. L. (2017). Term versus preterm cord blood cells for the prevention of preterm brain injury. Pediatr Res. Jul 19. doi: 10.1038/pr.2017.170 (Epub ahead of print)

Li, J., Yawno, T., Sutherland, A., Gurung, S., Paton, M., McDonald, C. A., Tiwari, A., Pham, Y., Castillo-Melendez, M., Jenkin, G., & Miller, S. L. Preterm umbilical cord blood derived mesenchymal stem/stromal cells protect preterm white matter brain development against hypoxia-ischemia. (Submitted to Stem Cells Translational Med. November, 2017)

Publications associated with this thesis

Gopagondanahalli, K. R., **Li, J.**, Fahey, M. C., Hunt, R. W., Jenkin, G., Miller, S. L., & Malhotra, A. (2016). Preterm Hypoxic–Ischemic Encephalopathy. Front Pediatr, 4(114) 1-10

Yawno, T., Sabaretnam, T., **Li, J.**, McDonald, C., Lim, R., Jenkin, G., Wallace, E. M., & Miller, S. L. (2017). Human Amnion Epithelial Cells Protect Against White Matter Brain Injury After Repeated Endotoxin Exposure in the Preterm Ovine Fetus. Cell Transplant, 26(4), 541-553.

Yawno, T., Mahen, M., Li, J., Fahey, M., Jenkin, G., & Miller, S. (2017). The Beneficial Effects of Melatonin Administration Following Hypoxia-Ischemia in Preterm Fetal Sheep. Frontiers in Cellular Neuroscience, 11(296) DOI:10.3389/fncel.2017.00296

Tiwari, A., Prasanna, N. L., Deane, J., McDonald, C., **Li, J.**, Pham, Y., Sutherland, A., Jenkin, G., & Kirkland, M. (2017). Controlling the Effective Oxygen Tension Experienced by Cells Using a Dynamic Culture Technique for Hematopoietic Ex Vivo Expansion. Current Protocols in Stem Cell Biology. (*accepted, In Press*)

Abstracts/Conference+Proceedings

Li J, Yawno T, Sutherland A, Loose J, Nitsos I, McDonald C, Jenkin G, Miller S. Cord blood cell therapy to reduce preterm brain injury, Australasian Society for Stem Cell Research/ International Society for Cellular Therapy, Lorne October 2014.

Li J, Yawno T, Sutherland A, Loose J, Nitsos I, Wong F, Jenkin G, Miller S. Cord blood cell therapy to reduce preterm brain injury. Early vs late administration. Perinatal Association of Australia and New Zealand, Melbourne, April 2015.

Li J, Yawno T, Sutherland A, Loose J, Nitsos I, Wong F, Jenkin G, Miller S. Umbilical cord blood cell administration is neuroprotective following preterm hypoxic-ischemic insult. Fetal and Neonatal Physiological Society, Vancouver, August 2015

Li J, Yawno T, Sutherland A, Loose J, Nitsos I, Wong F, Jenkin G, Miller S. Early administration of cord blood cells reduces preterm brain injury following hypoxia-ischemia, Neuroscience 2015, Chicago, October 2015

Li J, Yawno T, Sutherland A, Gurung S, Paton M, McDonald C, Tiwari A, Jain K, Miller S, Jenkin G. Umbilical cord blood derived mesenchymal stromal cells protect against preterm white matter brain injury following hypoxia-ischaemia. Australasian Society for Stem Cell Research, Cambridge September 2016.

Li J, Yawno T, Sutherland A, Gurung S, Paton M, McDonald C, Tiwari A, Jain K, Miller S, Jenkin G. Umbilical cord blood derived mesenchymal stromal cells protect against preterm white matter brain injury following hypoxia-ischaemia. Australasian Society for Stem Cell Research, Margaret River December 2016.

Abbreviations and Symbols

°C Degrees Celsius

± Plus or minus

7-AAD 7-Aminoactinomycin D

ABG Arterial blood gas

AF Alexa Fluor

ANOVA Analysis of variance

BBB Blood–brain-barrier

BDNF Brain-derived neurotrophic factor

BE Base excess

BrdU Bromodeoxyuridine

CA1 Cornu ammonis 1 of the hippocampus

CBF Cerebral blood flow

CFSE Carboxyfluorescein succinimidyl ester

CN Caudate nucleus

CNS Central nervous system

CP Cerebral palsy

CNPase Cyclic nucleotide 3′-phosphodiesterase

CXCL Chemokine (C-X-C motif) ligand

d Day

DAB Diaminobenzidine

DAMPs Danger-associated molecular patterns

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

EDTA Ethylene diamine tetra-acetic acid

ELISA Enzyme-linked immunosorbent assay

EPCs Endothelial progenitor cells

EPO Erythropoietin

ESCs Embryonic stem cells

FACS Fluorescence activated cell sorting

FHR Fetal heart rate

FITC Fluoresceinisothiocyanate isomer-I

g Gram

GA Gestational age

GDNF Glial cell line-derived neurotrophic factor

GFAP Glial fibrillary acidic protein antibody

GM1 Ganglioside

GVHD Graft-versus-host disease

h Hour

hAECs human Amnion epithelial cells

HI Hypoxia-ischemia

HIE Hypoxic-ischemic encephalopathy

HLA Human leukocyte antigen

HSCs Hematopoietic stem cells

i.v. Intravenous

IC Internal capsule

ICAM-1 Intercellular adhesion molecule-1

IL-6 Interleukin-6

IFN-γ Interferon-γ

Iba-1 Ionized calciumbinding adaptor molecule 1

IP Intraperitoneal

iPS cells Induced pluripotent stem cells

IT Intrathecal

IU International unit

IUGR Intrauterine growth restriction

IV Intravenous

IVen Intraventricular

IVH Intraventricular haemorrhage

kg Kilogram

KHCO Potassium bicarbonate

LPS Lipopolysaccharide

M Molar

MAP Mean arterial pressure

MCAO Middle cerebral artery occlusion

MDA Malondialdehyde

MHC Major histocompatibility complex

MNCs Mononuclear cells

MSCs Mesenchymal stromal cells

mg Milligram

min Minutes

ml Millilitre

mm Millimetre

mM Millimolar

mmHg Millimetres of mercury

MRI Magnetic resonance imaging

NA Not applicable

NaBH4 Sodium borohydride

NGF nerve growth factor

NSE Neuron-specific enolase

NF-κB Nuclear factor-kappaB

NH4Cl Ammonium chloride

NIH National Institutes of Health

NK Neural killer

NPCs Neural progenitor cells

NT Neurotrophin

NGS Normal goat serum

O2 Oxygen

OGD Oxygen/glucose deprivation

Olig2 Oligodendrocyte transcription factor 2

OPCs Oligodendrocytes progenitor cells

P Postnatal day

PaCO2 Partial pressure of carbon dioxide

PAMPs Pathogen-associated molecular patterns

PaO2 Partial pressure of oxygen

PBS Phosphate buffered saline

PCB Preterm cord blood

PE Phycoerythrin

pH Potential of hydrogen

PHH Post-hemorrhagic hydrocephalus

PVL periventricular leukomalacia

PVWM Periventricularwhite matter

RNS Reactive nitrogen species

ROS Reactive oxygen species

rpm Revolution per minute

SEM Standard error of the mean

SVZ subventricular zone

TBARS Thiobarbituric acid reactive substances

TCB Term cord blood

TLRs Toll-like receptors

TNC Total nucleated cell

TNF-α Tumor necrosis factor-α

Tregs Regulatory T cells

TUNEL Terminal deoxynucleotidyl transferase dUTP nick end labeling

UC Umbilical cord

UCB Umbilical cord blood

UCBCs Umbilical cord blood cells

UCO Umbilical cord occlusion

USA United States of America

VACM-1 Vascular cell adhesion molecule-1

VEGF Vascular endothelial growth factor

WMI White matter injury

Chapter 1 Literature Review

Could cord blood therapy reduce preterm brain injury?

Jingang Li[1], Courtney McDonald[1], Michael Fahey[1,3], Graham Jenkin[1,2], Suzanne L Miller[1,2*]

[1]The Ritchie Centre, MIMR-PHI Institute, Clayton, VIC Australia

[2]The Department of Obstetrics and Gynaecology, Monash University, Clayton, VIC, Australia

[3]The Department of Paediatrics, Monash University, Clayton, VIC, Australia

This chapter reviewed the current knowledge about the neuroprotective actions of UCB cells and their potential to ameliorate preterm brain injury. This is an unaltered version of the review paper published in *Frontiers in Neurology, 2014 Oct 9;5:200.*

Declaration for Thesis Chapter 1 — Could cord blood cell therapy reduce preterm brain injury?

Declaration by candidate

In the case of Chapter [insert chapter number], the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experimental design and execution, data analysis, manuscript preparation	70

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Courtney McDonald	writing section, editing manuscript
Michael Fahey	writing section, editing manuscript
Graham Jenkin	editing manuscript
Suzanne Miller	writing section, editing manuscript

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	███████	Date 15/11/2017
Main Supervisor's Signature		Date 15/11/2017

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Could cord blood cell therapy reduce preterm brain injury?

Jingang Li[1], Courtney A. McDonald[1], Michael C. Fahey[1,2], Graham Jenkin[1,3] and Suzanne L. Miller[1,3]*

[1] *The Ritchie Centre, MIMR-PHI Institute, Clayton, VIC, Australia*
[2] *Department of Paediatrics, Monash University, Clayton, VIC, Australia*
[3] *Department of Obstetrics and Gynaecology, Monash University, Clayton, VIC, Australia*

Major advances in neonatal care have led to significant improvements in survival rates for preterm infants, but this occurs at a cost, with a strong causal link between preterm birth and neurological deficits, including cerebral palsy (CP). Indeed, in high-income countries, up to 50% of children with CP were born preterm. The pathways that link preterm birth and brain injury are complex and multifactorial, but it is clear that preterm birth is strongly associated with damage to the white matter of the developing brain. Nearly 90% of preterm infants who later develop spastic CP have evidence of periventricular white matter injury. There are currently no treatments targeted at protecting the immature preterm brain. Umbilical cord blood (UCB) contains a diverse mix of stem and progenitor cells, and is a particularly promising source of cells for clinical applications, due to ethical and practical advantages over other potential therapeutic cell types. Recent studies have documented the potential benefits of UCB cells in reducing brain injury, particularly in rodent models of term neonatal hypoxia–ischemia. These studies indicate that UCB cells act via anti-inflammatory and immuno-modulatory effects, and release neurotrophic growth factors to support the damaged and surrounding brain tissue. The etiology of brain injury in preterm-born infants is less well understood than in term infants, but likely results from episodes of hypoperfusion, hypoxia–ischemia, and/or inflammation over a developmental period of white matter vulnerability. This review will explore current knowledge about the neuroprotective actions of UCB cells and their potential to ameliorate preterm brain injury through neonatal cell administration. We will also discuss the characteristics of UCB-derived from preterm and term infants for use in clinical applications.

Keywords: preterm birth, low birth weight, brain damage, white matter injury, oligodendrocytes, cerebral palsy, umbilical cord blood, stem cells, hypoxia–ischemia, inflammation, periventricular leukomalacia

BACKGROUND

Impressive advances in perinatal and neonatal care have led to substantial improvements in survival rates for preterm infants born at <37 weeks gestation. However, survival of preterm infants may occur at a cost, with a strong causal link between preterm birth and subsequent neurological motor and cognitive deficits, including cerebral palsy (CP). In particular, more extremely preterm babies now survive than ever before with these infants at the greatest risks of short and long-term neurodevelopmental deficits (1). Despite the known etiological link between preterm birth and neuromotor and neuro-cognitive dysfunctions, there are currently no specific neuroprotective treatments available for preterm infants.

Stem, or stem-like cells, have drawn attention from scientists and the general public due to their potential to induce tissue repair and/or regeneration. Umbilical cord blood (UCB)-derived cells offer ethical and practical advantages over other stem-like cells given that collection can be obtained from the discarded placenta at birth. Such cells possess multiple proven [as per cord blood hematopoietic stem cells (HSCs)] and potential therapeutic uses, including recent evidence that UCB cells may mitigate newborn brain damage arising from term neonatal hypoxic-ischemic encephalopathy (HIE). However, despite the heightened neurological risks associated with preterm births, the potential use of UCB cells in preterm neonates has not yet been actively investigated. This article briefly describes the background, etiology, and pathophysiological mechanisms of brain injury in preterm infants, and summarizes current research on the use of UCB cells for therapeutic use in term and preterm perinatal brain injury. Potential implications for future clinical trials of UCB cell therapy in preterm infants are discussed.

PRETERM BIRTH AND CHILDHOOD NEUROLOGICAL DEFICITS

In 2010, 14.9 million babies worldwide were born preterm, accounting for approximately 11% of all births, with the rates and burden of preterm birth significantly increased in both low and high-income birth settings compared to the previous decade (2). Of all preterm births in the developed world, 16% are born before 32 weeks of gestation or weigh <1500 g (2), with this population of *very preterm* infants (born 28 to <32 weeks) or *extremely preterm* infants (<28 weeks) at the greatest risks for long-term physical and neurological morbidities. Indeed, in developed countries, preterm births account for 70% of neonatal deaths and up to 75% of neonatal morbidity (3), with the risks of death or disability profoundly increased in middle- or low-income birth settings, reflecting decreased resources for neonatal intensive care (4). In addition, in developed countries most preterm babies now survive

as a result of advances in neonatal intensive care such that the survival rate for extremely preterm infants is 90% (2).

Cerebral palsy is the most common physical disability of childhood, occurring in 2–2.5/1000 live births in developed countries. This rate is increased to approximately 90–100/1000 babies that were born at <32 weeks gestation (5, 6). Indeed, 35–50% of children with established CP were born preterm (7, 8). The major overt neurological manifestations of brain injury observed in children that were born preterm are spastic motor deficits, commonly accompanied by intellectual deficits. Less severe disturbances of motility, cognition, and behavior occur in 25–50% of survivors (9).

The economic cost of preterm birth and CP are high due to the need for neonatal intensive care and ongoing long-term complex health care. The National Institute of Medicine estimated that the lifetime cost of all preterm births is $26.2 billion USD per year in the USA (10). The financial burden of CP in the USA has been separately costed and estimated at $11.5 billion USD (11) and is indicative of the large financial burden association with preterm birth and CP. This is in addition to the significant burden placed on families and society who care for children and adults with CP. There is therefore an enormous demand to prevent or reduce brain injury in preterm infants, to reduce the subsequent neurodevelopmental sequelae, and consequently decreasing the large socio-economical burden.

The complications associated with preterm birth and brain injury are complex and involve multiple overlapping adverse pathways, but it is clear that preterm birth is strongly associated with damage to the white matter of the immature brain. Therefore, an understanding of white matter injury (WMI) is a critical component required for the treatment of preterm brain injury.

WHITE MATTER INJURY

Fetal brain maturation and functional development involves a series of organizational processes including neurogenesis, cell migration, cell differentiation, synaptogenesis, and axonal myelination. The development of white matter requires mature oligodendrocyte glial cells to produce myelin and ensheath the axons of neurons, and thus oligodendrocytes play a crucial role in fast signal transmission along neurons and throughout the brain. Injury to these cells impairs, usually irreversibly, myelination. Oligodendrocytes develop according to a well-defined lineage. Pre-oligodendrocytes are the predominating oligodendroglial cell at gestational age 24–32 weeks in humans. They are exquisitely vulnerable to pro-inflammatory cytokines, excitotoxicity, oxygen free radical attack, and hypoxic stress, and rapidly undergo apoptosis under adverse conditions (12–15). It is believed that this selective vulnerability of the pre-oligodendrocytes in preterm infants restricts the number and functional ability of mature oligodendrocytes to undergo the process of laying down of white matter and formation of myelin fibers, thus causing very preterm and extremely preterm infants to be most susceptible to WMI (9, 16, 17). Thus, preserving oligodendrocytes and their precursor cells is fundamental to reducing injury to the developing white matter of the brain. Most commonly, preterm brain injury is evident in the periventricular white matter adjacent to the lateral ventricles, so-called periventricular leukomalacia (PVL). WMI is detectable in at least 50% of infants born very preterm or extremely preterm,

and is a strong indicator of long-term neurological adverse outcome. Nearly 90% of preterm infants who later develop spastic CP have evidence of WMI (9). Half of the children identified as having WMI will have cognitive and/or behavioral and/or attention deficits (18–20). Clinical imaging studies demonstrate that myelin loss (hypomyelination) and disorganization of major white matter fiber tracts correlate with functional impairments in children with CP and PVL (21, 22).

Pathologically, WMI is a condition demonstrated by coagulation and necrosis of white matter near the lateral ventricles, accompanied by gliosis (23). The periventricular area is vulnerable to ischemia in the preterm brain, which, in part, is anatomically due to poor vascularization and immature cerebrovascular autoregulation (24, 25). This may result in focal PVL. But, in many cases, WMI is widespread and incorporates periventricular, subcortical, and callosal white matter, as well as the internal capsule. WMI, and in particular PVL, has two distinct histopathological appearances, described as either cystic or non-cystic (diffuse) WMI. Cystic WMI typically affects all types of cells and is therefore considered the more severe type and is closely linked with CP, whereas diffuse PVL mainly targets pre-oligodendrocytes and is considered less severe but nonetheless is linked to cognitive and behavioral impairments, and CP (9). It is generally considered that the gray matter is not as susceptible to preterm insults as is white matter, but the pre-oligodendrocytes also present in gray matter are not spared, leading to damage involving the cerebral cortex, thalamus, and basal ganglia (26, 27).

There is a growing understanding of the etiology of preterm brain injury, likely involving one or more interactions between fundamental immaturity of the brain, vulnerability of white matter developmental processes, and the adverse effects of two principal upstream insults: hypoxia–ischemia and infection/inflammation. Hypoxia–ischemia and infection/inflammation are relatively common in the preterm period, and have profound adverse effects on white matter development (28–30).

HYPOXIA–ISCHEMIA AND WHITE MATTER INJURY

After an hypoxic-ischemic insult, microglia and macrophages within the brain's white matter exhibit immunoreactivity for interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), and infiltrate to lesion sites. Astrocytes become hypertrophied and diffuse gliosis is evident within 24 h. Loss of oligodendroglial lineage cells and impairment of myelinogenesis is evident within 10 days following hypoxia–ischemia (9, 31–35). When the insult has been prolonged or severe, brain injury is exacerbated through influx of cytokines and chemokines via the damaged blood–brain-barrier (BBB), thereby further increasing inflammatory mediators within the brain (36).

In response to a significant hypoxic-ischemic insult, secondary pathways of injury are also initiated and evolve over days. These adverse pathways include mitochondrial dysfunction, excitotoxicity, apoptosis, oxidative stress, and initiation of additional inflammatory processes (37). A further adverse effect of hypoxia–ischemia is the disruption of normal growth and differentiation factors driving brain development, decreasing concentrations of signaling proteins and nutrients that include neurotrophic factors vital for inhibition of programed cell death (38, 39). For example,

brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5 play important roles in promoting neuronal growth and differentiation, connective plasticity, and neuronal survival through their interaction with tyrosine kinase β-receptors, but these are each affected in the preterm brain in response to hypoxia–ischemia (40). Additional neuron and glial cell loss occurs over days and weeks after a sentinel insult, resulting from chronic deprivation of neurotrophic factors, decreased synaptic input from neighboring cells, and loss or recruitment failure of local neural and glial stem and progenitor cells (41, 42). The severity and duration of neurotrophic factor deprivation directly correlates to long-term neurological outcome (38, 42).

In addition, activated astrocytes and microglia mediate the release of reactive oxygen species (ROS) and reactive nitrogen species (RNS), leading to increased protein nitration and oxidative stress in response to hypoxia–ischemia and brain inflammation (43). In the context of preterm birth, a compromised intrauterine environment may induce excess release of free radicals and, combined with the transition to an extra-uterine high oxygen environment, may overwhelm endogenous antioxidant enzymes, resulting in preferential death of pre-oligodendrocytes and contributing to the development of WMI within the preterm brain (43–46).

INFECTION/INFLAMMATION AND WHITE MATTER INJURY

Fetal and neonatal exposure to infection and/or inflammation is recognized as a principal contributor to preterm birth and WMI. Maternal intrauterine infection including chorioamnionitis is associated with increased levels of pro-inflammatory cytokines (IL-6, IL-8, TNF-α, and IL-1b) in the amniotic fluid and cord blood (47–49) and is one of the most important causes of preterm birth <30 weeks of gestation (50). Maternal intrauterine infection presents a significant risk for WMI and CP (51, 52). Neonatal sepsis is also a risk factor for WMI in infants that were born preterm (53, 54).

Adverse inflammatory stimuli during fetal or neonatal life induce a systemic and central nervous system (CNS) response via activation of innate and adaptive immune systems. Microglia are the primary mediators of the brain's immune response, mediating the pro- and anti-inflammatory response to remove pathogens, via binding of toll-like receptors (TLRs) with ligands, pathogen-associated molecular patterns (PAMPs), and/or danger-associated molecular patterns (DAMPs) (55, 56). However, prolonged microglial activation can cause brain injury (55). Microglia are at their peak density in white matter during the WMI-vulnerable period (57), making them fundamental in producing WMI (44). Lipopolysaccharide (LPS), an endotoxin of gram-negative bacteria and a form of PAMP, and binds receptors including TLR-4 and CD14 on microglia, initiating a signal transduction cascade that ultimately activates transcription factors such as nuclear factor-kappaB (NF-κB). In turn, this leads to up-regulation of cytokines, chemokines, and complement proteins, and over time this response can sensitize the developing brain to secondary insults thereby contributing to sustained CNS inflammation. Cytokines may directly act upon oligodendrocytes to induce cell death, as evidenced by in vitro studies on human oligodendrocytes where TNF-α and interferon-γ (IFN-γ) induced

dose-dependent cell necrosis (58). In vivo administration of high-dose LPS to preterm fetal sheep results in significant cerebral hemodynamic changes that cause cerebral ischemia and PVL-like fetal brain injury (59), while low-dose LPS, insufficient to cause fetal hypoxia, induces diffuse WMI and microglial invasion, where the degree of microglial activation is correlated to the presence of WMI (60).

The downstream pathways that result from hypoxia–ischemia, or inflammatory stimuli, are complex and are not mutually exclusive. Hypoxia–ischemia and increased ROS are known to induce an inflammatory reaction and, conversely, pro-inflammatory mediators lead to the generation of free radicals and oxidative stress. This interaction is driven by NF-κB. In normal-state resting cells, the NF-κB protein complex remains within the cytoplasm, bound to inhibitory IκB protein. Pro-inflammatory cytokines, LPS and viruses cause proteolysis of IκB, allowing dissociation from NF-κB, and the nuclear translocation of NF-κB where it activates gene transcription (61). Additionally, tissue hypoxia and oxidative stress can modulate NF-κB release (61). Thus, hypoxia–ischemia can induce inflammation via microglial activation, and conversely infection/inflammation can induce hypoxia–ischemia through hypotension (62). Indeed, preterm infants have been shown to have higher risk of WMI when chorioamnionitis and placental perfusion deficits are present together (63).

LIMITATIONS OF CURRENT TREATMENTS OF WHITE MATTER INJURY

Although there have been major clinical and scientific advances in neonatal care over the last decade, currently only antenatal corticosteroid are proven to reduce the risk of intraventricular hemorrhage (IVH) (64). Other strategies in preterm infants, such as use of erythropoietin, melatonin, indomethacin, antenatal magnesium sulfate, therapeutic hypothermia, or delayed cord clamping remain at the experimental investigation stage and are not of proven benefit (65, 66). Thus, current management for preterm brain injury has, until now, been restricted to supportive strategies.

One of the biggest hurdles for identifying neuroprotective strategies for preterm infants is the multi-faceted etiology of the brain damage. As described in the section above, the primary antenatal causal factors that may induce brain injury include maternal/fetal infection and/or chronic placental perfusion insufficiency (67, 68). Postnatal factors may exacerbate or cause brain injury, including repetitive subacute/chronic hypoxia–ischemia due to poor lung function and ventilation, and free radical imbalance following oxygen reperfusion in response to a high oxygen extra-uterine environment or oxygen administration (44, 46, 69). Neonatal chronic cerebral hypoperfusion, hypotension, hypocarbia, or symptomatic persistent ductus arteriosus (70, 71), IVH with or without post-hemorrhagic ischemia or hydrocephalus (72), infection (53, 54), hypoglycemia, and glucocorticoid administration are also involved in the progression of brain injury (68, 73). Moreover, even preterm birth without exacerbating factors can result in subtle white matter pathology (69). Thus, it can be appreciated that unlike term neonatal HIE, these insults do not necessarily occur around the time of delivery, and it may therefore be difficult to recognize the timing of the onset of a sentinel (or

exacerbating) insult. It would therefore be likely that preterm brain injury would be best treated with a therapy, or therapies, with multiple neuroprotective mechanisms and with a long therapeutic window. Any therapy should be targeted at WMI as the predominant neuropathology. Additionally, such a treatment would be aimed at one or more of the following – reducing inflammation and free radical attack, halting the progression of cell death programing, and/or replacing damaged oligodendrocytes in order to remodel areas of WMI and normalize myelination. We will present data to support the therapeutic potential of neonatally administered UCB-derived cells, for protection and repair of the preterm brain.

STEM CELLS

Stem cells are characterized by their ability to undergo self-renewal and to differentiate into multiple cell types. In general, stem cells can be classified into three major categories on the basis of their source, namely embryonic stem cells (ESCs), fetal-derived stem cells, and adult stem cells. ESCs are pluripotent, are able to generate cells from all three germ layers and can be maintained in culture indefinitely (74), providing a limitless source of precursor cells for the regeneration of damaged tissue. However, due to the pluripotent nature of ESCs they are also tumorigenic and transplantation of these cells currently presents significant safety concerns (75), and has cautioned their use in clinical trials. ESCs are also obtained from embryros, presenting ethical issues. With recent advances, it has become possible to reprogram somatic cells and generate induced pluripotent stem (iPS) cells (76). These cells may be able to overcome some of the limitations of ESCs, i.e., ethical issues, and enable the generation of patient-specific iPS cell lines. However, they are also tumorigenic (77), and this issue remains unresolved. Adult stem cells, or stem-like cells, include mesenchymal stromal cells (MSCs), that can be obtained from a number of sources including bone marrow, adipose tissue, and dental pulp, and neural progenitor cells (NPCs) that are found in the subventricular zone of the brain and comprise multipotent stem/progenitor cells that can differentiate down the neural lineage, including to neurons and glial cells (78). NPCs can be isolated from the adult brain and expanded for several passages whilst retaining their undifferentiated state. Lastly, fetal-derived stem-like cells can be obtained from placental tissue (79) and UCB (80) and include placental and umbilical cord MSCs (UC-MSCs), amnion epithelial cells (AECs), HSCs, and endothelial progenitor cells (EPCs) (81). Given these cells are isolated from fetal tissue, they tend toward greater differentiation and expansion potential than adult stem cells. Fetal-derived stem cells can be easily isolated from tissue that is routinely discarded at birth, they are abundant due to the large number of births each year, and their collection raises no ethical concerns.

Stem-like cells sourced from placenta and umbilical cord (UC) have been studied pre-clinically for treatment of a variety of diseases including multiple sclerosis (82, 83), stroke (84, 85), bronchopulmonary dysplasia (86, 87), and CP (88, 89) and may be beneficial for reducing disease burden in these conditions. For MSCs alone, there are currently >400 clinical trials listed on clinical trials.gov (search: "mesenchymal stem cell"). However, there are numerous clinics around the world that are already capitalizing

on the *promise* of stem cell treatment and are offering stem cell therapies for financial gain to families of those with conditions including CP. This has been coined stem cell tourism (90). It is therefore imperative that well-planned and controlled pre-clinical and clinical trials are conducted to establish the safety, short-, and long-term efficacy, and mechanisms by which stem cell therapies may provide benefit, which in turn will enable treating clinicians and patients to make informed decisions regarding the use of stem cell treatments.

UMBILICAL CORD BLOOD

Umbilical cord blood is a rich source of HSCs, accounting for 0.5–1.0% of mononuclear cells (MNCs) in term UCB (91), used to treat patients with abnormal hematopoietic conditions, childhood leukemia, or metabolic diseases (92). HSCs are positive for CD34 and CD45, and defined by their capacity to self-renew and give rise to multiple blood lineages. Traditionally, bone marrow-derived HSCs were used to treat these conditions, however, UCB is easier to obtain, less expensive and less likely to trigger a deleterious immune response or rejection in the recipient (93). HSCs from human UCB (hUCB) are also more primitive than bone marrow-derived HSCs, have longer telomeres, have a higher colony-forming capacity and can repopulate blood lineages over a long period of time (94, 95). Given these advantages, more than 3000 hUCB transplants are now performed each year for blood and other disorders (96). Other strong advantages for the use of UCB for transplants include that it can be tissue typed, screened for viral biomarkers, processed and banked, allowing the supply for both urgent and directed transplants (97) and the volume and number of cells that can be attained is generally very good. In term births, a large volume of UCB can be collected [38–42 weeks: 102 ± 30 ml, containing $11.3 \pm 6.2 \times 10^8$ total nucleated cell (TNC)] (91). However, in preterm birth, or in pregnancy complications [such as intrauterine growth restriction (IUGR)], there is reduced UCB volume for collection [34–37 weeks: 90 ± 32 ml, $7.7 \pm 4.8 \times 10^8$ TNC; 25–33 weeks: 62 ± 31 ml, 3.3 ± 3.5 TNC] (91), which is problematic if UCB collection is required or requested. Furthermore, it is not known how antenatal complications, such as IUGR or chorioamnionitis, may change the composition of the stem and stem-like cells present in the UCB, and whether differences in cell composition may impact its therapeutic utility.

Umbilical cord blood is not only a useful source of HSCs, but also contains a number of other stem/progenitor cell types including MSCs and EPCs (80). Moreover, UCB is a rich source of immunosupressive cells, such as regulatory T cells (Tregs) (98). MSCs are multipotent adult progenitor cells that have a broad potential for repair of injured tissue. MSCs are characterized by their morphology, phenotype, and differentiation potential to form osteoblasts, chondrocytes, and adipocytes (82). MSCs are a plastic adherent cell population with the absence of CD34, CD45, and CD133, and are positive for CD13, CD29, CD44, CD73, and CD90 (80, 99). MSCs can indeed be isolated from UCB, but at a very low frequency and cellular fraction, with success rates for isolation ranging from 40 to 60% (99, 100) and, in one study, only 8% of UCB units could be effectively expanded into MSC-like colonies (100). While the frequency of MSCs is low in UCB (0.002% of MNCs in term UCB) (101), UCB–MSCs show a strong

proliferation capacity and can be maintained longer in culture than MSCs derived from other sources (99). Furthermore, following exposure to neural differentiation factors, hUCB–MSCs express a number of neural cell antigens, including glial fibrillary acidic protein (found in astrocytes), TuJ-1 (neural progenitor), vimentin, and nestin (102).

Endothelial progenitor cells, isolated from bone marrow, peripheral blood, and UCB can be differentiated into mature endothelial cells *in vitro* and, in animal models of ischemia, can incorporate into sites of active angiogenesis to stabilize and promote the growth of new blood vessels (103). While EPC classification remains contentious, they are generally characterized by the expression of CD133, CD34, and vascular endothelial growth factor (VEGF) receptor-2 (104). EPCs are estimated to make up 1–2% of HSC-containing CD34+ cell fraction in term UCB, representing 1 in 10^7 MNCs (105). Despite the low number, EPCs isolated from hUCB have been shown to have a stable endothelial phenotype and a higher proliferative capacity compared to those isolated from peripheral blood, making UCB a superior source for the isolation of EPCs. Studies are now being conducted to optimize the isolation of the three major cell types (EPCs, HSCs, and MSCs) from single UCB units (80).

Regulatory T cells should also be considered as potential useful cells to be isolated from UCB. Tregs are immunosupressive T cells that can maintain self-tolerance, prevent autoimmunity, inhibit rejection of transplants, and regulate the immune response to infectious disease (106). Tregs isolated from UCB exhibit a predominantly naïve phenotype, which is associated with a significantly enhanced proliferative potential compared to adult Tregs (107). It has been suggested that the low incidence of graft-versus-host-disease (GVHD) associated with UCB transplants is due to the presence of Tregs (108), adding to their importance for UCB transplants and their potential utility for treatment of inflammatory conditions.

Optimal selection of UCB units for HSC transplants includes determination of TNC content, CD34+ cell count, and HLA and blood group matching of the recipient and donor (97). However, there is sparse information related to other cells of interest within UCB. Given the increasing likelihood that children born preterm may request autologous UCB cell collection and treatment for brain injury (see below), it is imperative that we investigate the similarities and differences between term and preterm UCB. This knowledge will inform the design of clinical trials that will decide whether autologous or allogeneic UCB transplants will be best placed to treat neurological impairments in preterm-born infants. To date, it has been shown that the frequency of CD34+ cells in preterm neonates was twofold increased compared to those in term neonates (109) and, for a given gestational age, each 500 g increase in birth weight contributed to a 28% increase in CD34+ cell counts (110). In preterm infants, the immunophenotypic profile of UCB–CD34+ cells shows a significantly higher expression of CD33, and a lower expression of CD38, CD117, and HLA-DR, indicating preterm UCB has a higher percentage of primitive CD34+ subsets, while term UCB has a higher percentage of committed cells (111, 112). With specific regard to EPCs, preterm (28–34 weeks) UCB units have a fourfold increase in endothelial colony-forming cells compared to term UCB (113). However in compromised placental

conditions such as preeclampsia, EPCs are decreased in term UCB, and not different to those in preterm UCB (114). Similar to other cell types, MSC population is also richer in preterm UCB compared with term, with a significant inverse correlation between the gestational age and presence of MSCs (101, 115). Furthermore, studies to date have predominantly assessed cell number and not cell function over gestation, where functionality may be a more important marker of efficacy than absolute cell number. As has been shown with hAECs, while preterm cells have a high proliferative capacity, they are functionally immature and cannot differentiate into other cell types (116). The presence of Tregs has also been assessed over gestation, and is reportedly increased in preterm UCB compared to term UCB (107), but Tregs from preterm UCB secrete significantly less IFN-γ (117). Furthermore, Tregs in UCB from IUGR infants at term were decreased compared to those in UCB units from appropriately grown babies (118).

Most studies to date examining perinatal brain injury have utilized UCB–MNCs (**Table 1**), but UCB–MNCs is composed of a variety of cells of interest including immature T cells, B-cells, monocytes, and stem-like cells including HSCs, EPCs, and MSCs. The fraction or combination of UCB cells responsible for neural repair remains to be established. UCB–MSCs have attracted interest for some time because of their multilineage differentiation potential, strong capacity for immune modulation, and low immunogenicity. Indeed, expanded hUCB–MSC transplantation has shown promise in protecting against perinatal brain injury in pre-clinical animal studies (119–121). Despite this, the clinical application of purified UCB–MSCs is currently limited by their low numbers and low success rate for isolation. On the other hand, CD34+ cells have been shown to reduce brain injury in neonatal hypoxic-ischemic mice, with a transient augmentation of cerebral blood flow in the peri-infarct area (122). UCB–CD133+ cells, the fraction enriched for EPCs and HSCs, also reduces infarct volume in a rat model of stroke (123).

In addition to stem-like cells, other cellular fractions in UCB have also been shown to have potentially important neuroprotective roles. When hUCB–MNCs was depleted for CD14+ monocytes, there was no decrease observed in microglial activation or functional recovery following administration (124), suggesting that monocytes are essential for mediating the neuroprotective benefits of hUCB cells in hypoxic-ischemic rats. In addition, a further study showed that a single injection of hUCB-derived T cells (CD4+) induced endogenous NPC proliferation for 2 weeks and promoted increased neuronal cell survival in rats (125). The therapeutic effects of stem cells are now thought to be independent of tissue engraftment (89, 126–129), although many studies have shown that transplanted UCB cells can migrate selectively toward ischemic areas of damaged brain (127, 130). It is widely considered that regenerative effects of stem cells are principally derived from indirect paracrine and trophic effects, and increasing the regenerative capacity of the brain, rather than via direct cell replacement (38, 128, 129, 131, 132). However, it is important to note that the studies referred to above have utilized hUCB in a xenogeneic setting. As such, the ability of the transplanted cells to survive and differentiate may be compromised (133). To our knowledge, the ability of autologous UCB cells to home to the site of injury and differentiate into neurons or neuroglial cells has not

Table 1 | Outcome of umbilical cord blood interventions in neonatal hypoxia–ischemia.

Cell type	Animal model	Administration			Engraftment		Histology assessments		Functional assessments		Other	Reference
	Injury type	Timing	Dose	Route	Days	Results	Days	Outcomes	Days	Outcomes		
hUCB–MNCs	P7 rats, HI 80 min	24 h after HI	1×10^7 cells	IP	21 days	Many cells in ischemic hemisphere. No sign of transdifferentiation	NA	NA	21 days	Alleviation of spastic paresis		Meier 2006 (127)
	P7 rats, HI 120 min	24 h after HI	1×10^7 cells	IV jugular	21 days	Few cells in brain tissue	21 days	No change in volume of injured hemisphere	21 days	No change on spatial memory deficit		de Paula 2009 (192)
	P7 rats, HI 90 min	3 h after HI	2×10^6 cells	IP	2 days	Few cells in ischemic cortex and striatum	2 days	Decreased neuronal death in striatum, and microglial activation in cortex	4, 7 days	Improved developmental sensorimotor reflexes only at 4 days		Pimentel-Coelho 2010 (134)
	P7 rats, HI 150min	2–3 h after HI	1.5×10^4 cells (± mannitol)	IV jugular	14 days	Few cells in ischemic hippocampus	NA	NA	7, 14 days	20–25% improvement in rotarod and elevated body swing tests	Increased growth factors in brain, CA1 dendrites	Yasuhara 2010 (138)
	P7 rats, HI 80 min	24 h after HI	1×10^7 cells	IP	42 days	Many cells in peri-infarct area	42 days	No change in size of hemispheric lesion	42 days	Improved sensorimotor function, cortical maps, and receptive fields, and reduced hyperexcitability		Geissler 2011 (135)
	P7 rats, HI 80 min	24 h after HI	1×10^7 cells	IT	14 days	hUCB cells were localized in astrocyte-rich zone	2, 14, and 44 days	Decreased activation of microglia/macrophages and reactive astrogliosis, and reduced peri-lesional astrocytic wall	14, 44 days	Improved motor function (forelimb use bias, muscle strength and distal spasticity) both short- and long-term	Downregulation of Connexin 43	Wasielewski 2012 (136)
	P7 rats, HI 80 min	24 h after HI	1×10^7 cells	IP	NA	NA	2, 14 days	Decreased lesion-induced apoptosis, increased neurons	NA	NA	Increased the expression of proteins Tie-2, occludin, BDNF and VEGF in the lesioned brain	Rosenkranz 2012 (141)
	P7 rats, HI 120 min	2 h after HI	1×10^6, 1×10^7, 1×10^8 cells	IV jugular	7 days	Cells in the cortex and the hippocampus	8 weeks	No change in low-dose group. Decreased brain atrophy in medium- and high-dose groups	8 weeks	Cognitive improvement at the highest dose only		de Paula 2012 (133)
	P7 rats, HI 90 min	24 h after HI	1×10^7 cells	IV jugular	1, 3, and 10 weeks	Many cells were in ischemic periventricular region at 1 week, but very few at 3 and 10 weeks	10 weeks	No decrease in tissue loss volume, decreased neuronal loss in neocortex	10 weeks	Improved performance in a battery of behavioral tests		Bae 2012 (139)
	P7 rats, HI 120 min (+cyclosporin A)	24 h after HI	3×10^6 cells	IVen	NA	NA	24, 72 h, 7, 14 days	Decreased neuronal loss in cortex and CA1 of the hippocampus	NA	NA	Increased Shh and Gli1 protein levels	Wang 2014 (137)
hUCB–CD34+	P12 SCID mice, MCAO	48 h after HI	1×10^5 cells	IV femoral	24 h, 10 days	Few cells at 24 h, very few at 10 days	7 weeks	Decreased brain atrophy	9 days, 7 weeks	No effect on rotarod or open-field tests	Transient augmentation of CBF in peri-infarct area	Tsuji 2014 (122)

(Continued)

Table 1 | Continued

Cell type	Animal model	Administration			Engraftment		Histology assessment		Functional assessments		Other	Reference
	Injury type	Timing	Dose	Route	Days	Results	Days	Outcomes	Days	Outcomes		
hUCB–MSCs (passage 10)	P7 rats, HI 150 min (+cyclosporin A)	3 days after HI	1×10^5 cells	Intra-cerebral	7 days	Differentiation into astrocytes but not neurons	28 days	Reduced cortical neuronal loss	14, 21, 28 days	Improved neurological score		Xia 2010 (119)
(passage 5)	P10 rats, MCAO	6 h after HI	1×10^5 cells	IVen	NA	NA	28 days	Decreased apoptosis, microglial activation and astrogliosis in penumbra	28 days	Functional improvements (rotarod and cylinder test). GM1 enhanced the behavioral recovery	Improved survival and body weight gain, decreased lesion volume on MRI	Kim 2012 (120)
hUC-MSCs (passage 3)	P7 rats, HI 120 min (+cytarabine)	24 or 72 h after HI	5×10^6 cells (± GM1)	IP or IV jugular	35 days	More cells were in ischemic frontal cortex after iv than ip, with neural differentiation around infarct region	35 days	Decreased gliosis in ischemic regions	7 days, 20 days, 4 weeks	More improved locomotor function in animals given cells at 24 h than 72 h		Zhang 2014 (130)

BDNF, brain-derived neurotrophic factor; CA1, cornu ammonis 1 of the hippocampus; CBF, cerebral blood flow; GM1, ganglioside; HI, hypoxic–ischemia (unilateral ligation of the carotid artery followed by 8% oxygen systemic hypoxia); hUCB–MNCs, human umbilical cord-mononuclear cells; IP, intraperitoneal; IT, intrathecal; IV, intravenous; IVen, intraventricular; MCAO, middle cerebral artery occlusion; MSCs, mesenchymal cells; NA, not applicable; P, postnatal day; UC, umbilical cord; VEGF, vascular endothelial growth factor.

been investigated. Thus, the engraftment potential of UCB cells remains poorly characterized, and requires further investigation in studies using autologous transplantation.

NEUROPROTECTIVE PROPERTIES OF UMBILICAL CORD BLOOD

A number of studies have demonstrated significant and reproducible neuroprotective effects in rodent models of term neonatal hypoxia–ischemia using UCB–MNCs (127, 133–139), UCB–MSCs (119, 120), or UCB–CD34+ cells (122). Meier and colleagues first showed that intra-peritoneal administration of hUCB–MNCs alleviated spastic paresis in the Rice–Vannucci model of neonatal hypoxic-ischemic rats (127, 140). Following this, other rodent studies have shown that hUCB–MNCs induce significant improvements in sensorimotor performance (134–136, 138) and reduction in neuronal loss (133, 134, 137–139, 141). Recent studies also showed long-lasting neuroprotective effects of hUCB–MNCs in behavioral and cognitive outcomes at 8 and 10 weeks after ischemic insult (133, 139), with decreased brain atrophy (133). Animals treated with intra-cerebral hUCB–MSCs also demonstrated improved neurological function and tissue repair (119, 120).

From pre-clinical results obtained to date, we hypothesize that UCB cells may act in a neuroprotective manner via diverse actions, including anti-inflammatory effects, immunomodulation, and neurotrophic growth factor release to promote endogenous neurogenesis.

ANTI-INFLAMMATORY AND IMMUNO-MODULATORY ACTIONS OF UMBILICAL CORD BLOOD

A principal mechanism whereby UCB cells regulate neurological repair is via anti-inflammatory actions. UCB administration can dampen the expression of pro-inflammatory cytokines (IL-1α, IL-6, IL-1β, and TNF-α), enhance anti-inflammatory cytokines (IL-10), secrete chemotactic proteins (monocyte chemotactic protein 1), and modulate immune macrophage and T cell function (142, 143). As described above, hypoxia–ischemia induces an acute brain inflammatory response with activation of microglia and macrophages and reactive astrogliosis associated with peri-lesional up-regulation of connexin 43, the major astrocytic gap junction protein (144). Administration of hUCB–MNCs normalizes inflammatory balance, reduces microgliosis and astrogliosis (134, 136), and down-regulates connexin 43, which in turn restores BBB function to moderate inflammatory cell influx into the brain (136).

NEUROTROPHIC FACTOR ACTIONS OF UMBILICAL CORD BLOOD

Transplanted UCB–MNCs or MSCs reportedly enhance neurological recovery via secretion of a wide variety of trophic factors including BDNF, glial cell line-derived neurotrophic factor (GDNF), nerve growth factors NT-3 and NT-5, angiogenin, VEGF, fibroblast growth factor-2, and epidermal growth factor. Together, these act to promote endogenous neuronal growth and neurogenesis, angiogenesis, encourage remyelination, and synaptic connections, and decrease cellular apoptosis (141, 145–147). Transplantation of hUCB is associated with reduced levels of cleaved-caspase-3 protein in hypoxic-ischemic newborn rats, indicative of reduced apoptosis, with BDNF identified as playing

a role in inhibition of apoptosis and inflammation (141). Further, hUCB cell administration after hypoxia–ischemia increases expression of Tie-2 and occludin proteins, and increases expression of VEGF, indicating that UCB transplantation may increase endogenous angiogenesis and improved BBB integrity within the damaged brain. *In vivo*, MSCs provide trophic neuroprotection following injury by secreting physical tissue scaffold to surrounding tissues, while UCB–CD34+ cell transplantation enhances functional recovery and reduces both infarction and apoptosis in a rat model of spinal cord injury, mediated by the production of both VEGF and GDNF (148). UCB-derived CD133+ cells promote a threefold improvement in axonal regrowth, a 35% reduction in apoptosis and vascular and neuronal protection following hypoxia on organ co-culture of brain motor cortex cells and spinal cord from postnatal day 3 rats, suggesting that the trophic effects from CD133+ cells contributes to neuroprotection (149).

Hypoxia–ischemia stimulates endogenous proliferation of NPCs (150). However, if an insult is severe, brain damage still occurs, and the restorative proliferation of NPCs may be ameliorated, with activated NPCs failing to survive to mature neurons (150, 151) or differentiating into astrocytes (152). Recently UCB–MNC transplantation has been shown to promote the proliferation of endogenous NPCs, and reduce glial differentiation, an action mediated via the Sonic Hedgehog signaling pathway, resulting in the alleviation of brain injury in hypoxic-ischemic neonatal rats (137). These results support the ability of UCB cells to respond to insult with paracrine and trophic actions, initiating a regenerative environment mediated by resident cell populations within the brain.

PRETERM BRAIN INJURY ANIMAL MODELS AND CELL THERAPY

The majority of experimental studies described above that have investigated the neuroprotective actions of hUCB have been undertaken in rodent models of neonatal (term) hypoxic-ischemic brain injury (119, 120, 122, 127, 133–139). Injury to the human brain at the time of term birth induced by hypoxia–ischemia predominantly causes deep gray matter neuronal injury within the basal ganglia and hippocampus, together with injury to neighboring white matter – this is appropriately reflected in rodent and large animal studies of term hypoxia–ischemia. However, this distribution of injury is quite different in preterm WMI, reflecting susceptibility and region-specific effects following hypoxia–ischemia and other insults as brain maturation progresses.

Animal models exploring the injury profile and mechanisms of preterm pre-oligodendrocyte and WMI, have utilized either an hypoxic-ischemic insult, or exposure to LPS-induced inflammation. Excitotoxicity models such as administration of excitatory amino acid agonists quinolinic acid and ibotenate have also been used (26, 153). It is important to note that preterm infants mostly suffer hypoxic-ischemic insults that are subacute or chronic, in contrast to term infants where HIE is principally due to an acute severe insult (154). Irrespective of which experimental insult is utilized, the maturational age of the CNS is critical (26). In addition, the choice of species is important. It is well described that induction of predominant WMI is problematic in rats and mice due to the different CNS anatomy of

rodents that, besides being non-gyrencephalic and having a different vascular anatomy, demonstrates a much lower white/gray matter ratio than in humans. In contrast, the pattern of WMI in rabbits, cats, dogs, and sheep has a distribution and morphological appearance closer to that of human preterm brain injury induced by either hypoxia–ischemia or LPS administration (26, 59). The fetal rabbit brain myelinates with a similar perinatal time course to the human, and maturation of oligodendrocytes begins antenatally (155). *In utero*, hypoxia–ischemia to the preterm rabbit fetus causes postnatal hypertonic motor deficits that resembles CP, making rabbits a very good model for postnatal behavioral studies (156). Many further studies have been undertaken utilizing fetal sheep models of preterm WMI because of their abundance of cerebral white matter, their anatomic similarities to the preterm infant, and an ability to monitor the systemic and brain response to insult (37, 59, 157). However, because of cost, availability of antibodies/sequence data and genetically modified animals, rodent WMI studies are valued as complementary models (26). To date, only hAECs have been examined in a non-rodent (fetal sheep) model of preterm WMI. Yawno and colleagues demonstrated that administration of hAECs suppressed the up-regulation of activated microglia, and reduced gray and WMI in response to LPS in preterm fetal sheep (88) (**Table 2**).

PRETERM BRAIN INJURY AND UMBILICAL CORD BLOOD

Hall and colleagues demonstrated that in postnatal day 2 rats, intravenous hUCB–MNC administration preserves white matter structures following an hypoxic-ischemic insult. This timeframe corresponds to the period of white matter vulnerability in human preterm infants between 24 and 30 weeks of gestational age (158). Specifically, IV infusion of hUCB–MNCs at 48 h post-ischemia reduced WMI based on quantification of myelin basic protein. A direct protective effect of UCB–MNCs on oligodendrocyte injury induced by oxygen/glucose deprivation (OGD), which produces hypoxic-ischemic-like injury *in vitro*, was also identified (158). Although the data are limited, it appears that hUCB–MNCs have therapeutic potential for the protection of oligodendrocytes and thereby prevention of WMI in a premature rat model of ischemia.

ANTIEPILEPTIC EFFECTS OF UMBILICAL CORD BLOOD

The incidence of seizures in very low birth weight infants is 5.6%, while the occurrence in those infants identified as having PVL is 18.7% (159, 160). Seizures are typically observed in more severe cases of PVL and those born at lower gestational ages and birth weights (159, 160). Recent studies demonstrate the antiepileptic actions of hUCB–MNCs. Transplantation of hUCB–MNCs 90 min after the onset of status epilepticus in rats, induced by lithium and pilocarpine chloride, protected against neuronal loss in the hippocampus for up to 300 days. Additionally, MNC-transplanted rats had reduced frequency and duration of recurrent seizures, suggesting early administration could protect against the establishment of epilepsy (161). Furthermore in a single case of an infant with infantile spasms (West syndrome) and X-linked T/B + NK-severe combined immunodeficiency, allogeneic UCB transplantation together with topiramate and immune-modulating agents (corticosteroids, intravenous immunoglobulin,

Table 2 | Outcome of cell-based interventions in preterm brain injury.

Cell type	Animal model	Administration			Engraftment		Histology assessments		Functional assessments		Others	Reference
	Injury type	Timing	Dose	Route	Days	Results	Days	Outcomes	Days	Outcomes		
hUCB–MNCs	P2 rats, MCAO	48 h after stroke	1×10^6 cells	IV	NA	NA	4 days	Reduced white matter damage	NA	NA	UCB–MNCs directly reduced apoptosis of oligodendrocytes cultured under oxygen glucose deprivation *in vitro*	Hall 2008 (158)
hUCB–MNCs	P5 rats, excitotoxicity (ibotenate)	within 6 h or 24 h after injection	1×10^6 or 10^7 cells	IP or IV	5 days	No cells detected	5 days	No changes in lesion size, microglial activation, astrogliosis, or cell proliferation. Increased white matter damage with increased microglial activation by ip administration	NA	NA		Dalous 2013 (89)
hUC-MSCs (Passage 3)	P3 rats, HI 240 min	0, 1, 2 days after HI, once a day	1×10^6 cells, 3 times	IP	24 h	Cells migrated mainly toward the injured hemisphere	7, 18 days	Increased mature oligodendrocytes counts. Decreased astrocytosis and microglial activation	27 days	Improved exploratory behavior, mental stress and motor function		Zhu 2014 (174)
hUCB–MSCs	P4 rats, blood injection into lateral ventricle	P6	1×10^5 cells	IVen	NA	NA	28 days	Improvements of corpus callosal thickness and myelin basic protein expression reduction. Attenuation of astrogliosis and cell death	28 days	Improved behavioral tests (negative, geotaxis test and rotarod test)	Attenuation of post-hemorrhagic hydrocephalus development by MRI. Decreased inflammatory cytokines expression in CSF (IL-1α, IL-1β, IL-6, and TNF-α)	Ahn 2013 (121)
hAECs	117 days GA fetal sheep, LPS	0, 6 and 12 h after LPS	IT 1.8×10^8 cells, or IV 9×10^7 cells, or IT 9×10^7 + IV 9×10^7 cells	7d	Cells were detected in 2 of 14 fetal brains	7 days	Decreased activated microglia in the cortex, subcortical and periventricular white matter. Decreased apoptosis in the cortex and periventricular white matter	NA			Yawno 2013 (88)

CSF, cerebral spinal fluid; GA, gestational age; HI, hypoxic–ischemia (unilateral ligatiion of the carotid artery followed by 6% oxygen systemic hypoxia); hAECs, human amnion epithelial cells; hUCB–MNCs, human umbilical cord-mononuclear cells; IP, intraperitoneal; IT, intrathecal; IV, intravenous; IVen, intraventricular; MCAO, middle cerebral artery occlusion; MSCs, mesenchymal cells; MRI, magnetic resonance imaging; NA, not applicable; P, postnatal day; UC, umbilical cord; IL, interleukin; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α.

and tacrolimus) improved seizures, possibly contributed by an immuno-modulatory effect of UCB–MNCs (162).

SUPPRESSION OF EXCITOTOXICITY BY UMBILICAL CORD BLOOD

Suppression of excitotoxicity is a further important subject of investigation for protecting the developing brain. The potential therapeutic effects of stem cells in animal models of excitotoxic brain injury have been examined using the *N*-methyl-D-aspartate receptor agonist, quinolinic acid, to induce apoptosis and cleaved caspase-3 and excitotoxic damage in the neocortex, hippocampus, striatum, white matter, and subventricular zone, in the newborn mouse brain. Injection of human embryonic germ cell-derived NPCs partially restores the complement of striatal neurons, with engraftment of the transplanted cells in injured sites and their differentiation into neuronal and glial cells (163). In contrast, intra-peritoneal and intravenous hUCB–MNCs administration could not promote brain repair in ibotenate-induced excitotoxic brain lesions in neonatal rats. The authors of this recent study did, however, suggest that the intra-peritoneal injection of high amounts of hUCB–MNCs may have aggravated WMI, possibly due to systemic inflammation (89).

ANTIOXIDANT EFFECTS OF UMBILICAL CORD BLOOD

There is increasing evidence that stem cells, especially young cells, possess antioxidant potential, which may then contribute to anti-apoptotic effects (164–166). A recent paper showed that hUCB–NPCs, a neuronal phenotype differentiated from collagen-adherent hUCB–MNCs, induced neuroprotection via an antioxidant effect, decreasing free radical levels by 95% (167). Human MSCs *in vitro* also scavenge oxygen and nitrogen free radicals, constitutively express antioxidant enzymes, and themselves are highly resistant to oxidative stress-induced death (166). It is still unclear whether cells derived from UCB can mediate tissue oxidative stress *in vivo*. Since oxidative stress is known to play an important role in the progression of brain injury in preterm infants, this is a crucial consideration for the ability of UCB–MNCs to mediate the progression of preterm brain injury.

VASCULAR DEVELOPMENT, INTRAVENTRICULAR HEMORRHAGE, AND UMBILICAL CORD BLOOD

Preterm infants are highly vulnerable to IVH due to their maturation-dependent vascular vulnerability, localized to the area of the periventricular germinal matrix and possibly in part due to a coagulation system deficit of prematurity (9, 168). Preterm infants predominantly develop IVH in the first week after birth (169). While grades 1 and 2 IVH cause little neurological harm, >50% of infants with severe IVH (grades 3 and 4) die or develop post-hemorrhagic hydrocephalus (PHH). The incidence of severe IVH in very preterm infants ranges from <5 to 20% (170). IVH is observed in 25% of infants with PVL and worsens WMI by increasing the amount of iron that combines with harmful free radicals and inflammatory cytokines to exacerbate injury (171, 172). The incidence of IVH has declined with current neonatal intensive care practices, but it remains an important problem, for which there is no targeted treatment (170). A recent paper demonstrated that intraventricular administration of hUCB–MSCs attenuates brain damage after severe IVH in newborn rats. The anti-inflammatory effects of MSCs (i.e., reducing the expression of inflammatory cytokines, such as IL-1α, IL-1β, IL-6, and TNF-α) were hypothesized to contribute to the prevention of ventricular dilation and neuroprotection (121). In adult rats, IV administration of hUCB–MNCs also showed amelioration of neurologic deficits associated with intra-cerebral hemorrhage (173).

OLIGODENDROCYTES, MYELIN DEFICITS, AND UMBILICAL CORD BLOOD

Loss of pre-oligodendrocytes and hypomyelination are the principal characteristics of preterm WMI and therefore protection of oligodendrocyte lineage cells must be central to the development of any neuroprotective strategies for the preterm brain. Recently, Zhu and colleagues have shown that hUC–MSCs increase mature oligodendrocyte number and improve long-term functional outcomes following hypoxia–ischemia in postnatal day 3 rats, with engraftment of cells at lesion sites (174). It will, however be important to further elucidate whether UCB stem cells can reduce pre-oligodendrocyte injury and thereby restore myelination in fetal or neonatal animal models of WMI. There is some indirect evidence that UCB cell populations may infer benefit to oligodendrocytes and myelination. The rare but serious genetic disorders termed leukodystrophies cause degeneration of myelin and progressive neurological deterioration and, to date, the only known treatment option for leukodystrophies is early transplantation of HSCs (175). Experimentally, spontaneous myelin mutants have been used to study potential therapies. The most commonly used myelin mutant in transplant experiments is the shiverer mouse, which has a mutation in the myelin basic protein gene, and has been extensively used to study myelination by exogenous cell transplantation, including HSCs, MSCs, and oligodendrocytes progenitor cells (OPCs). OPCs can be isolated, differentiated, and expanded from both fresh and cryopreserved UCB (176, 177) offering a potential treatment option, and lay the foundations for future studies in this research field.

CLINICAL TRIALS FOR CEREBRAL PALSY

There are currently a number of clinical trials listed, or recently completed, for treatment of children with established CP. A pilot study from Hanyang University Medical Center, Republic of Korea examined 20 children aged 2–10 years with clinical CP, who were born either preterm or term, and administered peripheral autologous UCB–MNCs. Neurodevelopmental outcomes and neuroimaging studies were conducted up to 24 weeks after UCB administration, and compared with a pre-infusion baseline. Functional improvements were demonstrated in 25% of patients, and improvements in brain imaging outcomes were also noted in children with neurodevelopmental recovery. Side effects were identified in 25% of participants during infusion, treated successfully with antihistamines and hydration. Although not powered to demonstrate statistical benefit, the study showed the potential and safety of autologous UCB–MNC treatment in children (178). Between 2009 and 2012, Duke University in the USA treated 23 term newborns identified with HIE soon after birth with autologous UCB administration. The study was able to demonstrate feasibility and safety of autologous UCB re-administration in

combination with hypothermia, targeting UCB administration at 6 h. Neurodevelopmental outcomes were recorded at 1 year of age, however, a greater number of babies will be required to appropriately assess functional outcomes (179). Duke University is continuing to recruit for a larger study of autologous UCB administration for children with established spastic CP.

USE AND EFFICACY OF COMBINATION THERAPIES

A recent clinical study has assessed allogeneic UCB administered in combination with erythropoietin (EPO – itself the subject of neuroprotective trials), cyclosporine (an immunosuppressant), and rehabilitation therapy at Bundang CHA Hospital, Republic of Korea. Ninety-six children with CP aged 10 months to 10 years were treated with UCB cells, and improved cognitive and motor function were observed in all groups, including placebo, but with greater improvements in UCB + EPO children (180). Due to its design, this study does not separate the neuroprotective effects of EPO from UCB. However, combination therapy, which targets different mechanisms and therapeutic windows, may be a useful approach to treat preterm infants because of their multifactorial causes of injury and the inherent difficulties with identifying a therapeutic time frame in these infants. Indeed, ganglioside and mannitol have been shown to enhance the neuroprotective benefits of hUCB–MNCs and hUC–MSCs treatments following neonatal asphyxia in pre-clinical studies (130, 138). In contrast, despite recent promising neuroprotective outcomes of EPO in preterm cohort (181), routine clinical use of EPO, especially by high-dose, has always been hampered by its risk for retinopathy of prematurity (182). Melatonin, a powerful antioxidant shown to protect the developing brain by reducing oxidative stress following hypoxia–ischemia, with an absence of side effects, may be a candidate for co-administration with UCB (183). In term infants with HIE, hypothermia has been standard neuroprotective therapy for a number of years (184), and combination treatment of moderate hypothermia with MSCs significantly improves neuronal survival and mitochondrial activity after OGD exposure *in vitro* (185). Clinically, Cotten and colleagues recently showed that hypothermia and autologous UCB combination treatment is feasible and safe in term infants with HIE (179), and reflects that any treatment for term HIE must be considered in the context of therapeutic hypothermia. However, in very preterm and extremely preterm infants hypothermia is not currently recommended and may increase the risk of complications or death (186). A phase 1 clinical study of selected head cooling for preterm infants, born 32–35 weeks gestation, with neonatal HIE has recently been completed [NCT00620711], and the results are awaited with interest.

Other types of stem and stem-like cells may also be used for the combination therapy with UCB. UC, in addition to UCB, provides an abundant and non-invasive source of MSCs. These cells are neuroprotective in hypoxic-ischemic brain injury, and share similar *in vitro* immunosuppressive properties with bone marrow- and UCB-derived MSCs as well as mediating monocyte function to suppress T cell proliferation (130, 187). Importantly, hUC–MSCs also protect oligodendrocytes, reduce astrogliosis, and improve long-term functional outcomes in a model of preterm postnatal day 3 rats hypoxia–ischemia (174). Moreover, hUC–MSCs undergo successful cell expansion using animal serum-free culture medium, thereby removing safety concerns of animal-to-human viral transmission, further encouraging their potential for clinical application (188, 189). hAECs may present a useful therapy in combination with UCB, hypothermia, or alternate therapies. The proven anti-inflammatory properties of hAECs appear a principal mechanism to reduce preterm brain injury (88). They display both embryonic and pluripotent stem cells with abundant quantity, do not express MHC class molecules so have low immunogenicity, and do not form teratomas (190). Furthermore, the ready availability of hAECs without the need of expansion may enable them to be used for early autologous transplantation for preterm brain injury (88, 188, 189), with or without combination therapies.

OPTIMAL TIMING OF TRANSPLANTATION OF UMBILICAL CORD BLOOD

Recent experimental studies have been aimed at identifying the therapeutic window for UCB therapy. In adult rats who underwent middle cerebral artery occlusion-induced stroke, intravenous administration of hUCB–MNCs within 72 h resulted in an early functional recovery with lesion improvement, however cell administration at 120 h provided only minor functional recovery, and treatment at 14 days did not show any benefit (132). Whether a similar result can be obtained in an autologous or allogeneic setting is unknown. However, given that one of the primary benefits of UCB cells is their anti-inflammatory actions, it is likely that early intervention may be of greater benefit. Indeed, current ongoing clinical trials for neonatal HIE by National University Hospital, Singapore, and Duke University, USA are giving autologous UCB within the first 3 and 14 days, respectively after term birth asphyxia (NCT01649648 and NCT00593242). However, in preterm infants, it is difficult to know the timing of WMI that results in cystic PVL or diffuse WMI (9, 184, 191) and therefore either combination therapies, or cell preparations with multiple benefits would be most appropriate.

CLINICAL TRIALS FOR PRETERM BRAIN INJURY

No trials of neuroprotective UCB for use in treating WMI in preterm infants are currently registered in humans. A significant challenge in the design of a clinical trial for preterm infants is the question of which UCB cells to administer? As described above, it is becoming apparent that the type and quantity of specific cell types differs in preterm UCB from that in term UCB. A dose–response effect of UCB therapy for neonatal hypoxia–ischemia has been demonstrated (133, 192), but it is unclear whether a therapeutic quantity of cells can be derived from preterm UCB as the volumes obtained are low (see Umbilical Cord Blood above). Further to this, the ability to expand preterm UCB cells is not yet well described.

In preterm infants, as also discussed above, the timing of the onset, and chronic progression of WMI is usually not known. It is also not known whether to administer UCB cells before or after brain injury is identified. Administration following the identification of brain injury may provide better outcomes than administration in later childhood, due to the plasticity of the developing brain; although the evidence for this both in pre-clinical studies and clinical trials is sparse. In contrast, as cystic PVL or diffuse WMI tend to develop over days to weeks after birth (72, 169), early postnatal UCB administration "before defining

brain damage" in preterm and extremely low birth weight infants may be more efficacious. Indeed, two clinical trials administering autologous UCB to preterm infants in the first 5 or 14 days post-delivery, aiming to examine feasibility and efficacy for a variety of preterm complications, are currently underway (NCT02050971 and NCT01121328). However, in preterm infants susceptible to WMI at least until 32 weeks or more gestational age, a single administration of cells might not span an adequate period of brain protection. Thus, the need for repeated dose administration and expansion of UCB samples are further exemplified in a preterm cohort. Recently described *in vivo* cell tracking methodology using MRI, which enables the tracking of migration and distribution of magnetically labeled cells in tissues, may be useful for optimizing the time course for UCB treatment (193).

CONCLUSION

Taking into account the similarities and differences in preterm versus term brain injury, and limitations to date in stem cell studies for preterm WMI, it is apparent that a number of considerations apply before UCB treatment could be extended to infants born preterm. The clear advantage of undertaking UCB administration in a preterm cohort is the relative plasticity of the developing brain in immature infants, and potential for regeneration. However, there are current disadvantages that must be overcome. Studies to date suggest that early cell administration post-injury achieves favorable therapeutic outcomes, but a current lack of sensitive diagnostic tools and inability to accurately determine the onset of preterm brain injury remains problematic. Work to define the most appropriate time for therapeutic intervention is needed. Additionally, the specific cells present in UCB responsible for brain protection are not yet characterized and the complications of pregnancy that are often co-morbidities with preterm birth, such as uteroplacental inflammation or IUGR, may alter the cellular composition of UCB. A handful of published work suggests that preterm UCB cell number, cell total population and cell maturity is different to that in term UCB, which may not provide the expected benefit that has been observed using term hUCB in experimental animal and clinical studies. It is therefore currently not known whether autologous or allogeneic UCB cell administration would confer optimal benefit in a preterm cohort, or whether expansion of specific cell types should be considered and pursued. Thus it remains that UCB holds strong promise for the treatment of preterm brain injury in the neonate, but fundamental questions must be answered with appropriately designed experimental animal and clinical studies prior to large-scale randomized clinical trials for preterm brain injury.

ACKNOWLEDGMENTS

REFERENCES

1. Costeloe KL, Hennessy EM, Haider S, Stacey F, Marlow N, Draper ES. Short term outcomes after extreme preterm birth in England: comparison of two birth cohorts in 1995 and 2006 (the EPICure studies). *BMJ* (2012) **345**(7886):e7976. doi:10.1136/bmj.e7976
2. Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller AB, Narwal R, et al. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *Lancet* (2012) **379**(9832):2162–72. doi:10.1016/S0140-6736(12)60820-4
3. Wen SW, Smith G, Yang Q, Walker M. Epidemiology of preterm birth and neonatal outcome. *Semin Fetal Neonatal Med* (2004) **9**(6):429–35. doi:10.1016/j.siny.2004.04.002
4. Blencowe H, Cousens S, Chou D, Oestergaard M, Say L, Moller AB, et al. Born too soon: the global epidemiology of 15 million preterm births. *Reprod Health* (2013) **10**(Suppl):1. doi:10.1186/1742-4755-10-S1-S2
5. Larroque B, Ancel PY, Marret S, Marchand L, André M, Arnaud C, et al. Neurodevelopmental disabilities and special care of 5-year-old children born before 33 weeks of gestation (the EPIPAGE study): a longitudinal cohort study. *Lancet* (2008) **371**(9615):813–20. doi:10.1016/S0140-6736(08)60380-3
6. Robertson CMT, Watt MJ, Yasui Y. Changes in the prevalence of cerebral palsy for children born very prematurely within a population-based program over 30 years. *J Am Med Assoc* (2007) **297**(24):2733–40. doi:10.1001/jama.297.24.2733
7. Pharoah POD, Platt MJ, Cooke T. The changing epidemiology of cerebral palsy. *Arch Dis Child Fetal Neonatal Ed* (1996) **75**(3):F169–73. doi:10.1136/fn.75.3.F169
8. Thorngren-Jerneck K, Herbst A. Perinatal factors associated with cerebral palsy in children born in Sweden. *Obstet Gynecol* (2006) **108**(6):1499–505. doi:10.1097/01.AOG.0000247174.27979.6b
9. Volpe JJ. *Neurology of the Newborn*. Philadelphia: Elsevier Health Sciences (2008).
10. Behrman RE, Adashi EY, Allen MC, Caruso RL, Culhane J, Schetter CD, et al. Committee on understanding premature birth and assuring healthy outcomes. In: Behrman RE, Butler AS, editors. *Preterm Birth: Causes, Consequences, and Prevention*. Washington DC: Institute of Medicine of the National Academies (2007). p. 1–772.
11. Honeycutt A, Dunlap L, Chen H, Homsi G, Grosse S, Schendel D. Economic costs associated with mental retardation, cerebral palsy, hearing loss, and vision impairment United States, 2003. *MMWR Morb Mortal Wkly Rep* (2004) **53**(3):57–9.
12. Back SA. Perinatal white matter injury: the changing spectrum of pathology and emerging insights into pathogenetic mechanisms. *Ment Retard Dev Disabil Res Rev* (2006) **12**(2):129–40. doi:10.1002/mrdd.20107
13. Oka A, Belliveau MJ, Rosenberg PA, Volpe JJ. Vulnerability of oligodendroglia to glutamate: pharmacology, mechanisms, and prevention. *J Neurosci* (1993) **13**(4):1441–53.
14. Fern R, Möller T. Rapid ischemic cell death in immature oligodendrocytes: a fatal glutamate release feedback loop. *J Neurosci* (2000) **20**(1):34–42.
15. Dammann O, Leviton A. Maternal intrauterine infection, cytokines, and brain damage in the preterm newborn. *Pediatr Res* (1997) **42**(1):1–8. doi:10.1203/00006450-199707000-00001
16. Segovia KN, McClure M, Moravec M, Luo NL, Wan Y, Gong X, et al. Arrested oligodendrocyte lineage maturation in chronic perinatal white matter injury. *Ann Neurol* (2008) **63**(4):520–30. doi:10.1002/ana.21359
17. Buser JR, Maire J, Riddle A, Gong X, Nguyen T, Nelson K, et al. Arrested pre-oligodendrocyte maturation contributes to myelination failure in premature infants. *Ann Neurol* (2012) **71**(1):93–109. doi:10.1002/ana.22627
18. Volpe JJ. Cerebral white matter injury of the premature infant More common than you think. *Pediatrics* (2003) **112**(1 I):176–80. doi:10.1542/peds.112.1.176
19. Krägeloh-Mann I, Petersen D, Hagberg G, Vollmer B, Hagberg B, Michaelis R. Bilateral spastic cerebral palsy – MRI pathology and origin. Analysis from a representative series of 56 cases. *Dev Med Child Neurol* (1995) **37**(5):379–97. doi:10.1111/j.1469-8749.1995.tb12022.x
20. Whitaker AH, Van RossemR, Feldman JF, Schonfeld IS, Pinto-Martin JA, Tore C, et al. Psychiatric outcomes in low-birth-weight children at age 6 years: relation to neonatal cranial ultrasound abnormalities. *Arch Gen Psychiatry* (1997) **54**(9):847–56. doi:10.1001/archpsyc.1997.01830210091012
21. Koerte I, Pelavin P, Kirmess B, Fuchs T, Berweck S, Laubender RP, et al. Anisotropy of transcallosal motor fibres indicates functional impairment in children with periventricular leukomalacia. *Dev Med Child Neurol* (2011) **53**(2):179–86. doi:10.1111/j.1469-8749.2010.03840.x
22. Hoon AHJr, Stashinko EE, Nagae LM, Lin DD, Keller J, Bastian A, et al. Sensory and motor deficits in children with cerebral palsy born preterm correlate with diffusion tensor imaging abnormalities in thalamocortical pathways. *Dev Med Child Neurol* (2009) **51**(9):697–704. doi:10.1111/j.1469-8749.2009.03306.x

23. Khwaja O, Volpe JJ. Pathogenesis of cerebral white matter injury of prematurity. *Arch Dis Child Fetal Neonatal Ed* (2008) **93**(2):F153–61. doi:10.1136/adc.2006.108837

24. Takashima S, Tanaka K. Development of cerebrovascular architecture and its relationship to periventricular leukomalacia. *Arch Neurol* (1978) **35**(1):11–6. doi:10.1001/archneur.1978.00500250015003

25. Ballabh P, Braun A, Nedergaard M. Anatomic analysis of blood vessels in germinal matrix, cerebral cortex, and white matter in developing infants. *Pediatr Res* (2004) **56**(1):117–24. doi:10.1203/01.PDR.0000130472.30874.FF

26. Hagberg H, Peebles D, Mallard C. Models of white matter injury: comparison of infectious, hypoxic-ischemic, and excitotoxic insults. *Ment Retard Dev Disabil Res Rev* (2002) **8**(1):30–8. doi:10.1002/mrdd.10007

27. Pierson CR, Folkerth RD, Billiards SS, Trachtenberg FL, Drinkwater ME, Volpe JJ, et al. Gray matter injury associated with periventricular leukomalacia in the premature infant. *Acta Neuropathol* (2007) **114**(6):619–31. doi:10.1007/s00401-007-0295-5

28. Volpe JJ. Neurobiology of periventricular leukomalacia in the premature infant. *Pediatr Res* (2001) **50**(5):553–62. doi:10.1203/00006450-200111000-00003

29. Yoon BH, Park CW, Chaiworapongsa T. Intrauterine infection and the development of cerebral palsy. *BJOG* (2003) **110**(Suppl 20):124–7. doi:10.1046/j.1471-0528.2003.00063.x

30. Volpe JJ, Kinney HC, Jensen FE, Rosenberg PA. The developing oligodendrocyte: key cellular target in brain injury in the premature infant. *Int J Dev Neurosci* (2011) **29**(4):423–40. doi:10.1016/j.ijdevneu.2011.02.012

31. Arai Y, Deguchi K, Mizuguchi M, Takashima S. Expression of beta-amyloid precursor protein in axons of periventricular leukomalacia brains. *Pediatr Neurol* (1995) **13**(2):161–3. doi:10.1016/0887-8994(95)00149-A

32. Meng SZ, Arai Y, Deguchi K, Takashima S. Early detection of axonal and neuronal lesions in prenatal-onset periventricular leukomalacia. *Brain Dev* (1997) **19**(7):480–4. doi:10.1016/S0387-7604(97)00068-5

33. Rezaie P, Dean A. Periventricular leukomalacia, inflammation and white matter lesions within the developing nervous system. *Neuropathology* (2002) **22**(3):106–32. doi:10.1046/j.1440-1789.2002.00438.x

34. Kinney HC, Back SA. Human oligodendroglial development: relationship to periventricular leukomalacia. *Semin Pediatr Neurol* (1998) **5**(3):180–9. doi:10.1016/S1071-9091(98)80033-8

35. Sheldon RA, Chuai J, Ferriero DM. A rat model for hypoxic-ischemic brain damage in very premature infants. *Biol Neonate* (1996) **69**(5):327–41. doi:10.1159/000244327

36. Stoll G, Jander S, Schroeter M. Detrimental and beneficial effects of injury-induced inflammation and cytokine expression in the nervous system. *Adv Exp Med Biol* (2002) **513**:87–113.

37. Gunn AJ, Bennet L. Fetal hypoxia insults and patterns of brain injury: insights from animal models. *Clin Perinatol* (2009) **36**(3):579–93. doi:10.1016/j.clp.2009.06.007

38. Bennet L, Tan S, Van den Heuij L, Derrick M, Groenendaal F, van Bel F, et al. Cell therapy for neonatal hypoxia-ischemia and cerebral palsy. *Ann Neurol* (2012) **71**(5):589–600. doi:10.1002/ana.22670

39. Lorek A, Takei Y, Cady EB, Wyatt JS, Penrice J, Edwards AD, et al. 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**(6):699–706. doi:10.1203/00006450-199412000-00003

40. Narumiya S, Ohno M, Tanaka N, Yamano T, Shimada M. Enhanced expression of full-length TrkB receptors in young rat brain with hypoxic/ischemic injury. *Brain Res* (1998) **797**(2):278–86. doi:10.1016/S0006-8993(98)00385-0

41. Stone BS, Zhang J, Mack DW, Mori S, Martin LJ, Northington FJ. Delayed neural network degeneration after neonatal hypoxia-ischemia. *Ann Neurol* (2008) **64**(5):535–46. doi:10.1002/ana.21517

42. Barrett RD, Bennet L, Davidson J, Dean JM, George S, Emerald BS, et al. Destruction and reconstruction: hypoxia and the developing brain. *Birth Defects Res C Embryo Today* (2007) **81**(3):163–76. doi:10.1002/bdrc.20095

43. Haynes RL, Baud O, Li J, Kinney HC, Volpe JJ, Folkerth DR. Oxidative and nitrative injury in periventricular leukomalacia: a review. *Brain Pathol* (2005) **15**(3):225–33. doi:10.1111/j.1750-3639.2005.tb00525.x

44. Haynes RL, Folkerth RD, Keefe RJ, Sung I, Swzeda LI, Rosenberg PA, et al. Nitrosative and oxidative injury to premyelinating oligodendrocytes in periventricular leukomalacia. *J Neuropathol Exp Neurol* (2003) **62**(5):441–50.

45. Back SA, Luo NL, Mallinson RA, O'Malley JP, Wallen LD, Frei B, et al. Selective vulnerability of preterm white matter to oxidative damage defined by F2-isoprostanes. *Ann Neurol* (2005) **58**(1):108–20. doi:10.1002/ana.20530

46. Polglase GR, Miller SL, Barton SK, Kluckow M, Gill AW, Hooper SB, et al. Respiratory support for premature neonates in the delivery room: effects on cardiovascular function and the development of brain injury. *Pediatr Res* (2014) **75**(6):682–8. doi:10.1038/pr.2014.40

47. Yoon BH, Jun JK, Romero R, Park KH, Gomez R, Choi JH, et al. Amniotic fluid inflammatory cytokines (interleukin-6, interleukin-1β, and tumor necrosis factor-α), neonatal brain white matter lesions, and cerebral palsy. *Am J Obstet Gynecol* (1997) **177**(1):19–26. doi:10.1016/S0002-9378(97)70432-0

48. Yoon BH, Romero R, Yang SH, Jun JK, Kim IO, Choi JH, et al. Interleukin-6 concentrations in umbilical cord plasma are elevated in neonates with white matter lesions associated with periventricular leukomalacia. *Am J Obstet Gynecol* (1996) **174**(5):1433–40. doi:10.1016/S0002-9378(96)70585-9

49. Yoon BH, Romero R, Kim CJ, Jun JK, Gomez R, Choi JH, et al. Amniotic fluid interleukin-6: a sensitive test for antenatal diagnosis of acute inflammatory lesions of preterm placenta and prediction of perinatal morbidity. *Am J Obstet Gynecol* (1995) **172**(3):960–70. doi:10.1016/0002-9378(95)90028-4

50. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. *Lancet* (2008) **371**(9606):75–84. doi:10.1016/S0140-6736(08)60074-4

51. Resch B, Vollaard E, Maurer U, Haas J, Rosegger H, Müller W. Risk factors and determinants of neurodevelopmental outcome in cystic periventricular leucomalacia. *Eur J Pediatr* (2000) **159**(9):663–70. doi:10.1007/PL00008403

52. Bejar R, Wozniak P, Allard M, Benirschke K, Vaucher Y, Coen R, et al. Antenatal origin of neurologic damage in newborn infants. I. preterm infants. *Am J Obstet Gynecol* (1988) **159**(2):357–63. doi:10.1016/S0002-9378(88)80084-X

53. Silveira RC, Procianoy RS, Dill JC, da Costa CS. Periventricular leukomalacia in very low birth weight preterm neonates with high risk for neonatal sepsis. *J Pediatr* (2008) **84**(3):211–6. doi:10.2223/JPED.1777

54. Resch B, Neubauer K, Hofer N, Resch E, Maurer U, Haas J, et al. Episodes of hypocarbia and early-onset sepsis are risk factors for cystic periventricular leukomalacia in the preterm infant. *Early Hum Dev* (2012) **88**(1):27–31. doi:10.1016/j.earlhumdev.2011.06.011

55. Lehnardt S. Innate immunity and neuroinflammation in the CNS: the role of microglia in toll-like receptor-mediated neuronal injury. *Glia* (2010) **58**(3):253–63. doi:10.1002/glia.20928

56. Lehnardt S, Lachance C, Patrizi S, Lefebvre S, Follett PL, Jensen FE, et al. The toll-like receptor TLR4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the CNS. *J Neurosci* (2002) **22**(7):2478–86.

57. Billiards SS, Haynes RL, Folkerth RD, Trachtenberg FL, Liu LG, Volpe JJ, et al. Development of microglia in the cerebral white matter of the human fetus and infant. *J Comp Neurol* (2006) **497**(2):199–208. doi:10.1002/cne.20991

58. Jurewicz A, Matysiak M, Tybor K, Kilianek L, Raine CS, Selmaj K. Tumour necrosis factor-induced death of adult human oligodendrocytes is mediated by apoptosis inducing factor. *Brain* (2005) **128**(11):2675–88. doi:10.1093/brain/awh627

59. Peebles DM, Miller S, Newman JP, Scott R, Hanson MA. The effect of systemic administration of lipopolysaccharide on cerebral haemodynamics and oxygenation in the 0.65 gestation ovine fetus in utero. *BJOG* (2003) **110**(8):735–43. doi:10.1111/j.1471-0528.2003.02152.x

60. Duncan JR, Cock ML, Suzuki K, Scheerlinck JP, Harding R, Rees SM. Chronic endotoxin exposure causes brain injury in the ovine fetus in the absence of hypoxemia. *J Soc Gynecol Investig* (2006) **13**(2):87–96. doi:10.1016/j.jsgi.2005.12.003

61. Li N, Karin M. Is NF-kappaB the sensor of oxidative stress? *FASEB J* (1999) **13**(10):1137–43.

62. Duncan JR, Cock ML, Scheerlinck JP, Westcott KT, McLean C, Harding R, et al. White matter injury after repeated endotoxin exposure in the preterm ovine fetus. *Pediatr Res* (2002) **52**(6):941–9. doi:10.1203/00006450-200212000-00021

63. Kaukola T, Herva R, Perhomaa M, Pääkkö E, Kingsmore S, Vainionpää L, et al. Population cohort associating chorioamnionitis, cord inflammatory cytokines and neurologic outcome in very preterm, extremely low birth weight infants. *Pediatr Res* (2006) **59**(3):478–83. doi:10.1203/01.pdr.0000182596.66175.ee

64. Crowley PA. Antenatal corticosteroid therapy: a meta-analysis of the randomized trials, 1972 to 1994. *Am J Obstet Gynecol* (1995) **173**(1):322–35. doi:10.1016/0002-9378(95)90222-8

65. Robertson NJ, Tan S, Groenendaal F, van Bel F, Juul SE, Bennet L, et al. Which neuroprotective agents are ready for bench to bedside translation in the newborn infant? *J of Pediatr* (2012) **160**(4):544–52. doi:10.1016/j.jpeds.2011.12.052

66. Salmeen KE, Jelin AC, Thiet MP. Perinatal neuroprotection. *F1000Prime Rep* (2014) **6**:6. doi:10.12703/P6-6

67. Low JA. Determining the contribution of asphyxia to brain damage in the neonate. *J Obstet Gynaecol Res* (2004) **30**(4):276–86. doi:10.1111/j.1447-0756.2004.00194.x

68. Rees S, Harding R, Walker D. The biological basis of injury and neuroprotection in the fetal and neonatal brain. *Int J Dev Neurosci* (2011) **29**(6):551–63. doi:10.1016/j.ijdevneu.2011.04.004

69. Rees S, Inder T. Fetal and neonatal origins of altered brain development. *Early Hum Dev* (2005) **81**(9):753–61. doi:10.1016/j.earlhumdev.2005.07.004

70. Okumura A, Hayakawa F, Kato T, Itomi K, Maruyama K, Ishihara N, et al. Hypocarbia in preterm infants with periventricular leukomalacia: the relation between hypocarbia and mechanical ventilation. *Pediatrics* (2001) **107**(3):469–75. doi:10.1542/peds.107.3.469

71. Wiswell TE, Graziani LJ, Kornhauser MS, Stanley C, Merton DA, McKee L, et al. Effects of hypocarbia on the development of cystic periventricular leukomalacia in premature infants treated with high-frequency jet ventilation. *Pediatrics* (1996) **98**(5):918–24.

72. Volpe JJ. Brain injury in the premature infant: overview of clinical aspects, neuropathology, and pathogenesis. *Semin Pediatr Neurol* (1998) **5**(3):135–51. doi:10.1016/S1071-9091(98)80030-2

73. Vohr BR. Neurodevelopmental outcomes of extremely preterm infants. *Clin Perinatol* (2014) **41**(1):241–55. doi:10.1016/j.clp.2013.09.003

74. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. *Science* (1998) **282**(5391):1145–7. doi:10.1126/science.282.5391.1145

75. Roy NS, Cleren C, Singh SK, Yang L, Beal MF, Goldman SA. Functional engraftment of human ES cell-derived dopaminergic neurons enriched by coculture with telomerase-immortalized midbrain astrocytes. *Nat Med* (2006) **12**(11):1259–68. doi:10.1038/nm1495

76. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* (2007) **131**(5):861–72. doi:10.1016/j.cell.2007.11.019

77. Knoepfler PS. Deconstructing stem cell tumorigenicity: a roadmap to safe regenerative medicine. *Stem Cells* (2009) **27**(5):1050–6. doi:10.1002/stem.37

78. Payne N, Siatskas C, Bernard CCA. The promise of stem cell and regenerative therapies for multiple sclerosis. *J Autoimmun* (2008) **31**(3):288–94. doi:10.1016/j.jaut.2008.04.002

79. Ilancheran S, Michalska A, Peh G, Wallace EM, Pera M, Manuelpillai U. Stem cells derived from human fetal membranes display multilineage differentiation potential. *Biol Reprod* (2007) **77**(3):577–88. doi:10.1095/biolreprod.106.055244

80. Phuc PV, Ngoc VB, Lam DH, Tam NT, Viet PQ, Ngoc PK. Isolation of three important types of stem cells from the same samples of banked umbilical cord blood. *Cell Tissue Bank* (2012) **13**(2):341–51. doi:10.1007/s10561-011-9262-4

81. Castillo-Melendez M, Yawno T, Jenkin G, Miller SL. Stem cell therapy to protect and repair the developing brain: a review of mechanisms of action of cord blood and amnion epithelial derived cells. *Front Neurosci* (2013) **7**:194. doi:10.3389/fnins.2013.00194

82. Payne NL, Sun G, McDonald C, Layton D, Moussa L, Emerson-Webber A, et al. Distinct immunomodulatory and migratory mechanisms underpin the therapeutic potential of human mesenchymal stem cells in autoimmune demyelination. *Cell Transplant* (2013) **22**(8):1409–25. doi:10.3727/096368912X657620

83. Gordon D, Pavlovska G, Glover CP, Uney JB, Wraith D, Scolding NJ. Human mesenchymal stem cells abrogate experimental allergic encephalomyelitis after intraperitoneal injection, and with sparse CNS infiltration. *Neurosci Lett* (2008) **448**(1):71–3. doi:10.1016/j.neulet.2008.10.040

84. Li Y, Chen J, Chen XG, Wang L, Gautam SC, Xu YX, et al. Human marrow stromal cell therapy for stroke in rat: neurotrophins and functional recovery. *Neurology* (2002) **59**(4):514–23. doi:10.1212/WNL.59.4.514

85. Liu T, Wu J, Huang Q, Hou Y, Jiang Z, Zang S, et al. Human amniotic epithelial cells ameliorate behavioral dysfunction and reduce infarct size in

the rat middle cerebral artery occlusion model. *Shock* (2008) **29**(5):603–11. doi:10.1097/SHK.0b013e318157e845

86. Murphy S, Lim R, Dickinson H, Acharya R, Rosli S, Jenkin G, et al. Human amnion epithelial cells prevent bleomycin-induced lung injury and preserve lung function. *Cell Transplant* (2011) **20**(6):909–23. doi:10.3727/096368910X543385

87. Tropea KA, Leder E, Aslam M, Lau AN, Raiser DM, Lee JH, et al. Bronchioalveolar stem cells increase after mesenchymal stromal cell treatment in a mouse model of bronchopulmonary dysplasia. *Am J Physiol Lung Cell Mol Physiol* (2012) **302**(9):L829–37. doi:10.1152/ajplung.00347.2011

88. Yawno T, Schuilwerve J, Moss TJ, Vosdoganes P, Westover AJ, Afandi E, et al. Human amnion epithelial cells reduce fetal brain injury in response to intrauterine inflammation. *Dev Neurosci* (2013) **35**(2–3):272–82. doi:10.1159/000346683

89. Dalous J, Pansiot J, Pham H, Chatel P, Nadaradja C, D'Agostino I, et al. Use of human umbilical cord blood mononuclear cells to prevent perinatal brain injury: a preclinical study. *Stem Cells Dev* (2013) **22**(1):169–79. doi:10.1089/scd.2012.0183

90. Levine AD, Wolf LE. The roles and responsibilities of physicians in patients' decisions about unproven stem cell therapies. *J Law Med Ethics* (2012) **40**(1):122–34. doi:10.1111/j.1748-720X.2012.00650.x

91. Solves P, López M, Mirabet V, Blanquer A, Roig R, Perales A. Characteristics of umbilical cord blood units collected from preterm deliveries. *Gynecol Obstet Invest* (2009) **68**(3):181–5. doi:10.1159/000232382

92. Rogers I, Casper RF. Umbilical cord blood stem cells. *Best Pract Res Clin Obstet Gynaecol* (2004) **18**(6):893–908. doi:10.1016/j.bpobgyn.2004.06.004

93. Rocha V, Wagner JEJr, Sobocinski KA, Klein JP, Zhang MJ, Horowitz MM, et al. Graft-versus-host disease in children who have received a cord-blood or bone marrow transplant from an HLA-identical sibling. eurocord and international bone marrow transplant registry working committee on alternative donor and stem cell sources. *N Engl J Med* (2000) **342**(25):1846–54. doi:10.1056/NEJM200006223422501

94. Vaziri H, Dragowska W, Allsopp RC, Thomas TE, Harley CB, Lansdorp PM. Evidence for a mitotic clock in human hematopoietic stem cells: loss of telomeric DNA with age. *Proc Natl Acad Sci U S A* (1994) **91**(21):9857–60. doi:10.1073/pnas.91.21.9857

95. Nakahata T, Ogawa M. Hemopoietic colony-forming cells in umbilical cord blood with extensive capability to generate mono- and multi-potential hemopoietic progenitors. *J Clin Invest* (1982) **70**(6):1324–8. doi:10.1172/JCI110734

96. Tiwari A, Tursky ML, Kirkland MA, Pande G. Expansion of human hematopoietic stem/progenitor cells on decellularized matrix scaffolds. *Curr Protoc Stem Cell Biol* (2014) **1**(Suppl 28). doi:10.1002/9780470151808.sc01c15s28

97. Coldwell KE, Lee SJ, Kean J, Khoo CP, Tsaknakis G, Smythe J. Effects of obstetric factors and storage temperatures on the yield of endothelial colony forming cells from umbilical cord blood. *Angiogenesis* (2011) **14**(3):381–92. doi:10.1007/s10456-011-9222-4

98. Tolar J, Hippen KL, Blazar BR. Immune regulatory cells in umbilical cord blood: T regulatory cells and mesenchymal stromal cells. *Br J Haematol* (2009) **147**(2):200–6. doi:10.1111/j.1365-2141.2009.07781.x

99. Kern S, Eichler H, Stoeve J, Klüter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* (2006) **24**(5):1294–301. doi:10.1634/stemcells.2005-0342

100. Manca MF, Zwart I, Beo J, Palasingham R, Jen LS, Navarrete R, et al. Characterization of mesenchymal stromal cells derived from full-term umbilical cord blood. *Cytotherapy* (2008) **10**(1):54–68. doi:10.1080/14653240701732763

101. Jain A, Mathur N, Jeevashankar M, Mukhopadhyay A, Agarwal R, Deorari AK, et al. Does mesenchymal stem cell population in umbilical cord blood vary at different gestational periods? *Indian J Pediatr* (2013) **80**(5):375–9. doi:10.1007/s12098-012-0844-3

102. El-Badri NS, Hakki A, Saporta S, Liang X, Madhusodanan S, Willing AE, et al. Cord blood mesenchymal stem cells: potential use in neurological disorders. *Stem Cells Dev* (2006) **15**(4):497–506. doi:10.1089/scd.2006.15.497

103. Abbott NJ, Patabendige AA, Dolman DE, Yusof SR, Begley DJ. Structure and function of the blood-brain barrier. *Neurobiol Dis* (2010) **37**(1):13–25. doi:10.1016/j.nbd.2009.07.030

104. Hristov M, Erl W, Weber PC. Endothelial progenitor cells: mobilization, differentiation, and homing. *Arterioscler Thromb Vasc Biol* (2003) **23**(7):1185–9. doi:10.1161/01.ATV.0000073832.49290.B5

105. Larrivée B, Niessen K, Pollet I, Corbel SY, Long M, Rossi FM, et al. Minimal contribution of marrow-derived endothelial precursors to tumor vasculature. *J Immunol* (2005) 175(5):2890–9. doi:10.4049/jimmunol.175.5.2890

106. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* (2005) 6(4):345–52. doi:10.1038/ni1178

107. Takahata Y, Nomura A, Takada H, Ohga S, Furuno K, Hikino S, et al. CD25+CD4+ T cells in human cord blood: an immunoregulatory subset with naive phenotype and specific expression of forkhead box p3 (Foxp3) gene. *Exp Hematol* (2004) 32(7):622–9. doi:10.1016/j.exphem.2004.03.012

108. Hoffmann P, Edinger M. CD4+CD25+ regulatory T cells and graft-versus-host disease. *Semin Hematol* (2006) 43(1):62–9. doi:10.1053/j.seminhematol.2005.09.006

109. Meister B, Tötsch M, Mayr A, Widschwendter M, Huter O, Sperl W. Identification of CD34+ cord blood cells and their subpopulations in preterm and term neonates using three-color flow cytometry. *Biol Neonate* (1994) 66(5):272–9. doi:10.1159/000244117

110. Ballen KK, Wilson M, Wuu J, Ceredona AM, Hsieh C, Stewart FM, et al. Bigger is better: maternal and neonatal predictors of hematopoietic potential of umbilical cord blood units. *Bone Marrow Transplant* (2001) 27(1):7–14. doi:10.1038/sj.bmt.1702729

111. Cervera A, Lillo R, García-Sánchez F, Madero L, Madero R, Vicario JL. Flow cytometric assessment of hematopoietic cell subsets in cryopreserved preterm and term cord blood, influence of obstetrical parameters, and availability for transplantation. *Am J Hematol* (2006) 81(6):397–410. doi:10.1002/ajh.20598

112. Gasparoni A, Ciardelli L, Avanzini MA, Bonfichi M, di Mario M, Piazzi G, et al. Immunophenotypic changes of fetal cord blood hematopoietic progenitor cells during gestation. *Pediatr Res* (2000) 47(6):825–9. doi:10.1203/00006450-200006000-00024

113. Baker CD, Ryan SL, Ingram DA, Seedorf GJ, Abman SH, Balasubramaniam V. Endothelial colony-forming cells from preterm infants are increased and more susceptible to hyperoxia. *Am J Respir Crit Care Med* (2009) 180(5):454–61. doi:10.1164/rccm.200901-0115OC

114. Monga R, Buck S, Sharma P, Thomas R, Chouthai NS. Effect of preeclampsia and intrauterine growth restriction on endothelial progenitor cells in human umbilical cord blood. *J Matern Fetal Neonatal Med* (2012) 25(11):2385–9. doi:10.3109/14767058.2012.697228

115. Javed MJ, Mead LE, Prater D, Bessler WK, Foster D, Case J, et al. Endothelial colony forming cells and mesenchymal stem cells are enriched at different gestational ages in human umbilical cord blood. *Pediatr Res* (2008) 64(1):68–73. doi:10.1203/PDR.0b013e31817445e9

116. Lim R, Chan ST, Tan JL, Mockler JC, Murphy SV, Wallace EM. Preterm human amnion epithelial cells have limited reparative potential. *Placenta* (2013) 34(6):486–92. doi:10.1016/j.placenta.2013.03.010

117. Dirix V, Vermeulen F, Mascart F. Maturation of CD4+ regulatory T lymphocytes and of cytokine secretions in infants born prematurely. *J Clin Immunol* (2013) 33(6):1126–33. doi:10.1007/s10875-013-9911-4

118. Mukhopadhyay D, Weaver L, Tobin R, Henderson S, Beeram M, Newell-Rogers MK, et al. Intrauterine growth restriction and prematurity influence regulatory T cell development in newborns. *J Pediatr Surg* (2014) 49(5):727–32. doi:10.1016/j.jpedsurg.2014.02.055

119. Xia G, Hong X, Chen X, Lan F, Zhang G, Liao L. Intracerebral transplantation of mesenchymal stem cells derived from human umbilical cord blood alleviates hypoxic ischemic brain injury in rat neonates. *J Perinat Med* (2010) 38(2):215–21. doi:10.1515/JPM.2010.021

120. Kim ES, Ahn SY, Im GH, Sung DK, Park YR, Choi SH, et al. Human umbilical cord blood-derived mesenchymal stem cell transplantation attenuates severe brain injury by permanent middle cerebral artery occlusion in newborn rats. *Pediatr Res* (2012) 72(3):277–84. doi:10.1038/pr.2012.71

121. Ahn SY, Chang YS, Sung DK, Sung SI, Yoo HS, Lee JH, et al. Mesenchymal stem cells prevent hydrocephalus after severe intraventricular hemorrhage. *Stroke* (2013) 44(2):497–504. doi:10.1161/STROKEAHA.112.679092

122. Tsuji M, Taguchi A, Ohshima M, Kasahara Y, Sato Y, Tsuda H, et al. Effects of intravenous administration of umbilical cord blood CD34+ cells in a mouse model of neonatal stroke. *Neuroscience* (2014) 263:148–58. doi:10.1016/j.neuroscience.2014.01.018

123. Iskander A, Knight RA, Zhang ZG, Ewing JR, Shankar A, Varma NR, et al. Intravenous administration of human umbilical cord blood-derived AC133+ endothelial progenitor cells in rat stroke model reduces infarct volume: magnetic resonance imaging and histological findings. *Stem Cells Transl Med* (2013) 2(9):703–14. doi:10.5966/sctm.2013-0066

124. Womble TA, Green S, Shahaduzzaman M, Grieco J, Sanberg PR, Pennypacker KR, et al. Monocytes are essential for the neuroprotective effect of human cord blood cells following middle cerebral artery occlusion in rat. *Mol Cell Neurosci* (2014) 59:76–84. doi:10.1016/j.mcn.2014.01.004

125. Shahaduzzaman M, Golden JE, Green S, Gronda AE, Adrien E, Ahmed A, et al. A single administration of human umbilical cord blood T cells produces long-lasting effects in the aging hippocampus. *Age* (2013) 35(6):2071–87. doi:10.1007/s11357-012-9496-5

126. Saporta S, Kim JJ, Willing AE, Fu ES, Davis CD, Sanberg PR. Human umbilical cord blood stem cells infusion in spinal cord injury: engraftment and beneficial influence on behavior. *J Hematother Stem Cell Res* (2003) 12(3):271–8. doi:10.1089/152581603322023007

127. Meier C, Middelanis J, Wasielewski B, Neuhoff S, Roth-Haerer A, Gantert M, et al. Spastic paresis after perinatal brain damage in rats is reduced by human cord blood mononuclear cells. *Pediatr Res* (2006) 59(2):244–9. doi:10.1203/01.pdr.0000197309.08852.f5

128. Gornicka-Pawlak elB, Janowski M, Habich A, Jablonska A, Drela K, Kozlowska H, et al. Systemic treatment of focal brain injury in the rat by human umbilical cord blood cells being at different level of neural commitment. *Acta Neurobiol Exp* (2011) 71(1):46–64.

129. Prockop DJ. "Stemness" does not explain the repair of many tissues by mesenchymal stem/multipotent stromal cells (MSCs). *Clin Pharmacol Ther* (2007) 82(3):241–3. doi:10.1038/sj.clpt.6100313

130. Zhang X, Zhang Q, Li W, Nie D, Chen W, Xu C, et al. Therapeutic effect of human umbilical cord mesenchymal stem cells on neonatal rat hypoxic-ischemic encephalopathy. *J Neurosci Res* (2014) 92(1):35–45. doi:10.1002/jnr.23304

131. Seo JH, Cho SR. Neurorestoration induced by mesenchymal stem cells: potential therapeutic mechanisms for clinical trials. *Yonsei Med J* (2012) 53(6):1059–67. doi:10.3349/ymj.2012.53.6.1059

132. Boltze J, Schmidt UR, Reich DM, Kranz A, Reymann KG, Strassburger M, et al. Determination of the therapeutic time window for human umbilical cord blood mononuclear cell transplantation following experimental stroke in rats. *Cell Transplant* (2012) 21(6):1199–211. doi:10.3727/096368911X589609

133. de Paula S, Greggio S, Marinowic 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) 210:431–41. doi:10.1016/j.neuroscience.2012.03.009

134. Pimentel-Coelho PM, Magalhães 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(3):351–8. doi:10.1089/scd.2009.0049

135. 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(6):e20194. doi:10.1371/journal.pone.0020194

136. Wasielewski B, Jensen A, Roth-Härer 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. doi:10.1016/j.brainres.2012.05.066

137. Wang X, Zhao Y, Wang X. Umbilical cord blood cells regulate the differentiation of endogenous neural stem cells in hypoxic ischemic neonatal rats via the hedgehog signaling pathway. *Brain Res* (2014) 1560:18–26. doi:10.1016/j.brainres.2014.02.019

138. Yasuhara T, Hara K, Maki M, Xu L, Yu G, Ali MM, et al. 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(4):914–21. doi:10.1111/j.1582-4934.2008.00671.x

139. Bae SH, Kong TH, Lee HS, Kim KS, Hong KS, Chopp M, et al. Long-lasting paracrine effects of human cord blood cells on damaged neocortex in an animal model of cerebral palsy. *Cell Transplant* (2012) 21(11):2497–515. doi:10.3727/096368912X640457

140. Rice Iii JE, Vannucci RC, Brierley JB. The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Ann Neurol* (1981) 9(2):131–41. doi:10.1002/ana.410090206

141. Rosenkranz K. Transplantation of human umbilical cord blood cells mediated beneficial effects on apoptosis, angiogenesis and neuronal survival after hypoxic-ischemic brain injury in rats. *Cell Tissue Res* (2012) **348**(3):429–38. doi:10.1007/s00441-012-1401-0

142. Newman MB, Willing AE, Manresa JJ, Sanberg CD, Sanberg PR. Cytokines produced by cultured human umbilical cord blood (HUCB) cells: implications for brain repair. *Exp Neurol* (2006) **199**(1):201–8. doi:10.1016/j.expneurol.2006.04.001

143. Neuhoff S, Moers J, Rieks M, Grunwald T, Jensen A, Dermietzel R, et al. Proliferation, differentiation, and cytokine secretion of human umbilical cord blood-derived mononuclear cells in vitro. *Exp Hematol* (2007) **35**(7):1119–31. doi:10.1016/j.exphem.2007.03.019

144. Nakase T, Söhl G, Theis M, Willecke K, Naus CC. Increased apoptosis and inflammation after focal brain ischemia in mice lacking connexin43 in astrocytes. *Am J Pathol* (2004) **164**(6):2067–75. doi:10.1016/S0002-9440(10)63765-0

145. Bachstetter AD, Pabon MM, Cole MJ, Hudson CE, Sanberg PR, Willing AE, et al. Peripheral injection of human umbilical cord blood stimulates neurogenesis in the aged rat brain. *BMC Neurosci* (2008) **9**:22. doi:10.1186/1471-2202-9-22

146. Liao W, Zhong J, Yu J, Xie J, Liu Y, Du L, et al. Therapeutic benefit of human umbilical cord derived mesenchymal stromal cells in intracerebral hemorrhage rat: implications of anti-inflammation and angiogenesis. *Cell Physiol Biochem* (2009) **24**(3–4):307–16. doi:10.1159/000233255

147. Rosenkranz K, Meier C. Umbilical cord blood cell transplantation after brain ischemia-from recovery of function to cellular mechanisms. *Ann Anat* (2011) **193**(4):371–9. doi:10.1016/j.aanat.2011.03.005

148. Kao CH, Chen SH, Chio CC, Lin MT. Human umbilical cord blood-derived CD34+ cells may attenuate spinal cord injury by stimulating vascular endothelial and neurotrophic factors. *Shock* (2008) **29**(1):49–55. doi:10.1097/shk.0b013e31805cddce

149. Tanaka N, Kamei N, Nakamae T, Yamamoto R, Ishikawa M, Fujiwara H, et al. CD133+ cells from human umbilical cord blood reduce cortical damage and promote axonal growth in neonatal rat organ co-cultures exposed to hypoxia. *Int J Dev Neurosci* (2010) **28**(7):581–7. doi:10.1016/j.ijdevneu.2010.07.232

150. Brazel CY, Nuñez JL, Yang Z, Levison SW. Glutamate enhances survival and proliferation of neural progenitors derived from the subventricular zone. *Neuroscience* (2005) **131**(1):55–65. doi:10.1016/j.neuroscience.2004.10.038

151. Ikeda T, Iwai M, Hayashi T, Nagano I, Shogi M, Ikenoue T, et al. Limited differentiation to neurons and astroglia from neural stem cells in the cortex and striatum after ischemia/hypoxia in the neonatal rat brain. *Am J Obstet Gynecol* (2005) **193**(3):849–56. doi:10.1016/j.ajog.2005.01.029

152. Horie N, So K, Moriya T, Kitagawa N, Tsutsumi K, Nagata I, et al. Effects of oxygen concentration on the proliferation and differentiation of mouse neural stem cells in vitro. *Cell Mol Neurobiol* (2008) **28**(6):833–45. doi:10.1007/s10571-007-9237-y

153. Saliba E, Marret S. Cerebral white matter damage in the preterm infant: pathophysiology and risk factors. *Semin Neonatol* (2001) **6**(2):121–33. doi:10.1053/siny.2001.0043

154. Sie LT, van der Knaap MS, Oosting J, de Vries LS, Lafeber HN, Valk J. MR patterns of hypoxic-ischemic brain damage after prenatal, perinatal or postnatal asphyxia. *Neuropediatrics* (2000) **31**(3):128–36. doi:10.1055/s-2000-7496

155. Derrick M, Drobyshevsky A, Ji X, Tan S. A model of cerebral palsy from fetal hypoxia-ischemia. *Stroke* (2007) **38**(2 Pt 2):731–5. doi:10.1161/01.STR.0000251445.94697.64

156. Derrick M, Luo NL, Bregman JC, Jilling T, Ji X, Fisher K, et al. Preterm fetal hypoxia-ischemia causes hypertonia and motor deficits in the neonatal rabbit: a model for human cerebral palsy? *J Neurosci* (2004) **24**(1):24–34. doi:10.1523/JNEUROSCI.2816-03.2004

157. Back SA, Riddle A, Dean J, Hohimer AR. The instrumented fetal sheep as a model of cerebral white matter injury in the premature infant. *Neurotherapeutics* (2012) **9**(2):359–70. doi:10.1007/s13311-012-0108-y

158. Hall AA, Guyer AG, Leonardo CC, Ajmo CTJr, Collier LA, Willing AE, et al. Human umbilical cord blood cells directly suppress ischemic oligodendrocyte cell death. *J Neurosci Res* (2009) **87**(2):333–41. doi:10.1002/jnr.21857

159. Kohelet D, Shochat R, Lusky A, Reichman B; Israel Neonatal Network. Risk factors for seizures in very low birthweight infants with periventricular leukomalacia. *J Child Neurol* (2006) **21**(11):965–70. doi:10.1177/08830738060210111301

160. Kohelet D, Shochat R, Lusky A, Reichman B; Israel Neonatal Network. Risk factors for neonatal seizures in very low birthweight infants: population-based survey. *J Child Neurol* (2004) **19**(2):123–8. doi:10.1177/08830738040190020701

161. Costa-Ferro ZS, de Borba Cunha F, de Freitas Souza BS, Leal MM, da Silva AA, de Bellis Kühn TI, et al. Antiepileptic and neuroprotective effects of human umbilical cord blood mononuclear cells in a pilocarpine-induced epilepsy model. *Cytotechnology* (2014) **66**(2):193–9. doi:10.1007/s10616-013-9557-3

162. Motobayashi M, Inaba Y, Fukuyama T, Kurata T, Niimi T, Saito S, et al. Successful treatment for west syndrome with severe combined immunodeficiency. *Brain Dev* (2014). doi:10.1016/j.braindev.2014.01.012

163. Mueller D, Shamblott MJ, Fox HE, Gearhart JD, Martin LJ. Transplanted human embryonic germ cell-derived neural stem cells replace neurons and oligodendrocytes in the forebrain of neonatal mice with excitotoxic brain damage. *J Neurosci Res* (2005) **82**(5):592–608. doi:10.1002/jnr.20673

164. Madhavan L, Ourednik V, Ourednik J. Increased "vigilance" of antioxidant mechanisms in neural stem cells potentiates their capability to resist oxidative stress. *Stem Cells* (2006) **24**(9):2110–9. doi:10.1634/stemcells.2006-0018

165. Ito K, Hirao A, Arai F, Takubo K, Matsuoka S, Miyamoto K, et al. Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. *Nat Med* (2006) **12**(4):446–51. doi:10.1038/nm1388

166. Valle-Prieto A, Conget PA. Human mesenchymal stem cells efficiently manage oxidative stress. *Stem Cells Dev* (2010) **19**(12):1885–93. doi:10.1089/scd.2010.0093

167. Arien-Zakay H, Lecht S, Bercu MM, Tabakman R, Kohen R, Galski H, et al. Neuroprotection by cord blood neural progenitors involves antioxidants, neurotrophic and angiogenic factors. *Exp Neurol* (2009) **216**(1):83–94. doi:10.1016/j.expneurol.2008.11.006

168. Andrew M, Paes B, Milner R, Johnston M, Mitchell L, Tollefsen DM, et al. Development of the human coagulation system in the healthy premature infant. *Blood* (1988) **72**(5):1651–7.

169. Miller SP, Ferriero DM, Leonard C, Piecuch R, Glidden DV, Partridge JC, et al. Early brain injury in premature newborns detected with magnetic resonance imaging is associated with adverse early neurodevelopmental outcome. *J Pediatr* (2005) **147**(5):609–16. doi:10.1016/j.jpeds.2005.06.033

170. Heuchan AM, Evans N, Henderson Smart DJ, Simpson JM. Perinatal risk factors for major intraventricular haemorrhage in the Australian and New Zealand neonatal network, 1995-97. *Arch Dis Child Fetal Neonatal Ed* (2002) **86**(2):F86–90. doi:10.1136/fn.86.2.F86

171. Dommergues MA, Gallego J, Evrard P, Gressens P. Iron supplementation aggravates periventricular cystic white matter lesions in newborn mice. *Eur J Paediatrc Neurol* (1998) **2**(6):313–8. doi:10.1016/S1090-3798(98)80006-8

172. Gould SJ, Howard S, Hope PL, Reynolds EO. Periventricular intraparenchymal cerebral haemorrhage in preterm infants: the role of venous infarction. *J Pathol* (1987) **151**(3):197–202. doi:10.1002/path.1711510307

173. Nan Z, Grande A, Sanberg CD, Sanberg PR, Low WC. Infusion of human umbilical cord blood ameliorates neurologic deficits in rats with hemorrhagic brain injury. *Ann N Y Acad Sci* (2005) **1049**:84–96. doi:10.1196/annals.1334.009

174. Zhu LH, Bai X, Zhang N, Wang SY, Li W, Jiang L. Improvement of human umbilical cord mesenchymal stem cell transplantation on glial cell and behavioral function in a neonatal model of periventricular white matter damage. *Brain Res* (2014) **1563**:13–21. doi:10.1016/j.brainres.2014.03.030

175. van Egmond ME, Pouwels PJ, Boelens JJ, Lindemans CA, Barkhof F, Steenwijk MD, et al. Improvement of white matter changes on neuroimaging modalities after stem cell transplant in metachromatic leukodystrophy. *JAMA Neurol* (2013) **70**(6):779–82. doi:10.1001/jamaneurol.2013.629

176. 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**(6):722–9. doi:10.3109/14653249.2011.553592

177. Chua SJ, Bielecki R, Wong CJ, Yamanaka N, Rogers IM, Casper RF. Neural progenitors, neurons and oligodendrocytes from human umbilical cord blood cells in a serum-free, feeder-free cell culture. *Biochem Biophys Res Commun* (2009) **379**(1):217–21. doi:10.1016/j.bbrc.2008.12.045

178. Lee YH, Choi KV, Moon JH, Jun HJ, Kang HR, Oh SI, et al. Safety and feasibility of countering neurological impairment by intravenous administration of autologous cord blood in cerebral palsy. *J Transl Med* (2012) **10**(1):58. doi:10.1186/1479-5876-10-58

179. Cotten CM, Murtha AP, Goldberg RN, Grotegut CA, Smith PB, Goldstein RF, et al. Feasibility of autologous cord blood cells for infants with

hypoxic-ischemic encephalopathy. *J Pediatr* (2014) **164**(5):973–9. doi:10.1016/j.jpeds.2013.11.036

180. Min K, Song J, Kang JY, Ko J, Ryu JS, Kang MS, et al. Umbilical cord blood therapy potentiated with erythropoietin for children with cerebral palsy: a double-blind, randomized, placebo-controlled trial. *Stem Cells* (2013) **31**(3):581–91. doi:10.1002/stem.1304

181. Leuchter RH, Gui L, Poncet A, Hagmann C, Lodygensky GA, Martin E, et al. Association between early administration of high-dose erythropoietin in preterm infants and brain MRI abnormality at term-equivalent age. *JAMA* (2014) **312**(8):817–24. doi:10.1001/jama.2014.9645

182. Aher SM, Ohlsson A. Late erythropoietin for preventing red blood cell transfusion in preterm and/or low birth weight infants. *Cochrane Database Syst Rev* (2012) **9**:CD004868. doi:10.1002/14651858.CD004868.pub3

183. Miller SL, Yan EB, Castillo-Meléndez M, Jenkin G, Walker DW. Melatonin provides neuroprotection in the late-gestation fetal sheep brain in response to umbilical cord occlusion. *Dev Neurosci* (2005) **27**(2–4):200–10. doi:10.1159/000085993

184. Li J, Funato M, Tamai H, Wada H, Nishihara M, Iwamoto H, et al. Predictors of neurological outcome in cooled neonates. *Pediatr Int* (2013) **55**(2):169–76. doi:10.1111/ped.12008

185. Kaneko Y, Tajiri N, Su TP, Wang Y, Borlongan CV. Combination treatment of hypothermia and mesenchymal stromal cells amplifies neuroprotection in primary rat neurons exposed to hypoxic-ischemic-like injury in vitro: role of the opioid system. *PLoS One* (2012) **7**(10):e47583. doi:10.1371/journal.pone.0047583

186. de Almeida MF, Guinsburg R, Sancho GA, Rosa IR, Lamy ZC, Martinez FE, et al. Hypothermia and early neonatal mortality in preterm infants. *J Pediatr* (2014) **164**(2):271–5. doi:10.1016/j.jpeds.2013.09.049

187. Cutler AJ, Limbani V, Girdlestone J, Navarrete CV. Umbilical cord-derived mesenchymal stromal cells modulate monocyte function to suppress T cell proliferation. *J Immunol* (2010) **185**(11):6617–23. doi:10.4049/jimmunol.1002239

188. Julavijitphong S, Wichitwiengrat S, Tirawanchai N, Ruangvutilert P, Vantanasiri C, Phermthai T. A xeno-free culture method that enhances Wharton's jelly mesenchymal stromal cell culture efficiency over traditional animal serum-supplemented cultures. *Cytotherapy* (2014) **16**(5):683–91. doi:10.1016/j.jcyt.2013.07.012

189. Ding Y, Yang H, Feng JB, Qiu Y, Li DS, Zeng Y. Human umbilical cord-derived MSC culture: the replacement of animal sera with human cord blood plasma.

In vitro Cell Dev Biol Anim (2013) **49**(10):771–7. doi:10.1007/s11626-013-9663-8

190. Murphy S, Rosli S, Acharya R, Mathias L, Lim R, Wallace E, et al. Amnion epithelial cell isolation and characterization for clinical use. *Curr Protoc Stem Cell Biol* (2010) (Suppl 13):1E.6.1–25. doi:10.1002/9780470151808.sc01e06s13

191. Li J, Kobata K, Kamei Y, Okazaki Y, Nishihara M, Wada H, et al. Nucleated red blood cell counts: an early predictor of brain injury and 2-year outcome in neonates with hypoxic-ischemic encephalopathy in the era of cooling-based treatment. *Brain Dev* (2014) **36**(6):472–8. doi:10.1016/j.braindev.2013.06.012

192. de Paula S, Vitola AS, Greggio S, de Paula D, Mello PB, Lubianca JM, et al. 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**(6):631–5. doi:10.1203/PDR.0b013e31819ed5c8

193. Hansen L, Hansen AB, Mathiasen AB, Ng M, Bhakoo K, Ekblond A, et al. Characterisation of mesenchymal stromal cells labeled with ultrasmall superparamagnetic iron-oxide nanoparticles for clinical tracking studies. *Scand J Clin Lab Invest* (2014) **74**(5):437–46. doi:10.3109/00365513.2014.900698

1.2. Hypothesis and Aims of this Thesis

We **hypothesise** that cells derived from UCB are able to reduce preterm white matter brain injury following HI.

Specific Aims of this thesis were:

1) To demonstrate that allogeneic UCB derived cells are safe to administer to preterm fetal sheep.

2) To administer UCB derived cells after preterm brain injury induced by acute HI, to determine whether white matter brain injury is reduced.

3) To compare the effects of early and late administration of UCB derived cells after preterm brain injury, to determine the optimal timing of administration following HI.

4) To compare the efficacy of cells derived from term and preterm UCB for white matter protection in the preterm brain following HI.

5) To investigate the neuroprotective effects of ex-vivo expanded MSCs derived from preterm UCB in the preterm brain following acute HI.

Chapter 2

Preterm white matter brain injury is prevented by early administration of umbilical cord blood cells

Jingang Li, MD[1], Tamara Yawno, PhD[1], Amy Sutherland, PhD[1], Jan Loose[1], Ilias Nitsos, PhD[1], Robert Bischof, PhD[1], Margie Castillo-Melendez, PhD[1], Courtney A McDonald, PhD[1], Flora Y Wong, MD, PhD[1,3], Graham Jenkin, PhD[1,2], Suzanne L Miller, PhD[1,2*]

[1]The Ritchie Centre, Hudson Institute of Medical Research, Clayton, VIC Australia

[2]Department of Obstetrics and Gynaecology, Monash University, Clayton, VIC, Australia

[3]Department of Paediatrics, Monash Medical Centre, Clayton, VIC, Australia

We tested whether allogeneic administrations of UCB derived cells are neuroprotective following HI insult in a well-established fetal sheep model of preterm white matter injury. We also compared the neuroprotective effects of UCB cell administration at 12h vs 5d following acute HI. This is an unaltered version of the manuscript published in *Exp Neurol.* 2016, 283(Pt A), 179-187.

Declaration for Thesis Chapter 2 — Preterm white matter brain injury is prevented by early administration of umbilical cord blood cells.

Declaration by candidate

In the case of Chapter [insert chapter number], the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experimental design and execution, data analysis, manuscript preparation	75

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Tamara Yawno,	Animal surgery, data analysis, editing manuscript
Amy Sutherland	Animal surgery
Jan Loose	Data collection
Ilias Nitsos	Animal surgery
Robert Bischof	Data analysis
Margie Castillo-Melendez	Data analysis
Courtney McDonald	Data analysis
Flora Wong	Experimental design
Graham Jenkin	Experimental design, editing manuscript
Suzanne Miller	Experimental design, editing manuscript

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		Date 15/11/2017

Main Supervisor's Signature		Date 15/11/2017

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Research Paper

Preterm white matter brain injury is prevented by early administration of umbilical cord blood cells

Jingang Li [a], Tamara Yawno [a], Amy Sutherland [a], Jan Loose [a], Ilias Nitsos [a], Robert Bischof [a], Margie Castillo-Melendez [a], Courtney A. McDonald [a], Flora Y. Wong [a,c], Graham Jenkin [a,b], Suzanne L. Miller [a,b,*]

[a] The Ritchie Centre, Hudson Institute of Medical Research, Clayton, VIC, Australia
[b] Department of Obstetrics and Gynaecology, Monash University, Clayton, VIC, Australia
[c] Department of Paediatrics, Monash Medical Centre, Clayton, VIC, Australia

A B S T R A C T

Infants born very preterm are at high risk for neurological deficits including cerebral palsy. In this study we assessed the neuroprotective effects of umbilical cord blood cells (UCBCs) and optimal administration timing in a fetal sheep model of preterm brain injury. 50 million allogeneic UCBCs were intravenously administered to fetal sheep (0.7 gestation) at 12 h or 5 d after acute hypoxia-ischemia (HI) induced by umbilical cord occlusion. The fetal brains were collected at 10 d after HI. HI (n = 7) was associated with reduced number of oligodendrocytes (Olig2+) and myelin density (CNPase+), and increased density of activated microglia (Iba-1+) in cerebral white matter compared to control fetuses ($P < 0.05$). UCBCs administered at 12 h, but not 5 d after HI, significantly protected white matter structures and suppressed cerebral inflammation. Activated microglial density showed a correlation with decreasing oligodendrocyte number ($P < 0.001$). HI caused cell death (TUNEL+) in the internal capsule and cell proliferation (Ki-67+) in the subventricular zone compared to control ($P < 0.05$), while UCBCs at 12 h or 5 d ameliorated these effects. Additionally, UCBCs at 12 h induced a significant systemic increase in interleukin-10 at 10 d, and reduced oxidative stress (malondialdehyde) following HI ($P < 0.05$). UCBC administration at 12 h after HI reduces preterm white matter injury, via anti-inflammatory and antioxidant actions.

Crown Copyright © 2016 Published by Elsevier Inc. All rights reserved.

1. Introduction

Approximately 12% of infants are born preterm, and very preterm infants, born <32 weeks gestational age, are the highest risk group for neurological morbidities. White matter injury (WMI) is the most common brain damage in preterm infants, typically localized to the periventricular white matter (PVWM) area in a diffuse or focal pattern (Woodward et al., 2006). In all preterm infants with WMI, 5–15% will develop cerebral palsy, and 50% will have cognitive, behavioral or attention deficits (Volpe, 2003). Cerebral ischemia-reperfusion and inflammation are the two principal causes of WMI (Dammann and Leviton, 1997; Buser et al., 2012; Back et al., 2007), leading to neuropathologies that include disruption to oligodendrocyte development, deficits in axonal myelination, astrogliosis, and/or microglial activation (Khwaja and Volpe, 2008). Oligodendrocyte progenitor cells present between 23 and 32 weeks gestation in humans are particularly susceptible to inflammation and/or hypoxia-ischemia (Back and Volpe, 1997). The complexity of white

matter neuropathology, relative brain immaturity and inherent susceptibility of very preterm infants means that there are currently no neuroprotective treatments available for this vulnerable cohort.

Umbilical cord blood contains a diverse and rich mix of stem and progenitor cells with excellent potential for neurorepair, and is readily available (Castillo-Melendez et al., 2013; Li et al., 2014; Bennet et al., 2012). There is increasing evidence to demonstrate the beneficial effects of UCBCs in preventing or repairing HI-induced brain injury in term-equivalent newborn rats (postnatal day 7–10), when xenotransplanted human UCBCs are administered within 24 h after HI (Li et al., 2014; Bennet et al., 2012; Geissler, 2011; Pimentel-Coelho et al., 2010; Meier et al., 2006; Wasielewski et al., 2012). Human UCBCs transplanted intraperitoneally decrease apoptotic and necrotic cell death within the brain, mediated by reduced brain inflammation (Pimentel-Coelho et al., 2010; Geissler et al., 2011). While the white to gray matter ratio and pattern of brain injury is different in young rats (Rees and Inder, 2005), one study in preterm-equivalent (postnatal) rats supports that intravenous human UCBC administration may preserve white matter architecture following HI (Hall et al., 2009).

We tested the hypothesis that intravenously administered allogeneic UCBCs would protect the developing white matter of the preterm

* Corresponding author at: The Ritchie Centre, Hudson Institute of Medical Research, Department of Obstetrics and Gynaecology, Monash University, Level 3, 27-31 Wright St, Clayton 3168, VIC, Australia.

sheep brain following HI. We examined whether early (12 h) or late (5 d) administration of UCBCs after insult would have differential effects, and explored the mechanisms of UCBC action. UCBCs prevent neuronal degeneration when administered 12 h after birth asphyxia in term lambs (Aridas et al., 2015), but their role in white matter protection is not known, and this early administration may be unrealistic in clinical practice for brain injury in very preterm infants. Therefore we have selected a comparison between UCBC administration at 12 h versus 5 d. Prolonged umbilical cord occlusion (UCO) in preterm fetal sheep at 0.65–0.7 gestation induces significant WMI mediated by hypoxemia, inflammation, excitotoxicity, and oxidative stress (Bennet et al., 2007; Ferreiro, 2006). Accordingly, we induced a period of severe HI in preterm fetal sheep at a time corresponding to 28–32 weeks gestation of human white matter development (Bennet et al., 2007), to examine the neuroprotective benefits and preferred timing of UCBC therapy.

2. Materials and methods

2.1. Animals and surgery

Surgery was performed on 38 pregnant Merino-Border Leicester ewes of known gestational age bearing single (n = 34) and twin fetuses (n = 8) at 97.5 ± 0.1 d gestation (term = ~147 d). All surgical and experimental procedures were approved by the Monash Medical Centre Animal Ethics Committee (MMCA/2013/17). Anesthesia was induced with intravenous (iv) 20 mg/kg sodium thiopentone (Bomac Laboratories, New Zealand) and maintained with 2% isoflurane (Abott, Australia) via an endotracheal tube. Under aseptic conditions, the ewe underwent a laparotomy to exteriorize the fetus, and polyvinyl catheters (0.8 mm inner diameter, 1.2 mm outer diameter, Dural Plastics, Australia) were inserted into the fetal femoral artery for monitoring arterial blood pressure and obtaining blood samples, and fetal vein for administering UCBCs or saline. An inflatable balloon occluder (16HD, In Vivo Medical, USA) was placed around the umbilical cord to induce HI. The fetus was returned to the uterus, and the uterine and abdominal incisions sutured in layers. All catheters were exteriorized through the maternal flank, and the muscle layers closed separately. A maternal jugular vein catheter was also implanted for antibiotic administration. Prior to surgery and for 3 days post-surgery, 500 mg engemycin (Coopers, Bendigo East, Australia) and 1 g ampicillin (Lennon Healthcare, St Leonards, Australia) were administered iv to the ewe.

Experiments were conducted 4–5 d postoperatively. Fetal catheters were maintained by continuous infusion of heparinized saline (50 IU/ml, 0.2 ml/h). Fetal heart rate and mean arterial pressure (MAP), corrected for amniotic fluid pressure, were monitored using pressure transducers during the experiment, and digitized and stored for off-line analysis (Power Lab, AD Instruments, Castle Hill, Australia).

2.2. Experiment protocol (Fig. 1A)

At 102.3 ± 0.2d gestation (0.7 gestation), animals were randomized into one of five groups: (1) control (sham-occlusion + iv saline, n = 10); (2) HI (HI + saline, n = 7); (3) HI + UCB@12 h (HI + 50 million UCBCs injected iv at 12 h after UCO, n = 6); (4) HI + UCB@5 d (HI + 50 million UCBCs at 120 h, n = 6); (5) control + UCB@12 h (sham-occlusion + 50 million UCBCs at 12 h, n = 5). The HI + UCB@ 12 h, HI + UCB@5 d and control + UCB@12 h groups used singleton fetuses only, while the HI and the control group used singletons and twins, and there was no difference in sex distribution (Table 1). HI was achieved by complete UCO, in which the balloon occluder was filled with 2.0–2.5 ml water for 23-25 min. The occlusion was discontinued at 25 min or sooner if the occlusion was >23 min duration and MAP had decreased to <8 mm Hg. Fetal arterial blood samples (approximately 0.5–1.5 ml) were collected 24 h before, during, and 6, 12, 24, 48, 72, 120, and 240 h after HI for blood gas parameters (ABL 700, Radiometer, Copenhagen, Denmark), cytokine and malondialdehyde concentrations. Plasma samples were stored at −80 °C until assays were performed.

2.3. Cell preparation

Umbilical cord blood was collected at cesarean-section of near-term lambs (141 d gestation). The umbilical cord was clamped and cord blood from the placental side was collected into heparinized syringes. UCBCs were isolated by centrifuging the blood at 3100 rpm for 12 min at room temperature, without brake. The buffy coat layer was collected, excess red blood cells were removed using red blood cell lysis buffer (155 mM NH4Cl, 10 mM KHCO$_3$, 0.1 mM EDTA in H$_2$O). The cells were resuspended in bovine fetal serum with 10% DMSO (Merck, Darmstadt, Germany), and cryopreserved in liquid nitrogen. The cells were thawed just prior to administration. Cell yield and viability were assessed using the Trypan blue dye exclusion method. 50 million viable cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) to enable tracking of the cells within the brain (Yawno et al., 2013). Cells were suspended in 2.5 ml sterile saline, and administered to the fetus (over 3 min) via the femoral vein.

2.4. Tissue collection and processing

At 10 d after UCO (112.5 ± 0.2 d gestation), the ewe and fetus were euthanized by iv overdose of sodium pentobarbital (Virbac, Peakhurst, Australia) to the ewe. The fetus was removed and fetal body weight
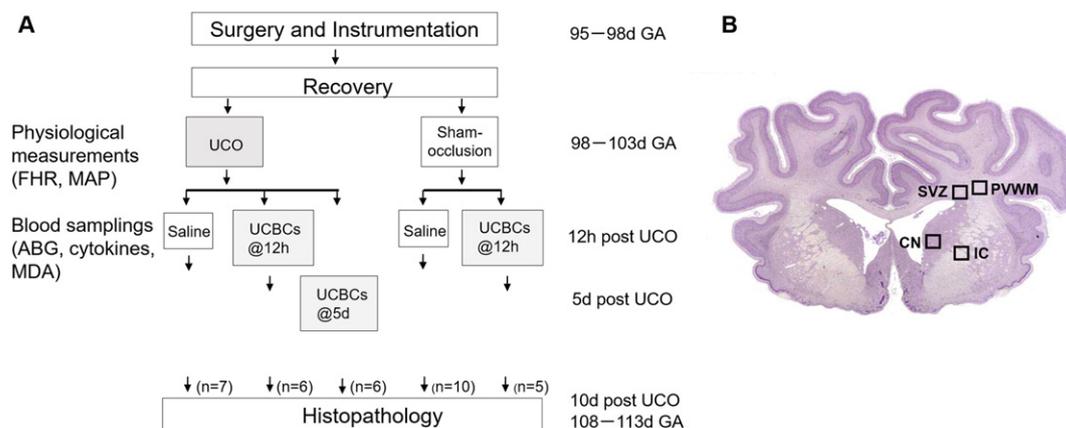

Fig. 1. A: Experimental timeline. ABG: arterial blood gas; FHR: fetal heart rate; GA: gestational age; HI: hypoxia-ischemia; MAP: mean arterial pressure; MDA: malondialdehyde; UCBCs: umbilical cord blood cells; UCO: umbilical cord occlusion. B: Representative lamb brain photomicrograph of anatomical regions assessed for histology (Sheep Ovis aries–Section 720). SVZ: subventricular zone; PVWM: periventricular white matter; IC: internal capsule; CN: caudate nucleus.

Table 1
Physiological outcomes.

Variables		Control	HI	HI + UCB@12 d	HI + UCB@5 d	Control + UCB@12 h
Samples included in analysis, n		10	7	6	6	5
Samples excluded due to death, n; (mortality, %)		1 (9)	3 (30)	2 (25)	2 (25)	0 (0)
Female, n (%)		7 (70)	3 (43)	3 (50)	3 (50)	2 (40)
Twin, n (%)		6 (60)	2 (29)	0 (0)[a]	0 (0)[a]	0 (0)[a]
UCO duration, min		0	24.4 ± 0.3[a]	24.2 ± 0.4[a]	24.3 ± 0.4[a]	0
Weight	Brain weight, g	34.5 ± 0.7	30.5 ± 0.9	29.8 ± 1.0 [a]	31.8 ± 0.9	36.5 ± 1.0
	Body weight, kg	2.0 ± 0.1	2.4 ± 0.1	2.1 ± 0.2	2.5 ± 0.2	2.1 ± 0.2
	Brain/body weight, g/kg	17.6 ± 0.7	13.6 ± 0.9[a]	14.4 ± 0.9	13.1 ± 0.9[a]	17.3 ± 1.0
FHR, bpm	Baseline	201 ± 9	201 ± 5	198 ± 4	192 ± 5	207 ± 4
	End of occlusion	194 ± 4	69 ± 3[a,b]	66 ± 7[a,b]	64 ± 5[a,b]	201 ± 6
	2–12 h	200 ± 3	201 ± 6	195 ± 9	206 ± 5	208 ± 5
	12–36 h	203 ± 4	202 ± 5	197 ± 4	195 ± 4	203 ± 5
	96–120 h	204 ± 2	197 ± 5	197 ± 3	205 ± 2	196 ± 4
	120–144 h	202 ± 4	192 ± 6	191 ± 2	194 ± 8	200 ± 4
MAP, mm Hg	Baseline	37.0 ± 2.4	35.9 ± 1.4	35.7 ± 0.7	36.7 ± 4.2	35.9 ± 0.5
	End of occlusion	36.4 ± 2.6	8.9 ± 0.9[a,b]	8.7 ± 2.0[a,b]	8.4 ± 1.8[a,b]	35.5 ± 0.5
	2–12 h	37.9 ± 1.7	37.3 ± 1.1	37.2 ± 1.4	38.7 ± 0.9	37.0 ± 1.1
	12–36 h	37.7 ± 2.1	37.7 ± 2.0	42.2 ± 2.0[b]	37.7 ± 2.0	36.5 ± 1.0
	96–120 h	37.2 ± 2.2	37.2 ± 1.7	38.6 ± 1.1	36.6 ± 1.9	36.0 ± 2.3
	120–144 h	37.6 ± 1.4	37.5 ± 1.4	39.2 ± 1.2	36.5 ± 2.0	36.1 ± 2.4
pH	Baseline	7.40 ± 0.02	7.36 ± 0.01	7.36 ± 0.02	7.37 ± 0.01	7.35 ± 0.01
	20 min	7.37 ± 0.01	6.87 ± 0.01[a,b]	6.84 ± 0.01[a,b]	6.89 ± 0.03[a,b]	7.35 ± 0.02
	12 h	7.38 ± 0.01	7.37 ± 0.01	7.35 ± 0.01	7.37 ± 0.01	7.35 ± 0.01
	120 h	7.37 ± 0.01	7.37 ± 0.01	7.37 ± 0.02	7.35 ± 0.01	7.36 ± 0.03
	240 h	7.38 ± 0.01	7.35 ± 0.01	7.37 ± 0.02	7.36 ± 0.01	7.37 ± 0.02
PaO$_2$, mm Hg	Baseline	23.2 ± 0.9	23.2 ± 0.8	23.5 ± 1.5	24.1 ± 2.2	25.1 ± 1.7
	20 min	23.7 ± 0.6	8.4 ± 1.4[a,b]	11.0 ± 2.0[a,b]	12.3 ± 2.2[a,b]	22.9 ± 1.6
	12 h	23.2 ± 0.3	23.9 ± 0.8	24.0 ± 1.3	26.7 ± 3.0	21.6 ± 1.1
	120 h	22.1 ± 1.0	23.8 ± 1.3	27.0 ± 2.3	27.8 ± 2.8	25.3 ± 0.5
	240 h	23.0 ± 1.1	22.1 ± 1.8	25.0 ± 2.0	27.0 ± 1.9	22.0 ± 1.8
PaCO$_2$, mm Hg	Baseline	38.3 ± 3.9	44.4 ± 2.6	46.9 ± 2.2	45.1 ± 0.5	43.4 ± 1.7
	20 min	43.8 ± 2.6	107.3 ± 7.8[a,b]	115.1 ± 8.9[a,b]	104.1 ± 5.0[a,b]	49.2 ± 1.4
	12 h	46.6 ± 2.5	46.2 ± 1.9	45.2 ± 2.7	46.5 ± 1.2	49.0 ± 0.7
	120 h	48.6 ± 1.5	47.4 ± 2.3	47.9 ± 2.2	45.6 ± 2.1	44.6 ± 2.6
	240 h	49.0 ± 1.4	50 ± 1.9	46.9 ± 2.9	49.7 ± 2.2	51.7 ± 2.6
BE, mmol/l	Baseline	−1.3 ± 1.9	−0.4 ± 1.5	0.7 ± 0.9	0.6 ± 0.3	−1.4 ± 0.9
	20 min	−0.3 ± 1.1	−16.0 ± 1.1[a,b]	−16.2 ± 0.9[a,b]	−12.2 ± 1.8[a,b]	1.3 ± 0.8
	12 h	1.6 ± 1.3	0.9 ± 1.0	0.3 ± 1.7	1.3 ± 0.4	1.1 ± 0.5
	120 h	2.2 ± 0.5	1.5 ± 1.3	1.7 ± 1.4	−1.0 ± 0.8	1.9 ± 0.4
	240 h	2.9 ± 0.4	1.4 ± 0.9	0.6 ± 0.1	1.9 ± 0.7	3.1 ± 0.5
Lactate, mmol/l	Baseline	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1
	20 min	0.8 ± 0.1	7.9 ± 0.8[a,b]	8.4 ± 0.8[a,b]	7.8 ± 0.5[a,b]	1.2 ± 0.1
	12 h	0.9	1.4 ± 0.2	2.0 ± 0.5	1.6 ± 0.2	1.1 ± 0.1
	120 h	1.0 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	1.2 ± 0.2
	240 h	1.0 ± 0.1	1.5 ± 0.3	0.9 ± 0.1	1.0 ± 0.1	1.2 ± 0.1

Mean (%) or mean ± standard error are presented for each group. One-way analysis of variance and post-hoc Tukey tests were carried out on comparisons between groups. [a]$P < 0.05$ vs control at the same time point or during the same time period. [b]$P < 0.05$ within group comparisons vs baseline. The start of occlusion was set as time zero. HI: hypoxia-ischemia; UCB: umbilical cord blood; FHR: fetal heart rate; MAP: mean arterial pressure; PaO$_2$: partial arterial pressure of oxygen; PaCO$_2$: partial arterial pressure of carbon dioxide; BE: base excess.

was recorded. The fetal brain was divided in half sagitally. The right cerebral hemisphere was cut transversely into 5 mm slices and immersion fixed in 10% formalin for 5 d. Subsequently, paraffin-embedded 10 μm coronal sections were cut at the level of the subventricular zone (SVZ) and the caudate nucleus (CN), and mounted on Superfrost/Plus slides (Thermoscientific, USA). Brain regions of interest were the SVZ, PVWM, internal capsule (IC), and CN (Fig. 1B). The left cerebral hemisphere was separated into anatomical regions, snap frozen in liquid nitrogen and stored at −80 °C for future assessment.

2.5. Immunohistochemistry

Rabbit polyclonal oligodendrocyte transcription factor 2 (Olig2, 1:1000; Millipore) and mouse-anti-human 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase, 1:200, Sigma Chemical) antibodies were used to identify oligodendrocyte lineage cells, and myelin fiber tracts, respectively. Apoptotic cell death was assessed with rabbit polyclonal anti-human/mouse active caspase-3 (1:1000; R&D Systems) and proliferating cells were visualized with rabbit anti-human Ki-67 antibody (1:100, Dako). Activated microglia were

identified via morphology using rabbit anti-ionized calcium binding adaptor molecule 1 (Iba-1, 1:500; Wako) (Yawno et al., 2013).

Sections were deparaffinized, and antigen retrieval performed by microwaving sections in 0.01 M citrate buffer (pH 6). Endogenous peroxidase activity was blocked by pretreating with 0.3% hydrogen peroxidase in 50% methanol or phosphate-buffered saline (PBS; 0.1 M) for 30 min. Nonspecific binding was blocked by incubation in 5% normal goat serum (Iba-1), 0.3% Triton X-100 in PBS (for Ki-67), or 5% bovine serum albumin (for Olig2, CNPase and caspase-3) for 1 h at room temperature. Sections were subsequently incubated overnight with the primary antibodies at 4 °C. All sections were treated with secondary antibody (1:200; biotinylated anti-rabbit or anti-mouse IgG antibody; Vector Laboratories) and staining was visualized by 3,3′-diaminobenzidine tetrahydrochloride (DAB, Pierce). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, for the detection of DNA fragmentation, was used as broadly detecting cell death including apoptosis/necrosis (Grasl-Kraupp et al., 1995). Manufacturer's instructions were followed (DeadEnd Colorimetric TUNEL System, Promega Corporation, Madison, USA). Negative control sections were included in each run.

2.6. Imaging and quantification

Slides were imaged at ×400 magnification under light microscopy (Olympus BX-41). The numbers of Olig2-, Ki-67-, and activated Iba-1-immunoreactive cells per field of view were counted by an investigator blinded to the treatment group. The densities of caspase-3-positive cells and CNPase-immunoreactive myelinated fibers were quantified using ImageJ (NIH). Immunohistochemical outcomes were assessed in 2 sections per animal and 3 fields of view per region on non-adjacent sections, with the results averaged per animal and then across animals in each group.

2.7. Double label immunohistochemistry

Double-label immunohistochemistry was performed on 2 sections from 2 fetal brains in each group to assess oligodendrocyte proliferation. Sections were first washed with sodium borohydride (NaBH4, 98%; 10 mg/ml) in PBS to reduce the auto-fluorescence, and treated with a serum-free protein blocker (DAKO) to prevent background staining, and then incubated with mouse anti-Olig2 (1:1000; Millipore) and rabbit anti-Ki-67 antibodies at 4 °C overnight. Immunoreactivity was visualized with Alexa Fluor 594 goat anti-mouse (Red, 1/1000; Molecular Probes) and Alexa Fluor 488 goat anti-rabbit (Green, 1/1000, Molecular Probes) and viewed with a fluorescence microscope (Olympus BX-41).

2.8. Cytokine assay

Plasma cytokine concentrations for pro-inflammatory IL-6 and TNF-α, and anti-inflammatory IL-10, were analyzed against recombinant cytokines via capture ELISA techniques using ovine-specific monoclonal antibodies (Liravi et al., 2015).

2.9. Malondialdehyde (MDA) assay

Plasma lipid peroxidation was assessed via the thiobarbituric acid reactive substances method to measure MDA in the sample, and MDA generated from lipid hydroperoxides by the hydrolytic conditions of the reaction (Miller et al., 2014). The manufacturer's protocol was followed (Cayman Chemical, Ann Arbor, USA).

2.10. Data analysis

All animals that completed the whole experimental course were included into data analyses. All assessments were conducted on coded slides or samples, with the examiner blinded to the experimental groups. Data are presented as mean ± standard error. Statistical analysis was performed with GraphPad Prism 6 (GraphPad Software, San Diego, USA). Differences between three or more groups were analyzed by a two-way analysis of variance (ANOVA) (histology data) or one-way ANOVA (fetal weight, arterial blood gas, physiology, plasma cytokine and MDA levels), followed by the Tukey post-hoc test when a significant difference was found. Differences between 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.

3. Results

The overall fetal survival rate for this study was 81%, with no significant differences in mortality between groups (Table 1). Mean fetal body weight at postmortem for all animals was 2.2 kg, with no significant group differences (Table 1). Brain weight for fetuses after HI, with or without UCBC treatment, was reduced by 20% compared to control fetuses. Brain/body weight ratio was reduced in HI and HI + UCB@ 5 d versus control groups ($P < 0.05$).

3.1. Physiological measures

HI resulted in severe hypotension and bradycardia, reduced fetal arterial pH, PaO$_2$ and base excess, and increased PaCO$_2$ and lactate levels, compared with control ($P < 0.05$; Table 1). There was no difference in the duration of UCO, or any physiological parameters following the insult between the 3 HI groups. Following reperfusion and reoxygenation, physiological parameters returned to baseline values. In HI + UCB@12 h fetuses, MAP was transiently elevated following UCBC administration ($P < 0.05$), but this was not observed within the HI + UCB@5 d and control + UCB@12 h animals. No intergroup differences in physiological and biochemical measures were found after 36 h.

3.2. Brain histopathology

3.2.1. White matter injury (WMI) induced by HI

We firstly confirmed that severe acute HI caused by UCO in preterm fetal sheep resulted in WMI. Ten days after insult, HI fetuses demonstrated a reduction in Olig2+ oligodendrocyte lineage cells, decreased 25–41% compared to control, within the SVZ and IC ($P < 0.05$), and PVWM ($P = 0.07$). The density of myelinated fiber tracts was reduced 25–30% within the PVWM and IC of HI fetuses, compared to control fetuses ($P < 0.05$; Fig. 2B); Fig. 3F shows that within the PVWM, the white matter tracts are sparse and disorganized following HI. TUNEL+ cell counts were highly variable across brain regions, with the IC of HI brains demonstrating the most profound TUNEL+ staining (Fig. 3J), and indeed the IC was the only region to show a significant increase in TUNEL+ cell density in HI fetuses compared to control ($P < 0.05$, Fig. 2C). HI was also associated with increased cell proliferation (Ki67+ cells) within the SVZ, compared to control ($P < 0.05$, Fig. 2D), and double label staining demonstrated that Ki-67 was substantially co-localized with Olig2+ oligodendrocyte lineage cells (Fig. 3W). Finally, HI resulted in significant inflammatory cell activation within the preterm brain, with increased Iba-1+ activated microglial density within the PVWM and IC ($P < 0.05$) compared to control fetal brains (Fig. 2E).

3.2.2. Effects of UCBCs at 12 h after HI

We next determined whether the intravenous administration of 50 million UCBCs at 12 h after HI had a neuroprotective effect within the white matter of the preterm brain. Two-way ANOVA demonstrated a significant overall protective effect of UCBCs administered at 12 h (Fig. 2A–E). Where HI alone reduced Olig2+ oligodendrocyte density compared to control, there was no difference in oligodendrocyte density in HI + UCB@12 h compared to control, and, indeed, oligodendrocyte density was significantly increased in UCB-treated brains within the IC, compared to HI ($P < 0.05$; Fig. 2A). Similarly, UCBC administration normalized axonal myelination, with no difference in the density of myelinated fiber tracts between control and HI + UCB@12 h across all white matter regions examined (Fig. 2B), and these tracts demonstrated appearance and organization similar to the control group (Fig. 3G). The administration of UCBCs at 12 h after HI protected against cell death, with no increase in TUNEL-positive staining in the IC in the HI + UCB@12 h compared to control animals (Fig. 2C), and there was also no increase in cell proliferation in the SVZ in UCB-treated animals (Fig. 2D). UCBC administration at 12 h demonstrated a profound anti-inflammatory effect within the brain after HI. Iba-1+ activated microglial density was decreased within the white matter in HI + UCB@12 h animals compared to HI alone ($P < 0.05$) and there was no difference between HI + UCB@12 h and control brains (Fig. 2E). Our results also demonstrate that UCBC administration to control fetuses (control + UCB@ 12 h) had no effect on white matter development, with no difference in oligodendrocyte density, myelination, cell death, proliferation, or activated microglia (Fig. 2A–E).
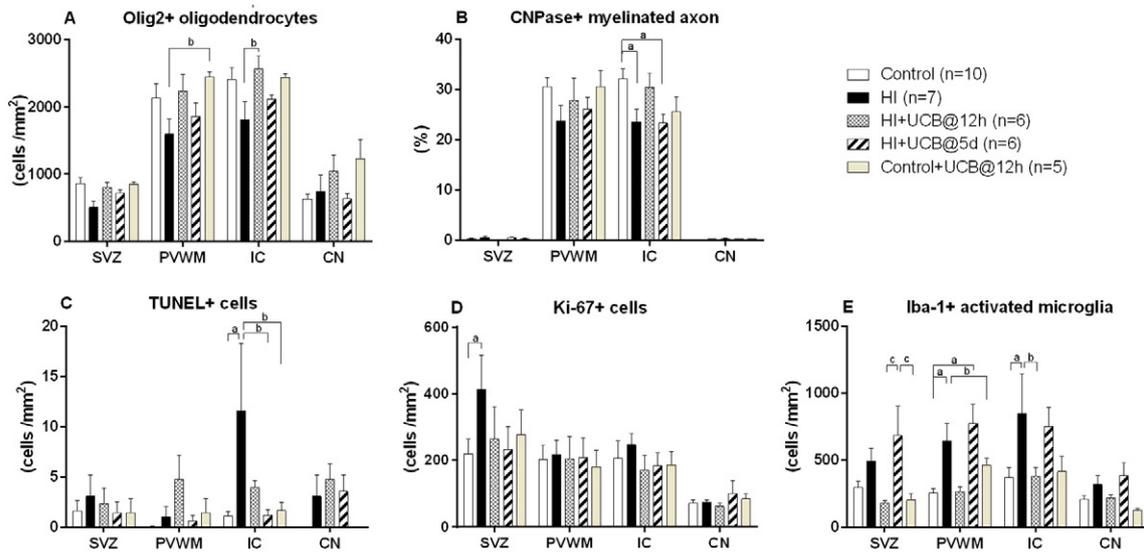
Fig. 2. Time-dependent effects of UCBCs on regional densities of Olig2+ oligodendrocytes (A), CNPase+ myelinated axon (B), TUNEL+ cell death (C), Ki67+ proliferating cells (D), and Iba-1+ activated microglia (E) in subventricular zone (SVZ), periventricular white matter (PVWM), internal capsule (IC) and caudate nucleus (CN). A and B: HI was associated with a reduced densities of Olig2+ oligodendrocytes and CNPase+ myelin fiber tracts ($P < 0.05$) in the white matter compared to control fetuses. UCBC administration at 12 h, but not 5 d, protected the white matter structures. C and D: HI fetuses showed increased cell death (TUNEL+ cells) in the IC, and increased cell proliferation in the SVZ compared to control fetuses. While UCBCs at 12 h and 5 d attenuated the effects. E: Activated microglia density (Iba-1+) was increased in the white matter of HI brains. However, UCBC administration at 12 h, but not 5 d, suppressed the microglial infiltration. Data are mean ± SEM, and were analyzed using ANOVA followed by post-hoc Tukey's pairwise comparisons for the individual brain region. *$P < 0.05$.

3.2.3. Delaying administration of UCBCs until 5 d after HI

Injury to the preterm brain is complex, and may take time to become clinically apparent. Therefore, lastly, we examined whether delaying UCBC administration until 5 d after HI was also neuroprotective. Our results show that there is an intermediate benefit of UCBC administration at 5 d after HI compared to treatment at 12 h. That is, where HI alone reduced oligodendrocyte density by 25–41% (SVZ, PVWM and IC), and cell administration at 12 h prevented this cell loss, delaying cell administration until 5 d resulted in ~15% loss of oligodendrocytes (Fig. 2A). Interestingly, hypomyelination persisted within the IC of the HI + UCB@5 d brains, compared to control ($P < 0.05$, ANOVA and Tukey test; Fig. 2B). There was no difference in TUNEL-, or Ki-67-positive cells in the white matter of HI + UCB@5 d versus controls (Fig. 2C, D). Fig. 2E shows that delaying UCBC administration until 5 d does not prevent microglial activation, with no difference between activated microglial densities in HI + UCB@5 d versus HI fetal brains.

We noted that the morphological appearance of Iba-1+ microglia was different between groups, with control brains exhibiting microglia with the characteristics of ramified (resting) microglia, with small cell bodies and long branching processes (Fig. 3Q, U). In contrast, microglia within the white matter of HI brains more commonly demonstrated morphology of activated or ameboid microglia (Fig. 3R, V). This observation of altered morphology with HI was largely reversed following UCBC administration at 12 h (Fig. 3S), but not at 5 d (Fig. 3T). There was a significant negative correlation between the density of activated microglia and oligodendrocytes within the PVWM ($R^2 = 0.46$, $P < 0.001$), and IC ($R^2 = 0.67$, $P < 0.001$; Fig. 4). There was no correlation between oligodendrocytes and TUNEL+ cell densities.

3.3. Distribution of UCBCs within the brain

The presence and localization of donor UCBCs within the fetal brains was determined by visualizing the CFSE-green fluorescent-positive cells within three adjacent brain sections in each animal. UCBCs were detected in 3 of 7 HI + UCB@12 h, 3 of 6 HI + UCB@5 d, and 3 of 5 control + UCB@12 h fetal brains. The number of cells observed was very small (0.7–2.1 cells per section without intergroup differences).

Where present, the cells were widely distributed across the brain section, with ~65% found in the white matter.

3.4. Plasma pro-inflammatory and anti-inflammatory cytokines

Circulating pro-inflammatory cytokines, TNF-α and IL-6 concentrations, showed no significant changes throughout the experiment in all groups. Anti-inflammatory IL-10 concentration at 10 d was significantly higher in HI + UCB@12 h animals compared to HI, HI + UCB@5 d, and control + UCB@12 h animals ($P < 0.05$; control 13.5 ± 2.0; HI 11.3 ± 1.4; HI + UCB@12 h 16.0 ± 1.4; HI + UCB@5 d 9.0 ± 1.2; control + UCB@12 h 7.1 ± 1.0 BU/ml; Fig. 5).

3.5. Plasma malondialdehyde

HI was associated with a significant increase of plasma MDA levels from 6 h, with a plateau from 12 to 48 h, and with a return to baseline at 72 h in the HI fetuses (Fig. 6). In HI + UCB@12 h fetuses, there was no difference in circulating MDA levels at 6 and 12 h compared to HI alone, but the administration of UCBCs at 12 h returned MDA levels to baseline by 24 h, such that concentrations were significantly reduced compared to HI fetuses at 48 h ($P < 0.05$).

4. Discussion

This study is the first to show that allogeneic UCBCs are neuroprotective following HI insult in a well-established fetal sheep model of preterm WMI, and early cell administration is optimal. Cell administration at 12 h after HI prevented WMI - oligodendrocyte loss and hypomyelination - but delaying administration until 5 d limited their protective benefits. We found that the neuroprotective benefits of UCBCs are likely mediated via anti-inflammatory and anti-oxidant actions. The anti-inflammatory effects of UCBCs appear critical, given the observation of a strong correlation between neuroinflammation and WMI.

Firstly, we confirmed that acute HI produced WMI in the preterm brain. HI induced an increase in TUNEL+ cells, where TUNEL reveals
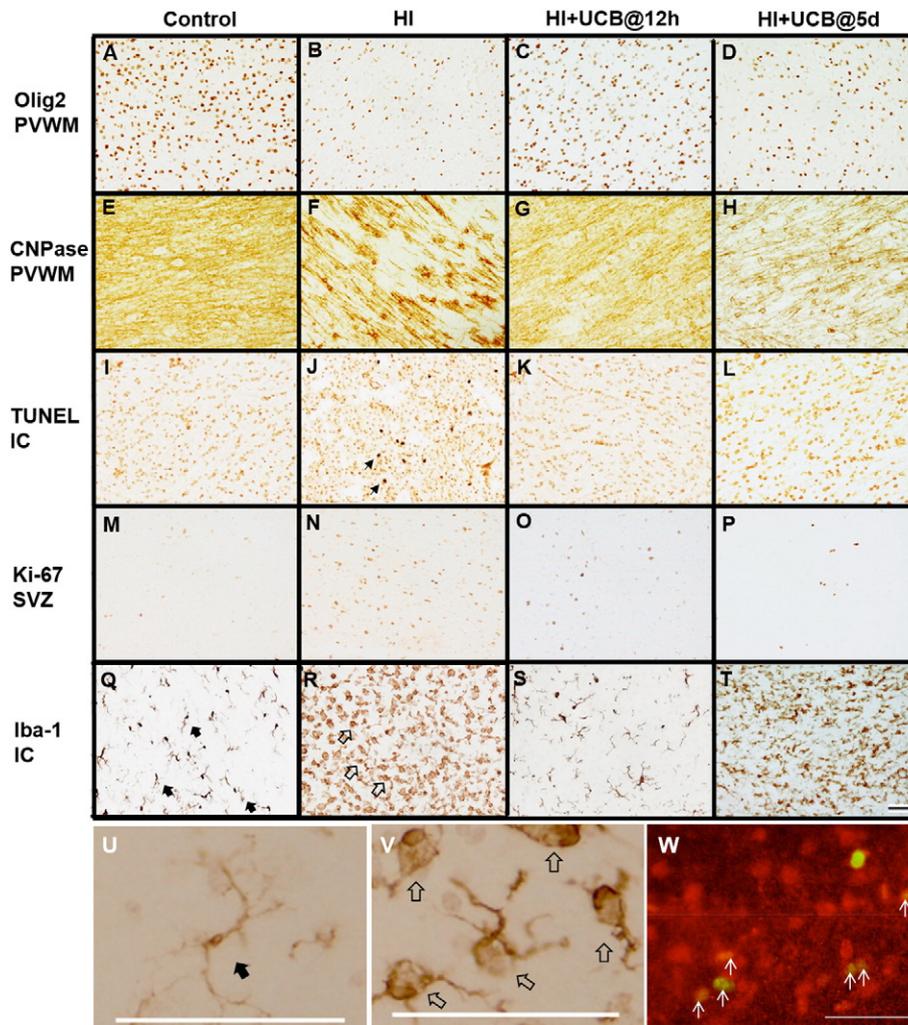
Fig. 3. Photomicrograph images showing Olig2 (A–D) and CNPase (E–H) staining in the periventricular white matter (PVWM), TUNEL (I–L) and Iba-1 (Q–T) staining in the internal capsule (IC), and Ki-67 (M–P) in subventricular zone (SVZ), in the brain of control, HI, HI + UCB@12 h, and HI + UCB@5 d fetuses. Thin black arrows show examples of TUNEL-positive cells. At high magnification, Iba-1 + inflammatory cells in control brains exhibited the characteristics of ramified (resting) microglia, with small cell bodies and long branching processes (Q, U; broad black arrow). However, the cells in HI group show loss of microglial branching and transformation into rounded macrophages (R, V; white arrows with black outline). The changes are to a large extent reversed following UCBC administration at 12 h (S), but not at 5 d (T). Double-label fluorescent immunostaining of Olig2 (red) and Ki-67 (green) with merged images in the SVZ of the brain in an HI fetus (W). White arrow shows example of cell with dual signal. Scale bar is 50 μm.

DNA fragmentation and is a useful, broad indicator of cell death (Grasl-Kraupp et al., 1995). We observed a variable pattern of TUNEL + staining at 10 d following HI, from negligible cell death through to a significant degree of cell death in the IC of some animals. Previous work has shown that TUNEL + staining is mostly resolved at 7d after HI (Renolleau et al., 1998), suggesting that we did not observe peak staining at 10 d post-HI. It was however encouraging to observe that UCBC administration at either 12 h or 5 d prevented TUNEL + cell death. HI induced an upregulation of cell proliferation in the SVZ, a germinal region of the brain associated with neurogenesis and gliogenesis
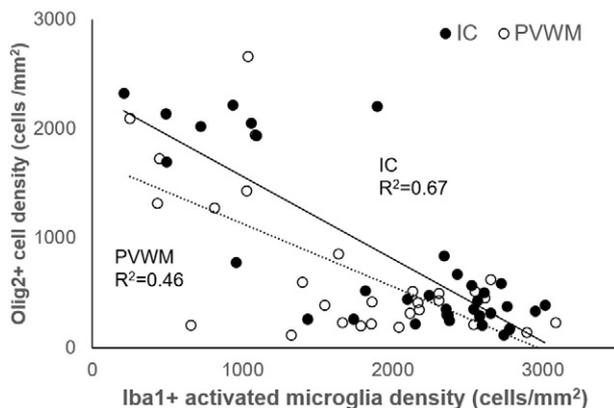
Fig. 4. Correlation analysis across all treatment groups between the number of oligodendrocyte (Olig2+) and activated microglia (Iba-1+) cells revealed a significant negative correlation in the PVWM ($R^2 = 0.46$, $P < 0.001$) and IC ($R^2 = 0.67$, $P < 0.001$). PVWM: periventricular white matter; IC: internal capsule.
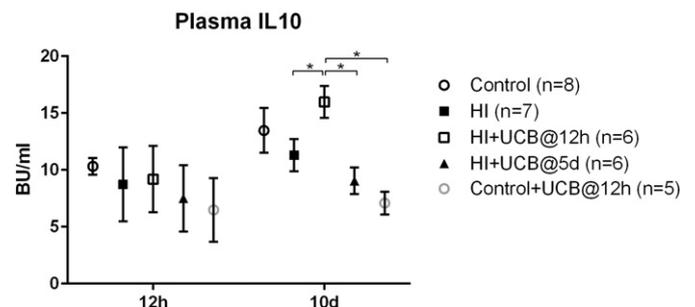
Fig. 5. The concentration of anti-inflammatory plasma IL-10 at 10 d was significantly higher in the HI + UCB@12 h group compared to HI, HI + UCB@5 d, and control + UCO@12 h groups. *$P < 0.05$. BU: biological unit.
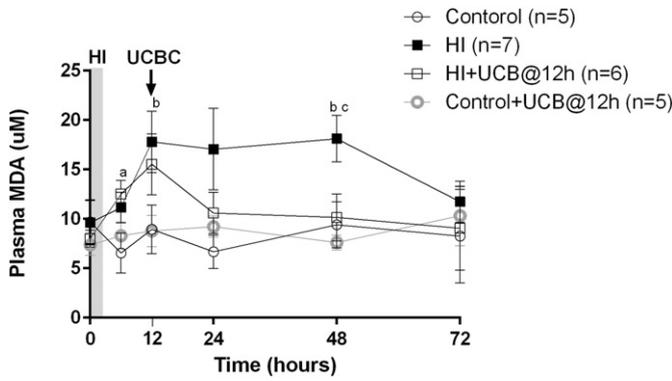
Fig. 6. Fetal plasma malondialdehyde (MDA) concentrations in control, HI, HI + UCB@12 h and Control + UCB@12 h groups. Hypoxia-ischemia (HI), induced by umbilical cord occlusion, is shown by the gray column, and the time point of umbilical cord blood cell (UCBC) administration is shown by black arrow. HI was associated with a significant increase in plasma MDA levels from 6 h, and a return to baseline at 72 h. In HI + UCB@ 12 h fetuses, there was no difference in plasma MDA levels at 6 and 12 h compared to HI alone, but the administration of UCBCs at 12 h returned MDA levels to baseline by 24 h, such that concentrations were significantly reduced compared to HI fetuses at 48 h ($P < 0.05$). [a]$P < 0.05$: control vs. HI + UCB@12 h; [b]$P < 0.05$: control + UCB@12 h vs. HI; [c]$P < 0.05$: HI vs. HI + UCB@12 h.

(Ong et al., 2005). We found that proliferating cells in this area predominantly expressed markers of oligodendrocytes. UCBC administration at 12 h or 5 d following HI attenuated the proliferative response. However, the timing of cell administration had a notable effect on white matter protection. Cell administration at 12 h after HI protected oligodendrocyte number and myelination density, but only partial protection was observed in the 5 d treatment cohort. The finding that UCBC treatment ameliorated oligodendrocyte degeneration and proliferation following acute insult is important, since selective death of pre-oligodendrocytes, aberrant proliferation and subsequent arrest of myelination are key features in chronic WMI (Buser et al., 2012; Baumann and Pham-Dinh, 2001). We used Olig2 and CNPase to identify oligodendrocyte lineage cells and myelinated fiber tracts respectively (Back et al., 2007), and did not characterize the maturational progression of oligodendrocytes in the current study. We can reasonably speculate that UCBC treatment at 12 h protected oligodendrocyte development, as evidenced by the density and organization of myelinated white matter tracts that was similar to the control group.

Our results support that the therapeutic effects of UCBCs on white matter protection are mediated by anti-inflammatory and anti-oxidant actions. We found very few labeled cells within the brain at 10 d after insult, with no difference in the presence or localization of donor cells administered at 12 h or 5 d. There has been contention regarding whether cell engraftment is necessary to mediate neuroprotection (Borlongan et al., 2004), but even with low numbers of donor cells within the brain, we found profound therapeutic effects of UCBCs against HI-induced preterm brain injury. Similarly, cell engraftment is not necessary to mediate the protective benefits of xenogeneic UCBC administration following stroke in adult rodents (Boltze et al., 2012). Our results support previous work to show that the neuroprotective actions of stem cells are primarily mediated by indirect systemic and neuroimmunomodulatory effects (Castillo-Melendez et al., 2013; Li et al., 2014; Bennet et al., 2012; Borlongan et al., 2004).

Acute HI induced a fetal inflammatory response, evidenced by elevated neuroinflammation. We show that UCBC administration at 12 h after HI moderates brain inflammation, decreasing activated microglial density and inducing a shift of microglial phenotype towards a resting state. This outcome is consistent with reports of reduced neuronal death and functional improvements, mediated by reduced microglial activation, following human UCBC treatment in neonatal rats (Pimentel-Coelho et al., 2010; Wasielewski et al., 2012). We now demonstrate that delaying UCBC administration until 5 d post-insult limits

UCBC suppressive effects on microglial activation, and their subsequent ability to preserve white matter. Microglial activation is one of the first steps in the process of neuroinflammation, and in turn is a critical contributor towards WMI (Czeh et al., 2011). A mouse model of neonatal WMI demonstrates a rapid microglial response that peaks within 3d of insult (Tahraoui et al., 2001). Activated microglia then exacerbate inflammation through the production of cytokines that initiate delayed cell degeneration, including oligodendrocyte death (Khwaja and Volpe, 2008; Deng et al., 2008). Indeed, in this study we show a strong correlation between upregulation of activated microglia and loss of oligodendrocytes in HI fetuses, causing subsequent hypomyelination. While the etiology of WMI in the human preterm brain is complex, it is appreciated that hypoxia and inflammation are consistent causal factors (Khwaja and Volpe, 2008), and both are mediated, at least in part, by the activation of microglia (Baburamani et al., 2014). Thus, preventing microglial activation with UCBC at 12 h appears to be a critical neuroprotective action. Additionally, UCBC administration at 12 h induced upregulation of systemic anti-inflammatory IL-10, which likely acts synergistically with suppression of neuroinflammation to protect against preterm WMI (McCarthy et al., 2010). These results are indicative of UCBCs preventing WMI predominantly via anti-inflammatory actions.

The neuroprotective effects of UCBCs may also be mediated by antioxidant actions. In the present study, UCBC administration at 12 h after HI decreased circulating MDA. MDA is a by-product of lipid peroxidation, upregulated under conditions of oxidative stress (Miller et al., 2014), and was increased in the fetal circulation at 6-12 h following HI. The preterm brain has a low anti-oxidant defense capability (Huertas et al., 1998), is highly susceptible to oxidative damage (Miller et al., 2014; Phillis, 1994), and increased lipid peroxides co-localize with pre-oligodendrocytes to induce cell death (Haynes et al., 2003). In vitro evidence supports that various stem cells have anti-oxidant properties (Madhavan et al., 2006; Valle-Prieto and Conget, 2010), however to date, evidence for antioxidant-mediated neuroprotection in vivo has been mixed (Li et al., 2014; Arien-Zakay et al., 2009). Activated microglia release reactive oxygen species within the brain following HI and inflammation (Haynes et al., 2005), which react with brain lipids to cause peroxidative damage. The efficacy of UCBC transplantation may partially depend on an anti-oxidant effect, acting synergistically with anti-inflammatory actions.

In the current study, cell administration at 5 d after HI was less effective than administration at 12 h. Unlike term hypoxic ischemic encephalopathy, the onset of WMI is usually unclear in preterm infants, and is likely to evolve chronically (Li et al., 2014; Li et al., 2013). Thus, a preferred neuroprotective treatment would be one with a prolonged therapeutic window, and with diverse therapeutic potential. Indeed, in this study UCBCs exhibited multiple mechanisms of action. Other studies have shown that UCBCs have therapeutic benefits when administered 48-72 h following acute injury (Bennet et al., 2012; Yu et al., 2009). The mononuclear cell fraction of UCB is composed of lymphocytes, monocytes, and three principal types of stem/progenitor cells; hematopoietic, endothelial and mesenchymal stem cells (MSCs) (Li et al., 2014). Individually, each cell type has neuroprotective potential (Castillo-Melendez et al., 2013; Li et al., 2014). In particular, monocytes, regulatory T-lymphocytes, and MSCs are shown to have anti-inflammatory properties (Li et al., 2014; Womble et al., 2014), and could mediate the neuroprotective effects observed in this study. We do not know the proportion of these cell types administered in the current study, but all studies to date demonstrate a very low yield of MSCs in human term UCB, and our work in sheep UCB suggests similar (unpublished observations). MSCs have received the greatest attention for prevention or repair of central nervous system injury, primarily due to their anti-inflammatory actions (Lei et al., 2015). It has been recently reported that the efficacy of human UCB-derived MSCs was time-dependent for reducing lung injury in preterm rats, wherein the cells were only effective when given within 3 days after insult (Chang et al., 2013). We suggest that the therapeutic time window of UCB therapy for

neuroprotection, particularly anti-inflammatory effects, is optimal within the first 3 days following the onset of WMI.

It should be noted that an advantage of undertaking UCB administration in a preterm cohort is the relative plasticity of the developing brain, and potential for regeneration. Our study finds that UCBCs administered at 5 d had limited protective effects, whereas it was recently reported that human UCBCs induce motor improvements in a rabbit model of cerebral palsy, even when cells were administered at 9d after HI insult (Drobyshevsky et al., 2015). That 5 d treatment with UCBCs was not neuroprotective for WMI in the current study may simply reflect that we did not allow the UCBCs at 5 d after insult sufficient time to provide a benefit. We acknowledge that undertaking all neuropathology assessments at 10 d after HI is a limitation of this study, and future studies should permit an extended period for neural regeneration. Nevertheless our observation that early administration of UCBC therapy is essential for optimal histological improvement is supported by other studies (Li et al., 2014; Wasielewski et al., 2012; Aridas et al., 2015; Boltze et al., 2012; Yu et al., 2009; Pimentel Coelho, 2010). Further evaluation in a larger number of animals, with a longer duration of study, is needed to assess the extended neuroprotective and regenerative abilities of UCB therapy. We do however suggest that preventing WMI is more straightforward than repairing WMI once injury has become apparent.

We utilized an allogeneic source of UCB, rather than autologous UCB, to avoid possible harm to fetuses caused by withdrawing a large volume of blood in utero, and because allogeneic UCB therapy is a realistic clinical option in infants born very or extremely preterm, where limited cord blood may be available (Surbek et al., 2000). We assumed that fetal weight was 1.6 kg when cells were administered (103 d gestation, from historical post-mortem data); thereby providing ~30 million cells/kg. However, questions remain, particularly around dose, and which UCBCs are optimal in this very preterm cohort; allogeneic or autologous cells, from term or preterm pregnancies? A further confounder for preterm infants is the lack of guidelines or sensitive diagnostic tools to advise which preterm infants may develop WMI and would benefit from UCBC therapy.

Hypomyelination and disorganization of major white matter fiber tracts correlate with functional deficits in children with periventricular leukomalacia and cerebral palsy. Our investigation demonstrates the therapeutic effects of UCBCs in preserving white matter architecture following HI in the preterm brain, with time-dependent efficacy. Neuroprotection with cord blood cell administration was mediated by diverse actions, including anti-inflammatory and antioxidant effects, and prevention of cell death. In particular, cord blood anti-inflammatory effects are likely to be a principal mechanism of action, given the strong association between neuroinflammation and WMI in preterm infants. UCBCs could provide significant protective benefit for preterm infants at high risk of WMI, and their neuroprotective benefit is optimized when cells are administered as early as possible.

Acknowledgment

References

Aridas, J.D., McDonald, C.A., Paton, M.C., et al., 2015. Cord blood mononuclear cells prevent neuronal apoptosis in response to perinatal asphyxia in the newborn lamb. J. Physiol.
Arien-Zakay, H., Lecht, S., Bercu, M.M., et al., 2009. Neuroprotection by cord blood neural progenitors involves antioxidants, neurotrophic and angiogenic factors. Exp. Neurol. 216, 83–94.
Baburamani, A.A., Supramaniam, V.G., Hagberg, H., Mallard, C., 2014. Microglia toxicity in preterm brain injury. Reprod. Toxicol. 48, 106–112.

Back, S.A., Volpe, J.J., 1997. Cellular and molecular pathogenesis of periventricular white matter injury. Ment. Retard. Dev. Disabil. Res. Rev. 3, 96–107.
Back, S.A., Riddle, A., McClure, M.M., 2007. Maturation-dependent vulnerability of perinatal white matter in premature birth. Stroke 38, 724–730.
Baumann, N., Pham-Dinh, D., 2001. Biology of oligodendrocyte and myelin in the mammalian central nervous system. Physiol. Rev. 81, 871–927.
Bennet, L., Roelfsema, V., George, S., Dean, J.M., Emerald, B.S., Gunn, A.J., 2007. The effect of cerebral hypothermia on white and grey matter injury induced by severe hypoxia in preterm fetal sheep. J. Physiol. 578, 491–506.
Bennet, L., Tan, S., Van Den Heuij, L., et al., 2012. Cell therapy for neonatal hypoxia-ischemia and cerebral palsy. Ann. Neurol. 71, 589–600.
Boltze, J., Schmidt, U.R., Reich, D.M., et al., 2012. Determination of the therapeutic time window for human umbilical cord blood mononuclear cell transplantation following experimental stroke in rats. Cell Transplant. 21, 1199–1211.
Borlongan, C.V., Hadman, M., Sanberg, C.D., Sanberg, P.R., 2004. Central nervous system entry of peripherally injected umbilical cord blood cells is not required for neuroprotection in stroke. Stroke 35, 2385–2389.
Buser, J.R., Maire, J., Riddle, A., et al., 2012. Arrested preoligodendrocyte maturation contributes to myelination failure in premature infants. Ann. Neurol. 71, 93–109.
Castillo-Melendez, M., Yawno, T., Jenkin, G., Miller, S.L., 2013. Stem cell therapy to protect and repair the developing brain: a review of mechanisms of action of cord blood and amnion epithelial derived cells. Front. Neurosci.
Chang, Y.S., Choi, S.J., Ahn, S.Y., et al., 2013. Timing of umbilical cord blood derived mesenchymal stem cells transplantation determines therapeutic efficacy in the neonatal hyperoxic lung injury. PLoS One 8.
Czeh, M., Gressens, P., Kaindl, A.M., 2011. The yin and yang of microglia. Dev. Neurosci. 33, 199–209.
Dammann, O., Leviton, A., 1997. Maternal intrauterine infection, cytokines, and brain damage in the preterm newborn. Pediatr. Res. 42, 1–8.
Deng, Y., Lu, J., Sivakumar, V., Ling, E.A., Kaur, C., 2008. Amoeboid microglia in the periventricular white matter induce oligodendrocyte damage through expression of proinflammatory cytokines via MAP kinase signaling pathway in hypoxic neonatal rats. Brain Pathol. 18, 387–400.
Drobyshevsky, A., Cotten, C.M., Shi, Z., et al., 2015. Human umbilical cord blood cells ameliorate motor deficits in rabbits in a cerebral palsy model. Dev. Neurosci. 37, 349–362.
Ferreiro, D.M., 2006. Can we define the pathogenesis of human periventricular white-matter injury using animal models? J. Child Neurol. 21, 580–581.
Geissler, M., 2011. Human umbilical cord blood cells restore brain damage induced changes in rat somatosensory cortex. PLoS One.
Geissler, M., Dinse, H.R., Neuhoff, S., Kreikemeier, K., Meier, C., 2011. Human umbilical cord blood cells restore brain damage induced changes in rat somatosensory cortex. PLoS One 6, e20194.
Grasl-Kraupp, B., Ruttkay-Nedecky, B., Koudelka, H., Bukowska, K., Bursch, W., Schulte-Hermann, R., 1995. In situ detection of fragmented DNA (tunel assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology 21, 1465–1468.
Hall, A.A., Guyer, A.G., Leonardo, C.C., et al., 2009. Human umbilical cord blood cells directly suppress ischemic oligodendrocyte cell death. J. Neurosci. 87, 333–341.
Haynes, R.L., Folkerth, R.D., Keefe, R.J., et al., 2003. Nitrosative and oxidative injury to premyelinating oligodendrocytes in periventricular leukomalacia. J. Neuropathol. Exp. Neurol. 62, 441–450.
Haynes, R.L., Baud, O., Li, J., Kinney, H.C., Volpe, J.J., Folkerth, R.D., 2005. Oxidative and nitrative injury in periventricular leukomalacia: a review. Brain Pathol. 15, 225–233.
Huertas, J.R., Palomino, N., Ochoa, J.J., et al., 1998. Lipid peroxidation and antioxidants in erythrocyte membranes of full term and preterm newborns. Biofactors 8, 133–137.
Khwaja, O., Volpe, J.J., 2008. Pathogenesis of cerebral white matter injury of prematurity. Arch. Dis. Child. Fetal Neonatal Ed. 93 (F153-F61).
Lei, J., Firdaus, W., Rosenzweig, J.M., et al., 2015. Murine model: maternal administration of stem cells for prevention of prematurity. Am. J. Obstet. Gynecol. 212 (639.e1-.e10).
Li, J., Funato, M., Tamai, H., et al., 2013. Predictors of neurological outcome in cooled neonates. Pediatr. Int. 55, 169–176.
Li, J., McDonald, C., Fahey, M.C., Jenkin, G., Miller, S.L., 2014. Could cord blood cell therapy reduce preterm brain injury? Front. Neurol. 5.
Liravi, B., Piedrafita, D., Nguyen, G., Bischof, R.J., 2015. Dynamics of IL-4 and IL-13 expression in the airways of sheep following allergen challenge. BMC Pulm. Med. 15.
Madhavan, L., Ourednik, V., Ourednik, J., 2006. Increased "vigilance" of antioxidant mechanisms in neural stem cells potentiates their capability to resist oxidative stress. Stem Cells 24, 2110–2119.
McCarthy, B.A., Reddi, A.S., Coakley, K.M., et al., 2010. Administration of human umbilical cord blood cells produces interleukin-10 (IL-10) in IL-10 deficient mice without immunosuppression. Curr. Stem Cell Res. Ther. 5, 13–16.
Meier, C., Middelanis, J., Wasielewski, B., et al., 2006. Spastic paresis after perinatal brain damage in rats is reduced by human cord blood mononuclear cells. Pediatr. Res. 59, 244–249.
Miller, S.L., Yawno, T., Alers, N.O., et al., 2014. Antenatal antioxidant treatment with melatonin to decrease newborn neurodevelopmental deficits and brain injury caused by fetal growth restriction. J. Pineal Res. 56, 286–294.
Ong, J., Plane, J.M., Parent, J.M., Silverstein, F.S., 2005. Hypoxic-ischemic injury stimulates subventricular zone proliferation and neurogenesis in the neonatal rat. Pediatr. Res. 58, 600–606.
Phillis, J.W., 1994. A "radical" view of cerebral ischemic injury. Prog. Neurobiol. 42, 441–448.
Pimentel Coelho, P.M., 2010. Human cord blood transplantation in a neonatal rat model of hypoxic-ischemic brain damage: functional outcome related to neuroprotection in the striatum (ORIGINAL RESEARCH REPORTS) (Report). Stem Cells Dev. 19, 351.

Pimentel-Coelho, P.M., Magalhães, E.S., Lopes, L.M., Deazevedo, L.C., Santiago, M.F., Mendez-Otero, R., 2010. 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. 19, 351–358.

Rees, S., Inder, T., 2005. Fetal and neonatal origins of altered brain development. Early Hum. Dev. 81, 753–761.

Renolleau, S., Aggoun-Zouaoui, D., Ben-Ari, Y., Charriaut-Marlangue, C., 1998. A model of transient unilateral focal ischemia with reperfusion in the P7 neonatal rat: morphological changes indicative of apoptosis. Stroke 29, 1454–1461.

Surbek, D.V., Glanzmann, R., Senn, H.P., Hoesli, I., Holzgreve, W., 2000. Can cord blood be used for autologous transfusion in preterm neonates? Eur. J. Pediatr. 159, 790–791.

Tahraoui, S.L., Marret, S., Bodénant, C., et al., 2001. Central role of microglia in neonatal excitotoxic lesions of the murine periventricular white matter. Brain Pathol. 11, 56–71.

Valle-Prieto, A., Conget, P.A., 2010. Human mesenchymal stem cells efficiently manage oxidative stress. Stem Cells Dev. 19, 1885–1893.

Volpe, J.J., 2003. Cerebral white matter injury of the premature infant - more common than you think. Pediatrics 112, 176–180.

Wasielewski, B., Jensen, A., Roth-Härer, A., Dermietzel, R., Meier, C., 2012. Neuroglial activation and Cx43 expression are reduced upon transplantation of human umbilical cord blood cells after perinatal hypoxic-ischemic injury. Brain Res. 1487, 39–53.

Womble, T.A., Green, S., Shahaduzzaman, M., et al., 2014. Monocytes are essential for the neuroprotective effect of human cord blood cells following middle cerebral artery occlusion in rat. Mol. Cell. Neurosci. 59, 76–84.

Woodward, L.J., Anderson, P.J., Austin, N.C., Howard, K., Inder, T.E., 2006. Neonatal MRI to predict neurodevelopmental outcomes in preterm infants. N. Engl. J. Med. 355, 685–694.

Yawno, T., Schuilwerve, J., Moss, T.J.M., et al., 2013. Human amnion epithelial cells reduce fetal brain injury in response to intrauterine inflammation. Dev. Neurosci. 35, 272–282.

Yu, G., Borlongan, C.V., Stahl, C.E., et al., 2009. Systemic delivery of umbilical cord blood cells for stroke therapy: a review. Restor. Neurol. Neurosci. 27, 41–54.

Chapter 3

Term versus preterm cord blood cells for the prevention of preterm brain injury

Jingang Li[1], Tamara Yawno[1], Amy Sutherland[1], Jan Loose[1], Ilias Nitsos[1], Beth J Allison[1], Robert Bischof[1], Courtney A McDonald[1], Graham Jenkin[1,2], Suzanne L Miller[1,2]*

[1]The Ritchie Centre, Hudson Institute of Medical Research, Clayton, VIC Australia

[2]Department of Obstetrics and Gynaecology, Monash University, Clayton, VIC, Australia

Following Chapter 2 we next compared the effects of cells derived from term and preterm UCB for white matter protection in the preterm brain following HI. This is an unaltered version of the manuscript accepted in *Pediatr Res.* 2017 July 19.

Declaration for Thesis Chapter 3—Term versus preterm cord blood cells for the prevention of preterm brain injury.

Declaration by candidate

In the case of Chapter [insert chapter number], the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experimental design and execution, data analysis, manuscript preparation	80

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Tamara Yawno,	Animal surgery, data analysis, editing manuscript
Amy Sutherland	Animal surgery
Jan Loose	Data collection
Ilias Nitsos	Animal surgery
Beth Allison	Animal surgery
Robert Bischof	Data analysis
Courtney McDonald	Data analysis
Graham Jenkin	Experimental design, editing manuscript
Suzanne Miller	Experimental design, editing manuscript

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	███████████	Date 15/11/2017

Main Supervisor's Signature		Date 15/11/2017

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

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

Jingang Li[1], Tamara Yawno[1], Amy Sutherland[1], Jan Loose[1], Ilias Nitsos[1], Beth J. Allison[1], Robert Bischof[1], Courtney A. McDonald[1], Graham Jenkin[1] and Suzanne L. Miller[1]

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)-a, 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.

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 neuro-protective benefits of human umbilical cord blood (UCB) cells in rodent models of term HI brain injury (2–6), and

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).

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 $-80\,^\circ$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 ×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)-a 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 ± 0.3**	24.2 ± 0.4**	24.3 ± 0.3**
Brain weight, g	34.5 ± 0.7	30.5 ± 0.9**	29.8 ± 1.0**	28.2 ± 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 ± 0.9**	14.4 ± 0.9**	14.0 ± 0.9**

HI, hypoxia–ischemia; PCB, preterm cord blood; TCB, term cord blood; UCO, umbilical cord occlusion.
Mean (%) or mean ± 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 ± 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 ameboid 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 co-localization 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-a, 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-a response at 24 h and resulted in decreased plasma TNF-a in HI+PCB when compared with the plasma TNF-a in HI+TCB ($P = 0.04$, **Table 2b**). In contrast,
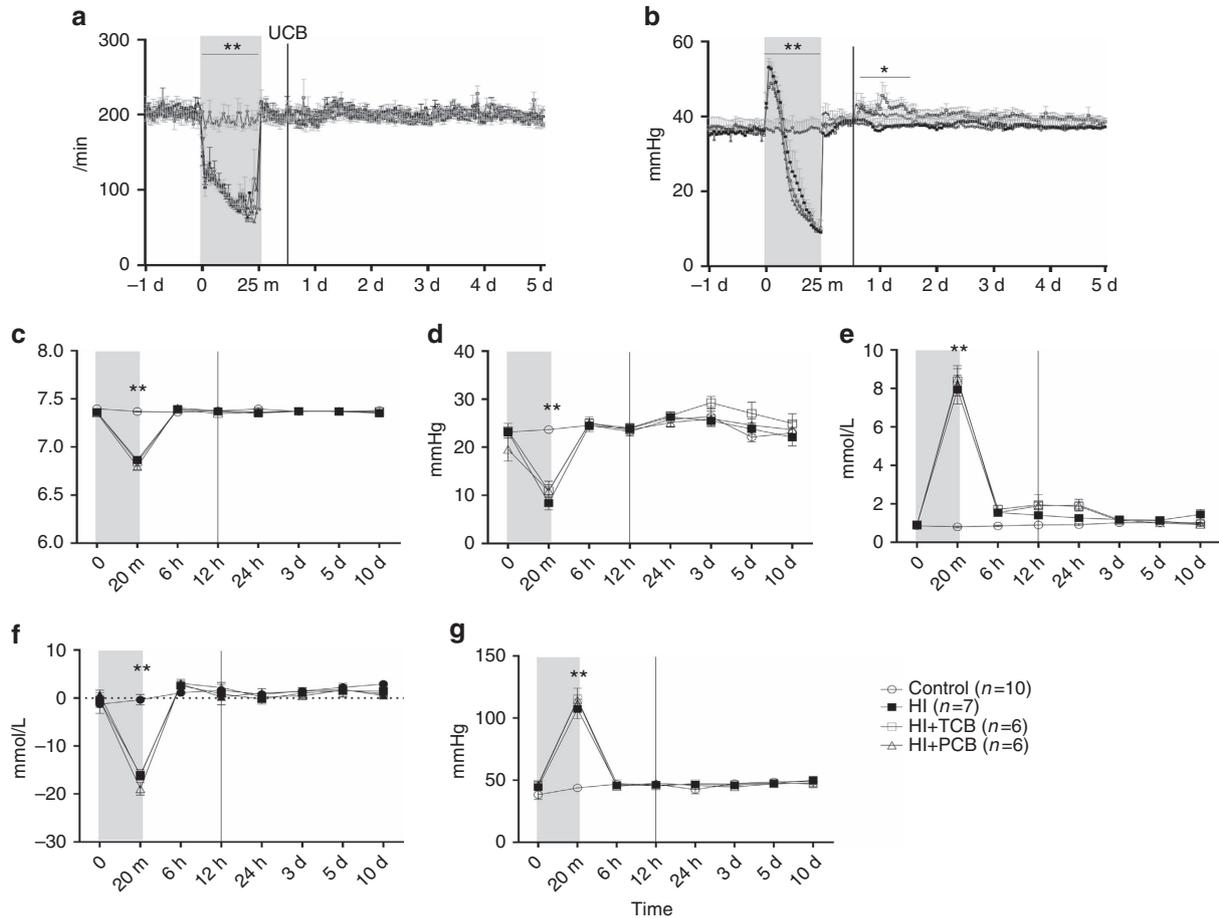
Figure 1. Physiological measurements during HI. HI, induced by umbilical cord occlusion, is shown by the gray columns and UCB cell administration is shown by vertical lines. HI resulted in severe hypotension (**a**) and bradycardia (**b**), markedly reduced fetal arterial pH (**c**), PaO_2 (**d**), and base excess (**f**), and increased $PaCO_2$ (**g**) and lactate (**e**) levels when compared with that in control groups ($P < 0.01$). Data are presented as mean ± 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.
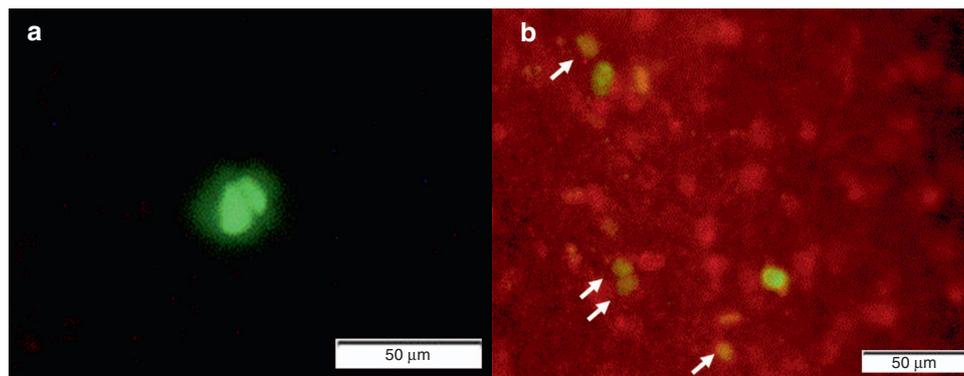
Figure 2. Fluorescent microscopy. (**a**) A representative microphotograph of carboxyfluorescein succinimidyl ester-labeled cells detected in the periventricular white matter of the preterm brain. (**b**) Double-label fluorescent immunostaining of Olig2 (red) and Ki-67 (green) with merged images in the SVZ of the brain in an HI fetus. White arrow denotes an example of a cell with dual signal. Bar = 50 μm. 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
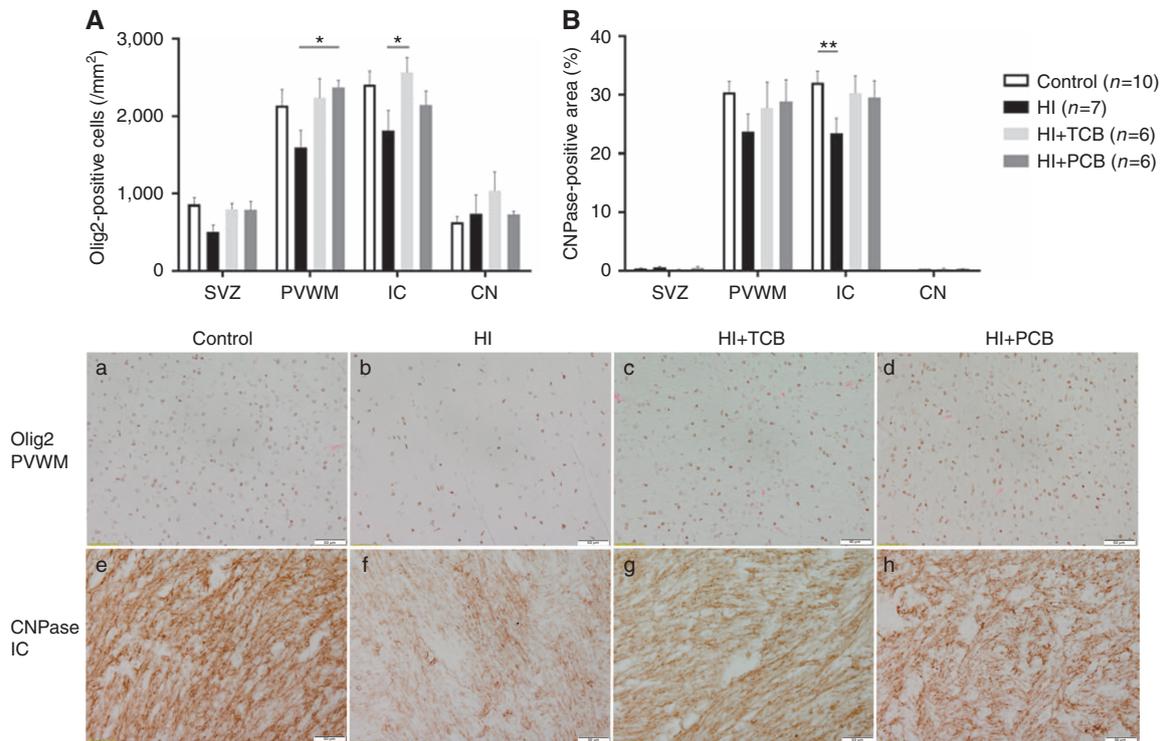
Figure 3. Effects of TCB and PCB cell on white matter cells following HI. The graphs of the number of Olig2+ oligodendrocyte (**A**) and density of oligodendrocyte and myelinating axons (CNPase+, **B**), and the representative photomicrograph images in control (**a**, **e**), HI (**b**, **f**), HI+TCB (**c**, **g**), and HI+PCB (**d**, **h**) groups. SVZ, PVWM, IC, and CN. Data are mean ± 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 µm. 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
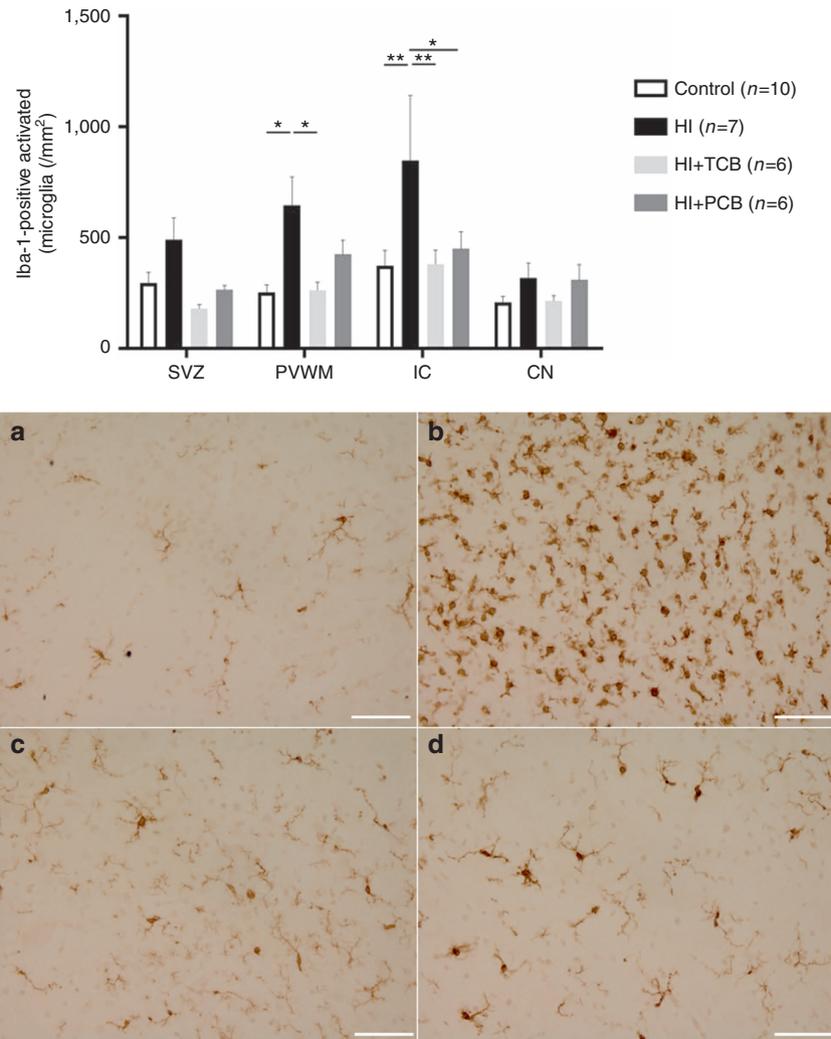
Figure 4. Effects of TCB and PCB cell following HI on the density of Iba-1+ activated microglia. The photomicrograph images show that Iba-1+ inflammatory cells in control brains exhibited the characteristics of ramified (resting) microglia, with small cell bodies and long branching processes (**a**). 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 ± 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 μm. 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-a response following HI (Table 2c). Suppressing pro-inflammatory cytokines (e.g., TNF-a 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
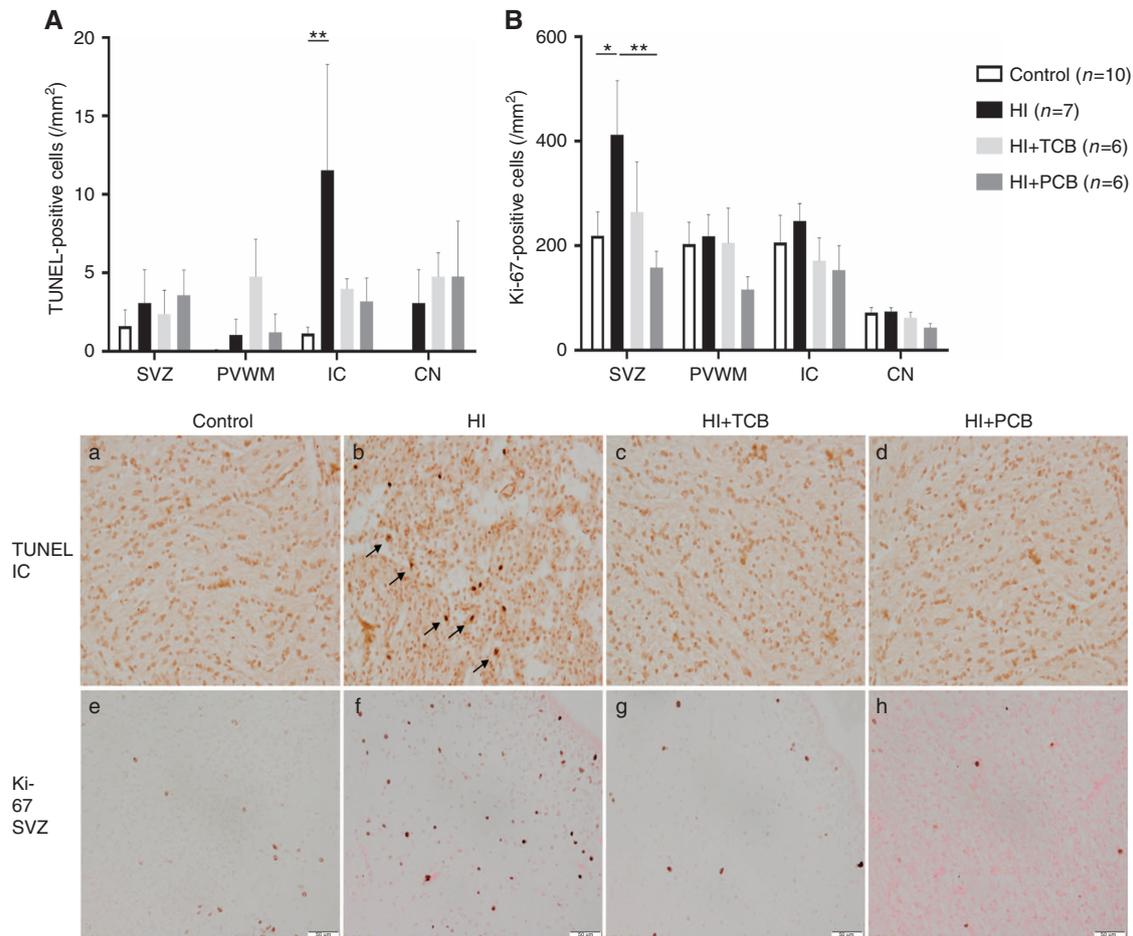
Figure 5. Effects of TCB cell and PCB cell on cell death and cell proliferation following HI. The graphs of the density of TUNEL+ cell death (**A**) and Ki-67+ proliferating cells (**B**), and the representative photomicrograph images in control (**a,e**), HI (**b,f**), HI+TCB (**c,g**), and HI+PCB (**d,h**) groups. Black arrows show examples of TUNEL-positive cells (**b**). Data are mean ± 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 μm. HI, hypoxia–ischemia; PCB, preterm cord blood; TCB, term cord blood; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Table 2a. MDA (μM)

	Control	HI	HI+TCB	HI+PCB
Base	9.7 ± 2.3	9.6 ± 2.3	8.0 ± 0.9	12.9 ± 3.4
6 h	6.5 ± 2.0	11.2 ± 2.0[a]	12.5 ± 1.4[a]	18.2 ± 9.6
12 h	9.0 ± 2.5	17.8 ± 2.5[a]	15.5 ± 3.1	16.6 ± 7.5
24 h	6.7 ± 1.7	17.1 ± 1.7[a]	10.6 ± 2.1	20.6 ± 9.5
48 h	9.4 ± 2.3	18.1 ± 2.3[a]	10.2 ± 2.4[b]	17.1 ± 3.0
72 h	8.3 ± 4.7	11.7 ± 4.7	9.1 ± 4.2	13.7 ± 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 ± 31 ml, 3.6×10^8 cells at 25–33 weeks vs. 102 ± 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 ± 0.5	4.0 ± 1.2	5.0 ± 0.9	1.6 ± 0.7[a]
10d	3.1 ± 1.0	2.7 ± 2.7	2.6 ± 1.0	1.3 ± 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 ± 0.7	7.0 ± 3.5	5.4 ± 4.1	2.6 ± 1.4
10d	13.5 ± 2.0	11.3 ± 1.4	16.0 ± 1.4[a]	12.0 ± 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-a 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.

Disclosure: The authors declare no conflict of interest.

REFERENCES
1. Volpe JJ. Cerebral white matter injury of the premature infant: more common than you think. Pediatrics 2003;112:176–80.
2. Pimentel-Coelho PM, Magalhães ES, Lopes LM, et al. 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;19:351–8.
3. Meier C, Middelanis J, Wasielewski B, et al. Spastic paresis after perinatal brain damage in rats is reduced by human cord blood mononuclear cells. Pediatr Res 2006;59:244–9.
4. Geissler M. Human umbilical cord blood cells restore brain damage induced changes in rat somatosensory cortex. PLoS ONE 2011;6:e20194.
5. Wasielewski B, Jensen A, Roth-Härer 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.
6. Li J, McDonald C, Fahey MC, Jenkin G, Miller SL. Could cord blood cell therapy reduce preterm brain injury? Front Neurol 2014;5:200.
7. Drobyshevsky A, Cotten CM, Shi Z, et al. Human umbilical cord blood cells ameliorate motor deficits in rabbits in a cerebral palsy model. Dev Neurosci 2015;37:349–62.
8. Hall AA, Guyer AG, Leonardo CC, et al. Human umbilical cord blood cells directly suppress ischemic oligodendrocyte cell death. J Neurosci Res 2009;87:333–41.
9. Li J, Yawno T, Sutherland A, et al. Preterm white matter brain injury is prevented by early administration of umbilical cord blood cells. Exp Neurol 2016;283:179–87.
10. Aridas JDS, McDonald CA, Paton MCB, et al. Cord blood mononuclear cells prevent neuronal apoptosis in response to perinatal asphyxia in the newborn lamb. J Physiol 2016;594:1421–35.

11. Trønnes H, Wilcox AJ, Lie RT, Markestad T, Moster D. Risk of cerebral palsy in relation to pregnancy disorders and preterm birth: a national cohort study. Dev Med Child Neurol 2014;56:779–85.

12. Novak I, Walker K, Hunt R, Wallace E, Fahey M, Badawi N. Concise review: stem cell interventions for people with cerebral palsy: systematic review with meta-analysis. Stem Cells Transl Med 2016;5:1014–25.

13. Boltze J, Schmidt UR, Reich DM, et al. Determination of the therapeutic time window for human umbilical cord blood mononuclear cell transplantation following experimental stroke in rats. Cell Transplant 2012;21:1199–211.

14. Solves P, López M, Mirabet V, Blanquer A, Roig R, Perales A. Characteristics of umbilical cord blood units collected from preterm deliveries. Gynecol Obstetr Invest 2009;68:181–5.

15. Javed MJ, Mead LE, Prater D, et al. Endothelial colony forming cells and mesenchymal stem cells are enriched at different gestational ages in human umbilical cord blood. Pediatr Res 2008;64:68–73.

16. Jain A, Mathur N, Jeevashankar M, et al. Does mesenchymal stem cell population in umbilical cord blood vary at different gestational periods? Indian J Pediatr 2013;80:375–9.

17. Cervera A, Lillo R, García-Sánchez F, Madero L, Madero R, Vicario JL. Flow cytometric assessment of hematopoietic cell subsets in cryopreserved preterm and term cord blood, influence of obstetrical parameters, and availability for transplantation. Am J Hematol 2006;81:397–410.

18. Gasparoni A, Ciardelli L, Avanzini MA, et al. Immunophenotypic changes of fetal cord blood hematopoietic progenitor cells during gestation. Pediatr Res 2000;47:825–9.

19. Surbek DV, Holzgreve W, Jansen W, et al. Quantitative immuno-phenotypic characterization, cryopreservation, and enrichment of second-and third-trimester human fetal cord blood hematopoietic stem cells (progenitor cells). Am J Obstetr Gynecol 1998;179:1228–33.

20. Czeh M, Gressens P, Kaindl AM. The yin and yang of microglia. Dev Neurosci 2011;33:199–209.

21. Hagberg H, Mallard C, Ferriero DM, et al. The role of inflammation in perinatal brain injury. Nat Rev Neurol 2015;11:192–208.

22. Buser JR, Maire J, Riddle A, et al. Arrested preoligodendrocyte maturation contributes to myelination failure in premature infants. Ann Neurol 2012;71:93–109.

23. Arien-Zakay H, Lecht S, Bercu MM, et al. Neuroprotection by cord blood neural progenitors involves antioxidants, neurotrophic and angiogenic factors. Exp Neurol 2009;216:83–94.

24. Valle-Prieto A, Conget PA. Human mesenchymal stem cells efficiently manage oxidative stress. Stem Cells Dev 2010;19:1885–93.

25. Ito K, Hirao A, Arai F, et al. Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. Nature 2004;431:997–1002.

26. Said EA, Dupuy FP, Trautmann L, et al. Programmed death-1–induced interleukin-10 production by monocytes impairs CD4(+) T cell activation during HIV infection. Nat Med 2010;16:452–9.

27. Christensen RD, Del Vecchio A, Henry E. Expected erythrocyte, platelet and neutrophil values for term and preterm neonates. J Maternal Fetal Neonatal Med 2012;25:77–9.

28. Prockop DJ, Youn Oh J. Mesenchymal stem/stromal cells (MSCs): role as guardians of inflammation. Mol Ther 2012;20:14–20.

29. Kang M, Min K, Jang J, et al. Involvement of immune responses in the efficacy of cord blood cell therapy for cerebral palsy. Stem Cells Dev 2015;24:2259–68.

30. Yu G, Borlongan CV, Stahl CE, et al. Systemic delivery of umbilical cord blood cells for stroke therapy: a review. Restorative Neurol Neurosci 2009;27:41–54.

31. Surbek DV, Glanzmann R, Senn HP, Hoesli I, Holzgreve W. Can cord blood be used for autologous transfusion in preterm neonates? Eur J Pediatrics 2000;159:790–1.

32. Hosono S, Hine K, Nagano N, et al. Residual blood volume in the umbilical cord of extremely premature infants. Pediatr Int 2015;57:68–71.

33. Kotiranta-Ainamo A, Apajasalo M, Pohjavuori M, Rautonen N, Rautonen J. Mononuclear cell subpopulations in preterm and full-term neonates: independent effects of gestational age, neonatal infection, maternal pre- eclampsia, maternal betamethason therapy, and mode of delivery. Clin Exp Immunol 1999;115:309–14.

34. Jones J, Stevens CE, Rubinstein P, Robertazzi RR, Kerr A, Cabbad MF. Obstetric predictors of placental/umbilical cord blood volume for transplantation. Am J Obstetr Gynecol 2003;188:503–9.

35. Larrivée B, Niessen K, Pollet I, et al. Minimal contribution of marrow-derived endothelial precursors to tumor vasculature. J Immunol 2005;175:2890–9.

36. Lee OK, Kuo TK, Chen WM, Lee KD, Hsieh SL, Chen TH. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 2004;103:1669–75.

37. Tsuji M, Taguchi A, Ohshima M, et al. Effects of intravenous administration of umbilical cord blood CD34+ cells in a mouse model of neonatal stroke. Neuroscience 2014;263:148–58.

38. Xia G, Hong X, Chen X, Lan F, Zhang G, Liao L. Intracerebral transplantation of mesenchymal stem cells derived from human umbilical cord blood alleviates hypoxic ischemic brain injury in rat neonates. J Perinatal Med 2010;38:215–21.

39. Shahaduzzaman M, Golden JE, Green S, et al. A single administration of human umbilical cord blood T cells produces long-lasting effects in the aging hippocampus. Age 2013;35:2071–87.

40. Womble TA, Green S, Shahaduzzaman M, et al. Monocytes are essential for the neuroprotective effect of human cord blood cells following middle cerebral artery occlusion in rat. Mol Cell Neurosci 2014;59:76–84.

41. Alves de Alencar Rocha AK, Allison BJ, Yawno T, et al. Early- versus late-onset fetal growth restriction differentially affects the development of the fetal sheep brain. Dev Neurosci 2017;39:141–155.

42. Trapp BD, Bernier L, Andrews SB, Colman DR. Cellular and subcellular distribution of 2',3'-cyclic nucleotide 3'-phosphodiesterase and its mRNA in the rat central nervous system. J Neurochem 1988;51:859–68.

Volume 00 | Number | Month   Pediatric RESEARCH **9**

Chapter 4

Preterm umbilical cord blood derived mesenchymal stem/stromal cells protect preterm white matter brain development against hypoxia-ischemia

Jingang Li[1], Tamara Yawno[1], Amy E Sutherland[1], Shanti Gurung[1], Madison Paton[1], Courtney McDonald[1], Abhilasha Tiwari[1], Yen Pham[1], Margie Castillo-Melendez[1], Graham Jenkin[1,2], Suzanne L Miller[1,2*]

[1]The Ritchie Centre, Hudson Institute of Medical Research, Clayton, VIC Australia

[2]Department of Obstetrics and Gynaecology, Monash University, Clayton, VIC, Australia

Following Chapter 2 and 3, this final chapter of results investigated the neuroprotective effects of ex-vivo expanded MSCs derived from preterm UCB in the preterm brain following acute HI. This is an unaltered version of the manuscript submitted to *Stem Cells Translational Medicine* on 5th November 2017. Evidence of submission is included below.

Declaration for Thesis Chapter 4—Preterm umbilical cord blood derived mesenchymal stem/stromal cells protect preterm white matter brain development against hypoxia-ischemia.

Declaration by candidate

In the case of Chapter [insert chapter number], the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experimental design and execution, data analysis, manuscript preparation	77

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors
Tamara Yawno,	Animal surgery, data analysis, editing manuscript	
Amy Sutherland	Animal surgery, data collection	
Shanti Gurung	data analysis	1
Madison Paton	data analysis	1
Courtney McDonald	Data analysis	
Abhilasha Tiwari	Data analysis	
Yen Pham	Data analysis	
Margie Castillo-Melendez	Data analysis	
Graham Jenkin	Experimental design, editing manuscript	
Suzanne Miller	Experimental design, editing manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	■■■■■■■■	Date 15/11/2017

Main Supervisor's Signature	■■■■■■■■	Date 15/11/2017

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

4.2. Formal acknowledgement of submission of manuscript to *Stem Cells Translational Medicine*

Stem Cells Translational Medicine Submission Received - SCTM-17-0256

SM Stem Cells Translational Medicine
2017/11/05 13:11

Dear Dr. Miller:

Thank you for submitting your paper to STEM CELLS Translational Medicine. This confirms receipt of your manuscript, "Preterm umbilical cord blood derived mesenchymal stem/stromal cells protect preterm white matter brain development against hypoxia-ischemia."

We appreciate receiving your submission and having the opportunity to consider your work. If you have any questions, please contact Terri Scott at ▮▮▮▮▮▮▮▮▮▮▮▮ or call ▮▮▮▮▮▮▮▮

Sincerely,

Anthony Atala, MD
Editor-in-Chief
Stem Cells Translational Medicine

Preterm umbilical cord blood derived mesenchymal stem/stromal cells protect preterm white matter brain development against hypoxia-ischemia

Original Research

Preterm umbilical cord blood derived mesenchymal stem/stromal cells protect

preterm white matter brain development against hypoxia-ischemia

Jingang Li[1], Tamara Yawno[1], Amy E Sutherland[1], Shanti Gurung[1], Madison Paton[1],

Courtney McDonald[1], Abhilasha Tiwari[1], Yen Pham[1], Margie Castillo-Melendez[1], Graham

Jenkin[1,2], Suzanne L Miller[1,2*]

[1]The Ritchie Centre, Hudson Institute of Medical Research, Clayton, VIC Australia

[2]Department of Obstetrics and Gynaecology, Monash University, Clayton, VIC, Australia

* Author for correspondence

Associate Professor Suzanne L Miller

The Ritchie Centre, Hudson Institute of Medical Research

Department of Obstetrics and Gynaecology, Monash University

Level 5 TRF, 27-31 Wright St Clayton

1

Li et al.   Preterm cord blood MSC protect the preterm brain

Key words: cell transplantation, mesenchymal stem cells, umbilical cord blood, oligodendrocytes, stem cell expansion, cytokines

Running title: Preterm cord blood MSC protect the preterm brain

Abstract length: 250 words

Manuscript word count: 4,963 words

Number of references: 67

Numbers of table: 1

Number of figures: 6

Author Contributions

JLi: conception and design, acquisition and analysis of data, manuscript writing; TY: acquisition and analysis of data, financial support; AS, MP: collection and/or assembly of

2

Li et al.　　　　　　　　　　　Preterm cord blood MSC protect the preterm brain

data; YP: administrative support, collection and/or assembly of data; AT, SG, MCM, CM:

Data analysis and interpretation; GJ: conception and design; SM: conception and design,

financial support, manuscript writing; all authors: final approval of manuscript.

Disclosure of Potential Conflicts of Interest

All authors indicated no potential conflicts of interest.

3

Li et al.   Preterm cord blood MSC protect the preterm brain

ABSTRACT

Introduction: Preterm infants are at high risk for white matter injury and subsequent

neurodevelopmental impairments. Mesenchymal stem/stromal cells (MSC) have

anti-inflammatory/immunomodulative actions and are of interest for neural repair in adults

and newborns. This study examined the neuroprotective effects of allogeneic MSC, derived

from preterm umbilical cord blood (UCB), in a preterm sheep model of white matter injury.

Methods: Quad-lineage differentiation, clonogenicity and self-renewal ability of UCB-derived

MSC were confirmed. Chronically instrumented fetal sheep (0.7 gestation) received either

25min hypoxia-ischemia (HI) to induce preterm brain injury, or sham-HI. Ten million MSC, or

saline, were administered iv to fetuses at 12h after HI. Fetal brains were collected 10d after

HI for histopathology and immunocytochemistry.

Results: HI induced white matter injury, as indicated by a reduction in CNPase-positive

myelin fiber density. HI also induced microglial activation (Iba-1) in the periventricular white

matter and internal capsule (P<0.05 vs control). MSC administration following HI preserved

myelination (P<0.05), modified microglial activation, and promoted macrophage migration

(CD163) and cell proliferation (Ki-67) within cerebral white matter (P<0.05). Cerebral

4

Li et al.   Preterm cord blood MSC protect the preterm brain

CXCL10 concentration was increased following MSC administration (P<0.05), which was

likely associated with macrophage migration and cell proliferation within the preterm brain.

Additionally, MSC administration reduced systemic pro-inflammatory cytokine TNFα at 3 d

post-HI (P<0.05).

Conclusions: UCB-derived MSC therapy preserved white matter brain structure following

preterm HI, mediated by a suppression of microglial activation, promotion of macrophage

migration and acceleration of self-repair within the preterm brain. UCB-derived MSC are

neuroprotective, acting via peripheral and cerebral anti-inflammatory and

immunomodulatory mechanisms.

5

Li et al.
Preterm cord blood MSC protect the preterm brain

INTRODUCTION

Infants born preterm have a high risk of brain injury, with surviving preterm infants commonly

demonstrating white matter injury (WMI) [1]. WMI is characterized by sparse or disorganized

axonal myelination, astrogliosis, and/or microglial activation, and can be detected in infants

born preterm via magnetic resonance imaging. Although WMI has a complex etiology, it is

well described that two relatively common upstream insults, hypoxia-ischemia (HI) and

inflammation, are principal contributors towards brain injury [2,3]. WMI is known to underlie

cerebral palsy, and 50% of infants with WMI will develop cognitive, behavioral and/or

attention deficits [1,4]. Due to the complex pathology and etiology of WMI, and the fragility of

extremely preterm infants, there are currently no effective treatments available to prevent or

repair WMI in preterm infants.

Mesenchymal stem/stromal cells (MSC) describe multipotent cells with

anti-inflammatory/immunomodulatory properties, trophic influences on tissue repair and

MSC are capable of differentiating into multiple lineages [5-7]. Due to their multi-potential

mechanisms of action and relative ease of proliferation *in vitro*, human and nonhuman

mammalian MSC have received attention as a therapeutic intervention for repair of

neurological deficits [7,8]. Recent studies show that transplantation of MSC in both neonatal

6

Li et al.   Preterm cord blood MSC protect the preterm brain

and adult animal models of brain damage promote functional and structural improvements

[9-15]. In neonatal rodent models of acute HI and stroke, MSC treatment improves behavioral

outcomes, and reduces tissue loss via neuronal and oligodendrocyte regeneration,

regardless of route of administration [9-12]. Early studies suggested that MSC might act

therapeutically to replace damaged cells, differentiating into neurons and oligodendrocytes

[16,17], but current knowledge supports that MSC principally act to modify the

microenvironment response to insult, thereby promoting endogenous repair processes [18].

Multiple sources of MSC are available including bone marrow, UCB, umbilical cord (UC),

and adipose tissue [12,19]. While there is currently one trial listed using UCB-derived MSC for

the treatment of cerebral palsy (Clin.Trials.Gov), it is generally accepted that UCB-MSC

studies are complicated by low yield and great variability in cell number obtained from

full-term UCB collection [20-22]. Interestingly, this may not be a factor in relation to preterm

birth, with the proportion of MSC in preterm UCB significantly increased when compared to

UCB collected from term birth [23-25] and, indeed, the number of MSC available for collection

increases with decreasing preterm age at birth [23,24]. Thus, collecting MSC from preterm

UCB, rather than term UCB, may be more appropriate for clinical application and treatment

of preterm WMI.

7

Li et al.   Preterm cord blood MSC protect the preterm brain

Accordingly, in this study we investigated the feasibility and efficacy for the administration of

allogeneic, ex-vivo expanded MSC derived from preterm UCB for neuroprotective use in a

well-established ovine fetal model of preterm WMI. Chronically instrumented preterm fetal

sheep were exposed to 25 minutes of acute hypoxia ischemia (HI) induced via umbilical

cord occlusion at 0.7 gestation, equivalent to brain development at 28-32 weeks in the

human [26]. We set out to determine whether UCB-derived MSC were neuroprotective for

preterm WMI, and to examine the mechanisms by which MSC mediate neuroprotective

benefits in a preclinical large animal model of preterm WMI.

MATERIALS AND METHODS

Ovine UCB-MSC isolation and expansion

This study was approved by the Monash Medical Centre Animal Ethics Committee

(MMCA/2013/17). Ovine UCB was collected from 5 normal preterm sheep undergoing

cesarean-section at ~118 d gestation. UCB samples were collected into 50ml Falcon tubes

containing heparin, and processed within 4h of collection. After centrifuging the blood at

3,100 rpm for 12 minutes at room temperature without brake, the buffy coat layer was

isolated, and 20-30 million UCB cells were plated in a 100-mm dish (Falcon) at 37°C in 5%

8

CO_2 in a humid atmosphere under aseptic conditions in medium containing DMEM/F-12,

10% fetal bovine serum, 100U/ml penicillin–streptomycin, 0.25ug/ml amphotericin B, and

2mM L-glutamine (Life Technologies). At 24h media was replaced, non-adherent cells

discarded, and adherent cells retained. Medium was changed at 7d intervals thereafter.

Between days 10-14, each colony consisting of spindle-shaped cells was harvested with

0.25% trypsin (Gibco) using cloning cylinders (Sigma-Aldrich), and transferred to individual

wells in 24-well tissue culture plates (Costar 3524) (passage 1). The cells were incubated

with medium replaced every 3d, and harvested by trypsinization when they reached 80–

90% confluence and replated (passage 2). From passage 2, the cells were incubated at

seeding densities of 5,000 cells/cm^2 in T75 or T175 culture flasks (Corning), and expanded.

After passage 3, the cells were resuspended in 10% fetal bovine serum and 10% DMSO

(Merck), and cryopreserved in liquid nitrogen until required for administration.

Serial Cloning Assay

The clonogenicity and self-renewal ability of the MSC were examined by serial cloning in

culture [27]. Several of the largest individual clones on culture plates were collected by

trypsinisation in cloning rings, and recloned. Cells were counted visually under a phase

contrast microscope using an ocular grid, seeded at 10-20 cells/cm^2 onto the 10cm dishes,

9

Li et al.   Preterm cord blood MSC protect the preterm brain

and cultured in standard medium, changed every 7d, to generate secondary clones.

Similarly, secondary, tertiary and quarternary clones were harvested and recloned as

previously described [27]. The cloning efficiency at each subcloning was assessed.

In vitro differentiation

The differentiation potential of the expanded ovine MSC (passage 3) into adipocytes,

osteoblasts, chondrocytes and myocytes was evaluated. For adipogenic, osteogenic and

myogenic differentiation, the cells were seeded separately at 5,000 to 10,000 cells/cm^2 on

coverslips (Thermo Scientific) in 24-well plates and cultured in specific differentiation

medium, as described previously for human and ovine MSC [27]. Briefly, to induce

chondrogenic differentiation, 5x10^5 cells were cultured in 15mL conical tubes following

centrifugation and cultured in chondrogenic medium to produce a 3D micromass culture [27].

Controls were ovine MSC cultured in standard medium. The cells were incubated for 4

weeks in differentiation or control media, replaced every 3d. Cells were then fixed on

coverslips and stained with 4% Alizarin Red (pH 4.1), 1% Oil Red O or by

immunohistochemistry using anti-α-smooth muscle actin antibody (3.6 μg/ml, clone 1A4;

Dako) for the detection of calcification, lipid vacuoles deposition and α-smooth muscle actin

expression to reveal osteogenic, adipogenic, and myogenic differentiation, respectively.

10

Chondrogenic micromass cultures were fixed, processed and paraffin embedded. Sections

were stained with 1% alcian blue (pH 2.5; Sigma-Aldrich) to detects acidic mucins[27]. Stained

cells or sections were examined using an Olympus BX41 microscope (Olympus), and

images taken using an Olympus DP25 digital camera (Olympus).

Surface phenotype

Freshly expanded MSC at passage 3 were phenotypically analyzed. The cells were

detached from the culture dish with trypsin, rinsed twice with phosphate buffered saline

(PBS), incubated with fluorochrome-conjugated antibody in the dark at room temperature for

20min, and rinsed twice with PBS. The following primary antibodies were used:

FITC-conjugated CD44 (0.1 mg/ml, mouse IgG1; LSBio), FITC-conjugated CD45 (0.1 mg/ml,

mouse IgG1; LSBio), AF647-conjugated CD73 (1 µg/ul, rabbit IgG; Bioss), PE-conjugated

CD90 (1 µg/ul, rabbit IgG; Bioss), FITC-conjugated CD105 (1 µg/ul, rabbit IgG; Bioss),

FITC-conjugated mouse anti-human CD146 (mouse IgG1, κ; BD Pharmingen),

PE-conjugated CD271 (1.25 µg/ml, mouse IgG1; R&D Systems). Cells were then incubated

with 7AAD staining solution (BD Pharmingen) for cell viability, and analyzed by FACS Canto

II analyzer (BD Biosciences). Isotype matched controls or unlabelled controls were included

for each antibody and used to set the electronic gates on the flow cytometer.

11

Li et al.   Preterm cord blood MSC protect the preterm brain

Chromosomal test

Karyotypic stability of the ovine MSC was examined at passage 3 by chromosomal analysis

(Cytogenetics laboratory, Australia).

Animals and Surgery

Surgery was performed on 24 pregnant cross-bred ewes (20 singles; 4 twins) at 97.4±0.2d

gestation (term=147d), as described previously [26]. Under general anesthesia, fetal polyvinyl

catheters (0.8mm inner diameter, 1.2mm outer diameter, Dural Plastics, Australia) were

inserted into the femoral artery and vein. An inflatable balloon occluder (16HD, In Vivo

Medical, USA) was placed around the umbilical cord and an amniotic catheter (1.5 mm inner

diameter, 2.7 mm outer diameter) inserted. All catheters were exteriorized through the

maternal flank. A maternal jugular vein catheter (1.5 mm inner diameter, 2.7 mm outer

diameter) was also implanted for antibiotic administration at the time of surgery. Prior to, and

for 3d after, surgery, 500mg engemycin and 1g ampicillin were given iv to the ewe. Fetal

catheters were maintained by continuous infusion of heparinized saline (50 IU/ml at 0.2

ml/h) and after 4-5d of recovery, fetal heart rate (FHR) and mean arterial pressure (MAP),

corrected for amniotic fluid pressure, were recorded continuously. All data were stored for

12

Li et al.   Preterm cord blood MSC protect the preterm brain

off-line analysis (Power Lab, AD Instruments, Australia).

Experiment protocol

At 102.2±0.3d gestation (0.7 gestation), animals were randomized into one of three groups:

(1) control (sham-occlusion + iv saline, n=10); (2) HI (HI + saline, n=7); (3) HI+MSC (HI + 10

million MSC injected iv at 12h after HI, n=6). HI was induced by complete umbilical cord

occlusion, in which the balloon occluder was filled with 2.0-2.5 ml water. The occlusion was

discontinued after 25 minutes, or sooner if the occlusion was >23 minutes duration and MAP

decreased to <8 mmHg. Cryopreserved MSC pooled from 5 different donors were thawed

just prior to administration. Cell yield and viability were assessed using Trypan blue dye

exclusion and 10 million viable cells were labeled with carboxyfluorescein succinimidyl ester,

suspended in 2 ml sterile saline, and administered to the fetus (over 2 minutes) via the

femoral vein catheter. Fetal arterial blood samples were collected 24h before, during, and 6,

12, 24, 48, 72, 120, and 240h after HI for blood gas analysis (ABL 700, Radiometer,

Denmark). Cytokine concentrations were assessed in selected plasma samples, which were

stored at -80 °C until assays were performed.

13

Li et al.   Preterm cord blood MSC protect the preterm brain

Tissue collection and processing

At 10d after UCO (112.4±0.3d gestation), the ewe and fetus(es) were euthanazed by iv

overdose of sodium pentobarbital (Virbac, Australia) to the ewe. Each fetus was removed

and fetal body weight was recorded. The fetal brain was removed from the skull and

weighed, divided in half sagitally and the right cerebral hemisphere was cut into 5 mm slices

and immersion fixed in 10% formalin for 5d. Subsequently, paraffin-embedded 10 μm

coronal sections were cut at the level of the subventricular zone (SVZ) and the caudate

nucleus (CN), and mounted on Superfrost Plus slides (Thermoscientific, USA). Brain

regions of interest were the SVZ, periventricular white matter (PVWM), internal capsule (IC),

and CN. The white matter tissue of the left cerebral hemisphere was isolated, and stored at

-80 °C.

Immunohistochemistry

Mouse polyclonal oligodendrocyte transcription factor 2 (Olig2, 1:1000; Millipore) and

mouse-anti-human 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase, 1:200, Sigma

Chemical) antibodies were used to detect oligodendroglial lineage cells and myelin sheaths

plus myelin producing (mature) oligodendrocytes. Proliferating cells were visualized with

rabbit anti-human Ki-67 antibody (1:100, Dako). Activated microglia were identified using

14

Li et al.   Preterm cord blood MSC protect the preterm brain

rabbit anti-ionized calcium binding adaptor molecule 1 (Iba-1, 1:500; Wako) [28].

Macrophages were visualized using mouse monoclonal anti-CD163 antibody (1:400,

Bio-Rad), and astrocytes were visualized using glial fibrillary acidic protein antibody (GFAP,

1:400; Sigma). Neutrophils were identified with rabbit polyclonal anti-neutrophil elastase

antibody (1:500, Abcam). Immunohistochemistry was performed following protocols used by

us previously [26]. Slides were imaged at x400 magnification under light microscopy (Olympus

BX-41). Numbers of Olig2-, Ki-67-, Iba-1-, CD163- and GFAP-immunoreactive cells per field

of view were counted. The percentage of CNPase-positive area, indicative of the density of

myelinated fibers, was quantified using ImageJ (NIH). The grade of the accumulation/

infiltration of neutrophil elastase-positive neutrophils associated with brain capillaries was

assessed using a scale as followed; 0-2 neutrophil elastase-positive polynuclear cells with

blood vessels per field of view scored 0; 3-10 neutrophil elastase-positive polynuclear cells

accumulated in blood vessels with or without co-staining of red blood cells scored 1 point;

>10 neutrophil elastase-positive polynuclear cells co-stained with red blood cells within or

around blood vessels scored 2 points. Immunohistochemical outcomes were assessed in 2

sections per animal and 3 fields of view per region on each section, with the results

averaged per animal and across all animals in each group.

15

Double label immunohistochemistry

Double-label immunohistochemistry was carried out on two adjacent sections from

HI+MSCs fetal brains by first blocking endogenous peroxidases with 0.3% hydrogen

peroxidase in 50% methanol and then washing sections with sodium borohydride (10

mg/ml) in 0.1 M PBS to reduce the autofluorescence that can occur with paraffin-embedded

sections. These sections were treated with a serum-free protein blocker (Dako) to prevent

background staining, and incubated with mouse monoclonal anti-Ki-67 (1:100), and

anti-Olig-2 (1:1000) or CNPase (1:200) or CD163 (1:400) to identify what cells are

proliferating. Immunoreactivity was visualized with Alexa Fluor 594 goat anti-mouse (1:800;

Molecular Probes) and Alexa Fluor 488 goat anti-rabbit (1:800; Molecular Probes), and

viewed with a fluorescent microscope (Olympus BX-41) at 400× magnification.

Cytokines, chemokines, and neurotrophic factors assay

Plasma cytokine concentrations for pro-inflammatory TNF-α and IL-6, and anti-inflammatory

IL-10 were analyzed against recombinant cytokines via capture ELISA techniques [29].using

ovine-specific monoclonal antibodies.

The concentrations of CXCL8 (IL8), CXCL9 (MIG), CXCL10 (IP10), IFNγ, IL-17A, IL-21,

16

Li et al.   Preterm cord blood MSC protect the preterm brain

TNFa, and VEGF-A in white matter tissue lysate were measured using an ovine cytokine

array (ovine QAO-CYT-1-1, RayBiotech), IL-3, IL-6, neuron-specific enolase (NSE), ICAM-1,

and VACM-1 were measured using a human custom array (Human custom QAA-CUST,

RayBiotech), and brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF)

were measured using ovine ELISA assay kits (Cloud- Clone).

Data analysis

Only animals that completed the whole experimental protocol were included in data

analyses. Assessments were conducted on coded slides or samples with the examiner

blinded to the experimental groups. Data are presented as mean ± standard error of the

mean. Statistical analysis was performed with GraphPad Prism 6 (GraphPad Software,

USA). Differences between groups were analyzed by a two-way analysis of variance

(ANOVA) (histology data) or one-way ANOVA (fetal weight, arterial blood gas, physiology,

cytokines and trophic factors concentrations) followed by the Tukey post hoc test when a

significant difference was found. Nonparametric data were analyzed with either the

Wilcoxon or Fisher's test. $P < 0.05$ was considered statistically significant.

17

Li et al.   Preterm cord blood MSC protect the preterm brain

RESULTS

MSC isolation, expansion and characterization

After 4-5d of incubation, UCB-derived cell colonies with the appearance of spindle-shape or

flat-polygonal morphology were observed. Thereafter, the cells from all five UCB samples

were expanded (10-300 colonies per dish; Figure 1A). The presumptive MSC were

harvested by trypsinization with cloning rings (Figure 1B), and replated at 10-14d of

incubation. A homogenous stromal cell layer was formed after subculture (Figure 1C), which

reached 70–80% confluence within 6-10d at passage 1. During passages 2 and 3, colony

growth was much faster than during passage 0 and 1. At the completion of passage 3 (4-5

weeks after initial seeding), over 100 million MSC, expressing normal karyotype (Figure 1D).

and with the capacity to differentiate into adipocytes, osteoblasts, chondrocytes and

myocytes (Figure 1F), were harvested from the 5 preterm ovine UCB samples.

Clonogenicity and self-renewal capacity was verified by serially cloning of the cells from 3 of

5 large colonies of UCB samples twice, and 2 of 5 samples cloned 4 times (Figure 1E).

FACS analysis for anti-CD146 antibody gave a high enrichment (43.5%) for MSC (passage

3), while other antibodies cross reacted with less than 5% of the MSC: CD44 (2.7%), CD45

(3.5%), CD73 (1.1%), CD90 (0.4%), CD105 (2.7%), CD271 (0.6%) (Figure 1G). These

levels of cross reaction correspond to those previously described for ovine MSC [27,30].

18

Li et al.   Preterm cord blood MSC protect the preterm brain

Physiological measures

Mean fetal body weight at postmortem for all animals was 2.2±0.1kg, with no differences between groups. Brain weight and brain/body weight for fetuses after HI, with or without MSC treatment, were significantly reduced compared to control fetuses (P<0.05; Table 1). The survival rate for fetuses in this study was 79%, with no significant differences in mortality between experimental groups.

HI resulted in hypotension, bradycardia, acidosis and hypoxia compared with the control group (P<0.05; Figure 2). There was no difference in the duration of the HI insult (Table 1), or any physiological parameters during, or immediately after occlusion, and following treatment, between the HI and HI+MSC groups (Figure 2).

Brain histopathology

Detection of transplanted MSC in the preterm brain 10 days after administration

Fluorescent MSC were observed in 3 of 7 HI+MSC fetal brains, but the number of cells observed was very low (2.0 cells per field of view). MSC did not appear to localize to a specific brain area.

19

Li et al.   Preterm cord blood MSC protect the preterm brain

White matter injury

In response to HI, CNPase-positive oligodendrocytes and myelin density was reduced at

day 10 within the PVWM and IC by 32% and 23%, respectively, compared to control fetuses

(P<0.05). The administration of MSC at 12h after HI decreased this hypomyelination.

(Figures 3B and 4A-C). No significant differences in the numbers of Olig2-positive

oligodendrocyte lineage cells were observed between groups (Figures 3A).

Neuroinflammation

HI induced microglial cell activation, as evidenced by a significant increase in Iba-1-positive

activated microglia within the PVWM and IC (P<0.05), compared to control fetuses. MSC

administration moderately reduced the number of Iba-1-positive activated microglia

following HI, with no significant difference observed in the numbers of activated microglia

between control and HI+MSC fetal brains (Figures 3C and 4D-F). The morphological

appearance of Iba-1+ microglia in control brains exhibited the characteristics of ramified

(resting) microglia, with small cell bodies and long branching processes (Figure 4D). In

contrast, microglia within white matter of HI brains (Figure 4E) and HI+MSC brains (Figure

4F), resembled ameboid (activated) microglia. MSC administration in HI fetuses induced an

20

Li et al.   Preterm cord blood MSC protect the preterm brain

increase of CD163-positive macrophages in all brain regions examined, particularly in

PVWM ($P<0.05$), which was not observed in control or HI fetuses (Figure 3D and 4G-I).

With respect to neutrophil elastase staining, neutrophils were rarely observed in control

brains (mean score 0.1). HI induced a significant colonization of neutrophils within the

vessels in the preterm brain (mean score 1.4) as evidenced by increased neutrophil

elastase-positive polynuclear cells with co-staining of red blood cells within blood vessels,

compared to controls. MSC administration did not alter the effect on neutrophils following HI

(score 1.2; Supplemental Figure).

No significant differences were observed in GFAP-positive astrocytic cell counts between

groups (data not shown).

Cell proliferation

Cell proliferation (Ki-67-positive cell counts) was significantly increased within all white

matter areas examined (SVZ, PVWM, and IC; $P<0.05$) in HI+MSC fetuses compared to

control and HI fetuses (Figure 3E and 4J-L). Within the white matter, we used double-label

immunohistochemistry to demonstrate that it was predominantly oligodendrocytes (Olig2+)

21

Li et al.   Preterm cord blood MSC protect the preterm brain

that were proliferating (Ki-67+) following HI+MSCs administration (Figure 4M and N).

Ki-67-positive cells were not co-localized with CD163-positive cells.

Cytokines, chemokines, and neurotrophic factors assay

HI induced an increased fetal plasma TNF-α concentration at 3d post-HI compared to

control (P<0.05); MSC administration prevented this increase (Figure 6). There was no

difference in plasma TNF-α concentrations at 10d between the three groups. No differences

in plasma IL-6 and IL-10 concentrations were found at any time points between groups (data

not shown).

CXCL10 concentration in white matter tissue lysate was increased in HI+MSC brains

compared with control and HI (p<0.05, Figure 5). IL-6 and NSE levels were increased in HI

and HI+MSC brains compared to controls (p<0.05), and IL-21 and IFNγ levels were

significantly decreased in HI and HI+MSC brains compared to controls (p<0.05), with no

difference between HI and HI+MSC. IL-17A and VEGF-A levels were significantly decreased

in HI+MSC animals compared to controls (p<0.05).

22

Li et al.   Preterm cord blood MSC protect the preterm brain

DISCUSSION

This study shows that allogeneic administration of ex-vivo expanded MSC derived from

preterm UCB are neuroprotective in a well-established fetal sheep model of HI-induced

preterm WMI. Our novel data show that preterm MSC treatment effectively reduce myelin

and mature oligodendrocyte loss following HI in the preterm brain. We show that the

neuroprotective benefits of MSC are mediated via induction of macrophage migration, and

cell proliferation, with suppression of microglial activation in the white matter, modulated

through systemic and cerebral cytokines.

Isolation, characterization, and expansion of ovine UCB-MSC

We successfully isolated, and expanded to passage 3, primary ovine cells with MSC

characteristics from five preterm sheep UCB samples to obtain sufficient cells for the target

dose. These cells were confirmed to have MSC-like characteristics, including plastic

adherence, morphology, in vitro differentiation capacity into 4 mesodermal lineages

(adipocytic, smooth muscle, chondrocytic, osteoblastic and myogenic differentiation),

clonogenicity and self-renewal ability [27]. Although the MSC used in this study showed a

limited sensitivity to undergo adipogenic differentiation; only cells from one in five UCB

samples showed adipogenic differentiation, with these findings in sheep UCB-MSC

23

Li et al.   Preterm cord blood MSC protect the preterm brain

consistent with observations in the human [31].   Our cells were also highly CD146-positive,

which is a putative MSC marker with interspecies cross-reactivity [30]. We chose to study

ovine UCB-MSC, in preference to human MSC, to replicate allogeneic cell administration as

might occur in the human situation and to avoid potential xenogeneic immune reactions.

Neuroprotective potential of MSC in the preterm brain

HI induced by 25 min umbilical cord occlusion was associated with a significant loss of

mature oligodendrocytes and hypomyelination of the white matter, as identified by reduced

CNPase-positive staining density in the PVWM and IC of the preterm brain. Mature

oligodendrocytes myelinate the axonal surface to increase axonal conduction velocity. The

developmental impairment of oligodendrocyte maturation and subsequent hypomyelination

is directly associated with adverse neurodevelopmental outcome in preterm infants [1,2]. In

this study, we showed that i.v. administration of MSC protected myelination and preserved

white matter structure. Previous reports have also demonstrated that treatment with MSC

improves neurological structure and function in both neonatal and adult rodent models of

ischemic brain damage [9-15]. Velthoven et al showed that treatment with MSC and

MSC+BDNF reduced white matter loss in a rat model of HI brain injury [32]. They also showed

that MSC treatment improves behavioral outcomes and induces neuronal and

24

Li et al.   Preterm cord blood MSC protect the preterm brain

oligodendrocyte regeneration in a mouse model of neonatal stroke [33]. In immature rat brains,

human UC-MSC also induced a considerable increase in myelin basic protein-labeled

mature oligodendrocytes post-HI [34]. However, the white to gray matter ratio, as well as the

developmental profile of young rodents are different from human, therefore, the pattern of

brain injury is also different from that in preterm infants [35,36]. In contrast, brain development

in sheep is morphologically and temporally similar to the human preterm brain. This study

was undertaken when the ovine fetal brain is developmentally similar to that of a 28-32 week

gestation human brain. Our study is the first evidence that, in a clinically relevant brain injury

model, allogeneic MSCs are effective at preserving white matter integrity. This is significant,

as the advantage of using MSC is that they have strong potential as an *off-the-shelf* product,

with commercial allogeneic MSC products now approved for use in humans [37], which would

allow early intervention for high risk extremely preterm infants.

Anti-inflammatory actions

WMI in infants born preterm is mediated by pro-inflammatory mechanisms [38], including

activation of resident brain microglia and astrocytes, invasion of mobilized peripheral

immune cells such as macrophages, lymphocytes and leucocytes, and stimulation of

cytokines and chemokines. In the current study, we confirm that HI induces

25

Li et al.   Preterm cord blood MSC protect the preterm brain

neuroinflammation and systemic immune system activation. MSC have frequently been

reported to have strong anti-inflammatory, immunomodulatory and regenerative capacities

in vitro [39-41]. However, *in vivo*, there is a paucity of data to demonstrate the mechanisms of

MSC-mediated neuroprotective effects in the developing brain [6]. Here we show that preterm

MSC only partially suppressed microglial activation. This finding is somewhat surprising

given that we have previously demonstrated that early administration of whole white blood

cell fraction of term and preterm UCB are neuroprotective for white matter development in

response to HI insult, principally mediated by suppression of microglial activation [26,42]. To

further examine the potential actions of MSC, we examined the presence of infiltrating

macrophages (CD163-positive) within the brain. Interestingly, macrophages were increased

in the SVZ, PVWM, and IC following MSC administration, but not in response to HI alone.

We noted that HI also significantly increased neutrophil infiltration within the preterm brain,

and MSC treatment did not suppress or modify this response. These findings indicate that

MSC protect white matter development against HI insult, principally mediated through an

immune-modification of macrophage activation within the white matter, and a partial

reduction of activated microglia. MSC are known to support monocyte differentiation into

macrophages [43], and macrophages are also recruited to inflammatory sites of injury to

moderate tissue damage and repair [44,45]. We observed an upregulation of brain CXCL10,

26

Li et al. Preterm cord blood MSC protect the preterm brain

which may play a regulatory role as a potent inflammatory chemoattractant for activated T

lymphocytes, produced by multiple cell types including monocytes, macrophages, and

endothelial cells [46]. Soluble factors secreted by macrophages induce a migratory MSC

phenotype, and can further enhance CXCL10 secretion from MSC [47]. We suggest that

increased CXCL10 and macrophage density within the preterm brain following MSC

administration, and in turn the interaction between macrophages and MSC, is central to

white matter repair following HI.

Cell proliferation

We found that treating with MSC following HI remarkably increased cell proliferation in all

white matter regions examined, compared to both control and HI groups. We confirmed that

Ki-67-positive cells were principally co-localised with Olig2 but not CNPase or CD163

staining, indicative that it was cells of oligodendrocyte lineage that were proliferating. This

result confirms previous work showing that MSC enhance formation of bromodeoxyuridine

(BrdU)-positive neurons and oligodendrocytes [48-50]. Treatment with either MSC or

MSC+BDNF induces long-lasting cell proliferation in injured brain regions, maintained to 28

days post-HI [33]. Another study also showed that injection of human MSC into the dentate

gyrus of the hippocampus of mice enhanced proliferation, migration, and neural

27

Li et al.   Preterm cord blood MSC protect the preterm brain

differentiation of the endogenous neural stem cells of the mouse and contributed to further

mode of tissue repair [51]. Taken together, our results demonstrate that MSC therapy in HI

animals ameliorated hypomyelination, potentially contributed by proliferation of

oligodendrocytes. This is a critical observation, since this pattern of WMI is the principal

neuropathological feature observed in infants born extremely preterm [52].

A potential role for macrophages induced by MSC

Interestingly, we also noted that CD163-positive macrophages and cerebral CXCL10

concentration were increased in response to MSC treatment. This is supported by other

studies to show that MSC achieve immunomodulation by supporting M2-macrophage

differentiation [53-55]. After central nervous system injury, inflammatory M1 macrophages

predominate over anti-inflammatory M2 macrophages. While overly simplistic, this M1/M2

balance is suggested as one of the most important mechanisms of injury/repair regulation [56].

In response to stimulation, MSC are activated by inflammatory mediators to polarize the M1

macrophages into an M2 phenotype, by means of three molecular pathways (prostaglandin

E2, tumor-necrosis-factor-inducible gene 6 protein, and progesterone receptor and

glucocorticoid receptors) [55,57]. The chemokine CXCL10 was also significantly upregulated

within cerebral tissue following MSC administration. This is a novel observation for the

28

Li et al.　　　　　　　　　　　　Preterm cord blood MSC protect the preterm brain

developing brain in response to MSC treatment, but given that CXCL10 is a known

chemoattractant to monocytes/macrophages, and has been shown to be upregulated by

MSCs when administered to a highly inflammatory environment [47,58], we postulate that the

neuroprotective actions of MSC are mediated via synergistic release of CXCL10 and the

modification of macrophage phenotype and function towards an anti-inflammatory or

reparative response.

Additionally, we show that MSC administration significantly suppressed the increase of

systemic TNF-α concentration at day 3 after HI. Within brain tissue, HI resulted in an

increased concentration of cerebral IL-6, and decreased concentrations of IFNγ and IL-21,

which were not modified by MSC. NSE is a glial cell specific isoenzyme, and known to

increase when the brain is damaged, via release from activated astrocytes [59]. Our results

found HI insult increased brain NSE, but no change was seen after MSC administration.

Additionally, we did not detect an increase in BDNF or NGF within the preterm brain

following MSC administration, although previous studies have reported that MSC can secret

a variety of trophic factors such as neurotrophins (BDNF, NGF), VEGF and insulin-like

growth factor, which can potentially contribute to endogenous repair [18,33,48,50,60].

29

Li et al.   Preterm cord blood MSC protect the preterm brain

Potential autologous cell therapy for preterm white matter injury

MSC are enriched in preterm UCB compared to term [23-25], and this is the first data to show

the therapeutic efficacy of preterm derived MSC. We have shown for the first time that MSC

isolated from preterm UCB are effective at reducing preterm WMI. This is an important

finding given that up to 50% of cerebral palsy patients are born preterm and this preterm

cohort is the most likely to require therapeutic intervention [61,62]. If an autologous cell therapy

is necessary then the optimal source of these cells would be from their own placental tissue.

Other placental cell therapy options include UCB mononuclear cells (MNC) [63], amnion

epithelial cells (AEC) [28,64,65], or UC-MSC [34]. Unfortunately, the volume of UCB that can be

obtained from preterm infants is reduced compared to term and restricts the number of

UCB-MNC available for re-administration [66]. As for AEC, it has been shown that cells

isolated from preterm amniotic membrane have a superior proliferation capacity, but do not

possess the same immunosuppressive and reparative capacity [67]. Although human

UC-MSC are shown to protect mature oligodendrocytes in a rat model of term HI [34], no data

to date is available regarding preterm UC-MSC. Our results show that preterm UCB-MSC

are highly proliferative and have both anti-inflammatory and neuroreparative properties

making them the ideal autologous cell therapy candidate for infants with preterm brain injury.

30

In conclusion, our findings support that preterm UCB-derived, expanded MSC respond to

signals provided by the intracerebral milieu activated in response to the HI insult. MSC

modulate the immune microenvironment of the injury within the brain's white matter by

regulating the activation of macrophages via release of chemokine and cytokines, and

moderating the neuroinflammatory response. In turn, MSC are neuroprotective for the

developing white matter of the preterm brain, protecting against hypomyelination by

maintaining oligodendrocyte development. The administration of MSC accelerated the

proliferation and differentiation of endogenous cells, including those cells that mediate

self-repair, resulting in white matter protection against HI insult in the preterm brain.

Previous reports also suggest that MSC inhibit T-cell activation and proliferation, modulate

B-cell and macrophage responses, suppress cytokine/chemokine release, and

downregulate pathogenic immune responses [39-41]. Further exploration of this important

interaction between MSC and macrophages in relation to other immune cells, especially T

lymphocytes, in the setting of preterm brain injury, may provide a more complete

understanding of the mechanisms of MSC-induced neuroprotection.

31

Li et al.   Preterm cord blood MSC protect the preterm brain

REFERENCES

1.   Volpe JJ. Cerebral white matter injury of the premature infant - More common than

      you think. *Pediatrics.* 2003;112(1 I):176-180.

2.   Volpe J. Neurology of the newborn. 5th edn. Elsevier, Philadelphia. 2008.

3.   Gopagondanahalli KR, Li J, Fahey MC, et al. Preterm Hypoxic–Ischemic

      Encephalopathy. *Frontiers in pediatrics.* 2016;4(114).

4.   Woodward LJ, Anderson PJ, Austin NC, et al. Neonatal MRI to predict

      neurodevelopmental outcomes in preterm infants. *New England Journal of Medicine.*

      2006;355(7):685-694.

5.   Gang EJ, Hong SH, Jeong JA, et al. In vitro mesengenic potential of human

      umbilical cord blood-derived mesenchymal stem cells. *Biochemical and Biophysical*

      Research Communications. 2004;321(1):102-108.

6.   Javazon EH, Beggs KJ, Flake AW. Mesenchymal stem cells: Paradoxes of

      passaging. *Experimental Hematology.* 2004;32(5):414-425.

7.   Trounson A, McDonald C. Stem Cell Therapies in Clinical Trials: Progress and

      Challenges. *Cell Stem Cell.* 2015;17(1):11-22.

8.   Barry FP, Murphy JM. Mesenchymal stem cells: Clinical applications and biological

      characterization. *International Journal of Biochemistry and Cell Biology.*

Li et al.   Preterm cord blood MSC protect the preterm brain

2004;36(4):568-584.

9.   van Velthoven CT, Kavelaars A, Heijnen CJ. Mesenchymal stem cells as a treatment

for neonatal ischemic brain damage. *Pediatric research.* 2012;71(4 Pt 2):474-481.

10.   van Velthoven CTJ, Kavelaars A, van Bel F, et al. Mesenchymal stem cell treatment

after neonatal hypoxic-ischemic brain injury improves behavioral outcome and

induces neuronal and oligodendrocyte regeneration. *Brain, Behavior, and Immunity.*

2010;24(3):387-393.

11.   Donega V, van Velthoven CTJ, Nijboer CH, et al. Intranasal Mesenchymal Stem Cell

Treatment for Neonatal Brain Damage: Long-Term Cognitive and Sensorimotor

Improvement. *PLoS ONE.* 2013;8(1).

12.   Kim ES, Ahn SY, Im GH, et al. Human umbilical cord blood-derived mesenchymal

stem cell transplantation attenuates severe brain injury by permanent middle

cerebral artery occlusion in newborn rats. *Pediatric Research.* 2012;72(3):277-284.

13.   Yasuhara T, Matsukawa N, Yu G, et al. Behavioral and histological characterization

of intrahippocampal grafts of human bone marrow-derived multipotent progenitor

cells in neonatal rats with hypoxic-ischemic injury. *Cell Transplantation.*

2006;15(3):231-238.

14.   Lim JY, Jeong CH, Jun JA, et al. Therapeutic effects of human umbilical cord

33

Li et al.   Preterm cord blood MSC protect the preterm brain

blood-derived mesenchymal stem cells after intrathecal administration by lumbar

puncture in a rat model of cerebral ischemia. *Stem Cell Research & Therapy.*

2011;2(5):38.

15.   Lee JS, Hong JM, Moon GJ, et al. Autologous mesenchymal stem cell

transplantation in stroke patients. *Stem Cells.* 2010;28.

16.   Shen LH, Li Y, Chen J, et al. One-year follow-up after bone marrow stromal cell

treatment in middle-aged female rats with stroke. *Stroke.* 2007;38(7):2150-2156.

17.   Messerli M, Wagner A, Sager R, et al. Stem cells from umbilical cord Wharton's jelly

from preterm birth have neuroglial differentiation potential. *Reproductive Sciences.*

2013;20(12):1455-1464.

18.   van Velthoven CTJ, Kavelaars A, van Bel F, et al. Regeneration of the ischemic brain

by engineered stem cells: Fuelling endogenous repair processes. *Brain Research*

Reviews. 2009;61(1):1-13.

19.   Xia G, Hong X, Chen X, et al. Intracerebral transplantation of mesenchymal stem

cells derived from human umbilical cord blood alleviates hypoxic ischemic brain

injury in rat neonates. *Journal of Perinatal Medicine.* 2010;38(2):215-221.

20.   Barachini S, Trombi L, Danti S, et al. Morpho-functional characterization of human

mesenchymal stem cells from umbilical cord blood for potential uses in regenerative

34

Li et al.   Preterm cord blood MSC protect the preterm brain

medicine. *Stem Cells and Development.* 2009;18(2):293-305.

21.   Kern S, Eichler H, Stoeve J, et al. Comparative analysis of mesenchymal stem cells

        from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells.*

        2006;24(5):1294-1301.

22.   Manca MF, Zwart I, Beo J, et al. Characterization of mesenchymal stromal cells

        derived from full-term umbilical cord blood. *Cytotherapy.* 2008;10(1):54-68.

23.   Javed MJ, Mead LE, Prater D, et al. Endothelial colony forming cells and

        mesenchymal stem cells are enriched at different gestational ages in human

        umbilical cord blood. *Pediatric Research.* 2008;64(1):68-73.

24.   Jain A, Mathur N, Jeevashankar M, et al. Does mesenchymal stem cell population in

        umbilical cord blood vary at different gestational periods? *Indian Journal of

        Pediatrics.* 2013;80(5):375-379.

25.   Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical

        cord blood. *British Journal of Haematology.* 2000;109(1):235-242.

26.   Li J, Yawno T, Sutherland A, et al. Preterm white matter brain injury is prevented by

        early administration of umbilical cord blood cells. *Exp Neurol.* 2016;283(Pt

        A):179-187.

27.   Letouzey V, Tan KS, Deane JA, et al. Isolation and characterisation of mesenchymal

35

Li et al.   Preterm cord blood MSC protect the preterm brain

stem/stromal cells in the ovine endometrium. *PLoS ONE.* 2015;10(5).

28.   Yawno T, Schuilwerve J, Moss TJM, et al. Human amnion epithelial cells reduce

fetal brain injury in response to intrauterine inflammation. *Developmental*

Neuroscience. 2013;35(2-3):272-282.

29.   Liravi B, Piedrafita D, Nguyen G, et al. Dynamics of IL-4 and IL-13 expression in the

airways of sheep following allergen challenge. *BMC Pulmonary Medicine.*

2015;15(1).

30.   Rozemuller H, Prins HJ, Naaijkens B, et al. Prospective isolation of mesenchymal

stem cells from multiple mammalian species using cross-reacting anti-human

monoclonal antibodies. *Stem Cells and Development.* 2010;19(12):1911-1921.

31.   Bieback K, Kern S, Kluter H, et al. Critical parameters for the isolation of

mesenchymal stem cells from umbilical cord blood. *Stem Cells.* 2004;22(4):625-634.

32.   van Velthoven CT, Kavelaars A, van Bel F, et al. Mesenchymal stem cell treatment

after neonatal hypoxic-ischemic brain injury improves behavioral outcome and

induces neuronal and oligodendrocyte regeneration. *Brain Behav Immun.*

2010;24(3):387-393.

33.   Van Velthoven CTJ, Sheldon RA, Kavelaars A, et al. Mesenchymal stem cell

transplantation attenuates brain injury after neonatal stroke. *Stroke.*

Li et al.   Preterm cord blood MSC protect the preterm brain

2013;44(5):1426-1432.

34.   Zhu Lh, Bai X, Zhang N, et al. Improvement of human umbilical cord mesenchymal

stem cell transplantation on glial cell and behavioral function in a neonatal model of

periventricular white matter damage. *Brain Research.* 2014.

35.   Rees S, Inder T. Fetal and neonatal origins of altered brain development. *Early

Human Development.* 2005;81(9):753-761.

36.   Hagberg H, Peebles D, Mallard C. Models of white matter injury: comparison of

infectious, hypoxic-ischemic, and excitotoxic insults. *Ment Retard Dev Disabil Res

Rev.* 2002;8(1):30-38.

37.   Miyamura K. Insurance approval of mesenchymal stem cell for acute GVHD in

Japan: need of follow up for some remaining concerns. *Int J Hematol.*

2016;103(2):155-164.

38.   Hagberg H, Mallard C, Ferriero DM, et al. The role of inflammation in perinatal brain

injury. *Nature Reviews Neurology.* 2015;11(4):192-208.

39.   Glenn JD, Whartenby KA. Mesenchymal stem cells: Emerging mechanisms of

immunomodulation and therapy. *World Journal of Stem Cells.* 2014;6(5):526-539.

40.   Bennet L, Tan S, Van Den Heuij L, et al. Cell therapy for neonatal hypoxia-ischemia

and cerebral palsy. *Annals of Neurology.* 2012;71(5):589-600.

37

Li et al.   Preterm cord blood MSC protect the preterm brain

41.   Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease.

Nature Reviews Immunology. 2008;8(9):726-736.

42.   Li J, Yawno T, Sutherland A, et al. Term versus preterm cord blood cells for the

prevention of preterm brain injury. *Pediatr Res.* 2017.

43.   Chiossone L, Conte R, Spaggiari GM, et al. Mesenchymal Stromal Cells Induce

Peculiar Alternatively Activated Macrophages Capable of Dampening Both Innate

and Adaptive Immune Responses. *Stem Cells.* 2016;34(7):1909-1921.

44.   Wang Y, Chen X, Cao W, et al. Plasticity of mesenchymal stem cells in

immunomodulation: Pathological and therapeutic implications. *Nature Immunology.*

2014;15(11):1009-1016.

45.   Shi C, Pamer EG. Monocyte recruitment during infection and inflammation. *Nature

Reviews Immunology.* 2011;11(11):762-774.

46.   Cassatella MA, Gasperini S, Calzetti F, et al. Regulated production of the

interferon-γ-inducible protein-l0 (IP-10) chemokine by human neutrophils. *European

Journal of Immunology.* 1997;27(1):111-115.

47.   Anton K, Banerjee D, Glod J. Macrophage-Associated Mesenchymal Stem Cells

Assume an Activated, Migratory, Pro-Inflammatory Phenotype with Increased IL-6

and CXCL10 Secretion. *PLoS ONE.* 2012;7(4):e35036.

38

Li et al.   Preterm cord blood MSC protect the preterm brain

48.   Chen J, Li Y, Katakowski M, et al. Intravenous bone marrow stromal cell therapy

reduces apoptosis and promotes endogenous cell proliferation after stroke in female

rat. *J Neurosci Res.* 2003;73(6):778-786.

49.   Li Y, Chen J, Zhang CL, et al. Gliosis and brain remodeling after treatment of stroke

in rats with marrow stromal cells. *Glia.* 2005;49(3):407-417.

50.   Ohtaki H, Ylostalo JH, Foraker JE, et al. Stem/progenitor cells from bone marrow

decrease neuronal death in global ischemia by modulation of inflammatory/immune

responses. *Proc Natl Acad Sci U S A.* 2008;105(38):14638-14643.

51.   Munoz JR, Stoutenger BR, Robinson AP, et al. Human stem/progenitor cells from

bone marrow promote neurogenesis of endogenous neural stem cells in the

hippocampus of mice. *Proceedings of the National Academy of Sciences of the*

United States of America. 2005;102(50):18171-18176.

52.   Volpe JJ, Kinney HC, Jensen FE, et al. The developing oligodendrocyte: Key cellular

target in brain injury in the premature infant. *International Journal of Developmental*

Neuroscience. 2011;29(4):423-440.

53.   Mantovani A, Biswas SK, Galdiero MR, et al. Macrophage plasticity and polarization

in tissue repair and remodelling. *Journal of Pathology.* 2013;229(2):176-185.

54.   Selleri S, Bifsha P, Civini S, et al. Human mesenchymal stromal cell-secreted lactate

39

Li et al.   Preterm cord blood MSC protect the preterm brain

induces M2-macrophage differentiation by metabolic reprogramming. *Oncotarget.*

2016;7(21):30193-30210.

55.   Xu C, Fu F, Li X, et al. Mesenchymal stem cells maintain the microenvironment of

central nervous system by regulating the polarization of macrophages/microglia

after traumatic brain injury. *The International journal of neuroscience.* 2017:1-12.

56.   Turtzo LC, Lescher J, Janes L, et al. Macrophagic and microglial responses after

focal traumatic brain injury in the female rat. *Journal of Neuroinflammation.* 2014;11.

57.   Prockop DJ. Concise review: Two negative feedback loops place mesenchymal

stem/stromal cells at the center of early regulators of inflammation. *Stem Cells.*

2013;31(10):2042-2046.

58.   Mao F, Xu WR, Qian H, et al. Immunosuppressive effects of mesenchymal stem

cells   in   collagen-induced   mouse   arthritis. *Inflammation   Research.*

2010;59(3):219-225.

59.   Li R, Fujitani N, Jia JT, et al. Immunohistochemical indicators of early brain injury: an

experimental study using the fluid-percussion model in cats. *The American journal of

forensic medicine and pathology.* 1998;19(2):129-136.

60.   Li Y, Chen J, Chen XG, et al. Human marrow stromal cell therapy for stroke in rat:

Neurotrophins and functional recovery. *Neurology.* 2002;59(4):514-523.

61.   Pharoah POD, Platt MJ, Cooke T. The changing epidemiology of cerebral palsy.

Archives   of   Disease   in   Childhood: Fetal   and   Neonatal   Edition.

1996;75(3):F169-F173.

62.   Thorngren-Jerneck K, Herbst A. Perinatal factors associated with cerebral palsy in

children born in Sweden. *Obstetrics and Gynecology.* 2006;108(6):1499-1505.

63.   Li J, McDonald C, Fahey MC, et al. Could cord blood cell therapy reduce preterm

brain injury? *Frontiers in Neurology.* 2014;5.

64.   Yawno T, Sabaretnam T, Li J, et al. Human Amnion Epithelial Cells Protect Against

White Matter Brain Injury After Repeated Endotoxin Exposure in the Preterm Ovine

Fetus. *Cell Transplant.* 2017;26(4):541-553.

65.   Leaw B, Zhu D, Tan J, et al. Human amnion epithelial cells rescue cell death via

immunomodulation of microglia in a mouse model of perinatal brain injury. *Stem Cell

Res Ther.* 2017;8(1):46.

66.   Solves P, López M, Mirabet V, et al. Characteristics of umbilical cord blood units

collected   from   preterm   deliveries.   *Gynecologic   and   Obstetric   Investigation.*

2009;68(3):181-185.

67.   Lim R, Chan ST, Tan JL, et al. Preterm human amnion epithelial cells have limited

reparative potential. *Placenta.* 2013;34(6):486-492.

41
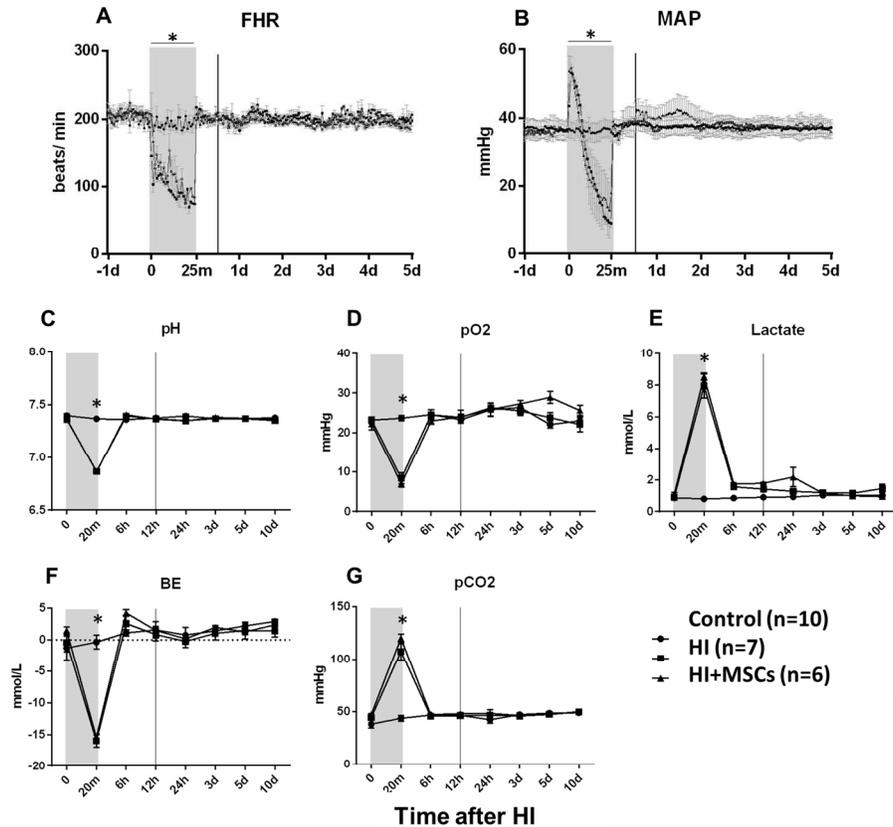
Figure 2. Physiological measurements during hypoxia-ischemia (HI).. HI resulted in severe bradycardia (A) and hypotension (B), markedly reduced fetal arterial pH (C), PaO2 (D) and base excess (F), and increased PaCO2 (G) and lactate levels (E) compared with control groups (P<0.05). The physiological parameters were rapidly returned to normal values after reperfusion (A & B). No intergroup differences in these physiological and biochemical measures were found between HI and HI+MSC groups. HI, induced by umbilical cord occlusion, is shown by the grey columns, UCB-MSC administration is shown by vertical lines. Data is presented as mean ± SEM. UCB-MSC: umbilical cord blood mesenchymal stem cells; MAP: mean arterial pressure; FHR: fetal heart rate; BE: base excess. *P<0.05 vs control.

150x140mm (300 x 300 DPI)
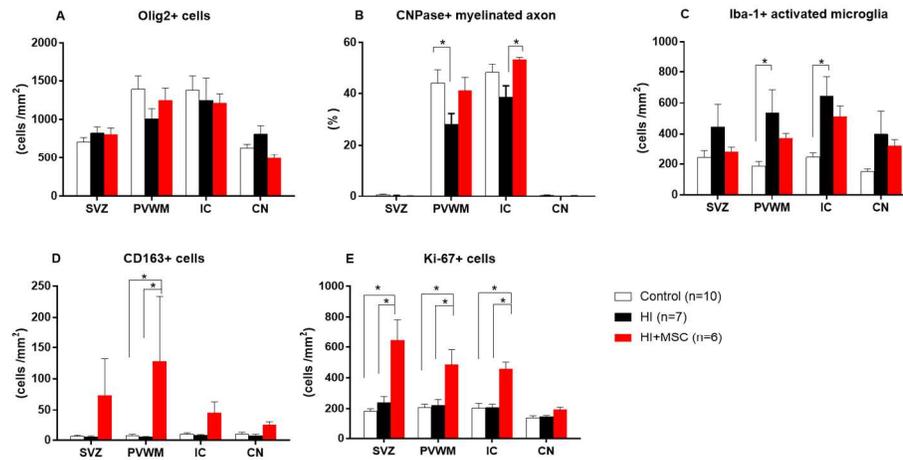
Figure 3. Effects of MSC on brain histopathology. The number of oligodendrocyte positive cells (A), CNPase+ meylinating axons (B), Iba-1+ positive cells (C), CD163+ positive cells (D), and Ki-67+ positive cells (E) in the subventricular zone (SVZ), periventricular white matter (PVWM), internal capsule (IC) and caudate nucleus (CN). Data are mean ± SEM, *P<0.05.

157x85mm (300 x 300 DPI)
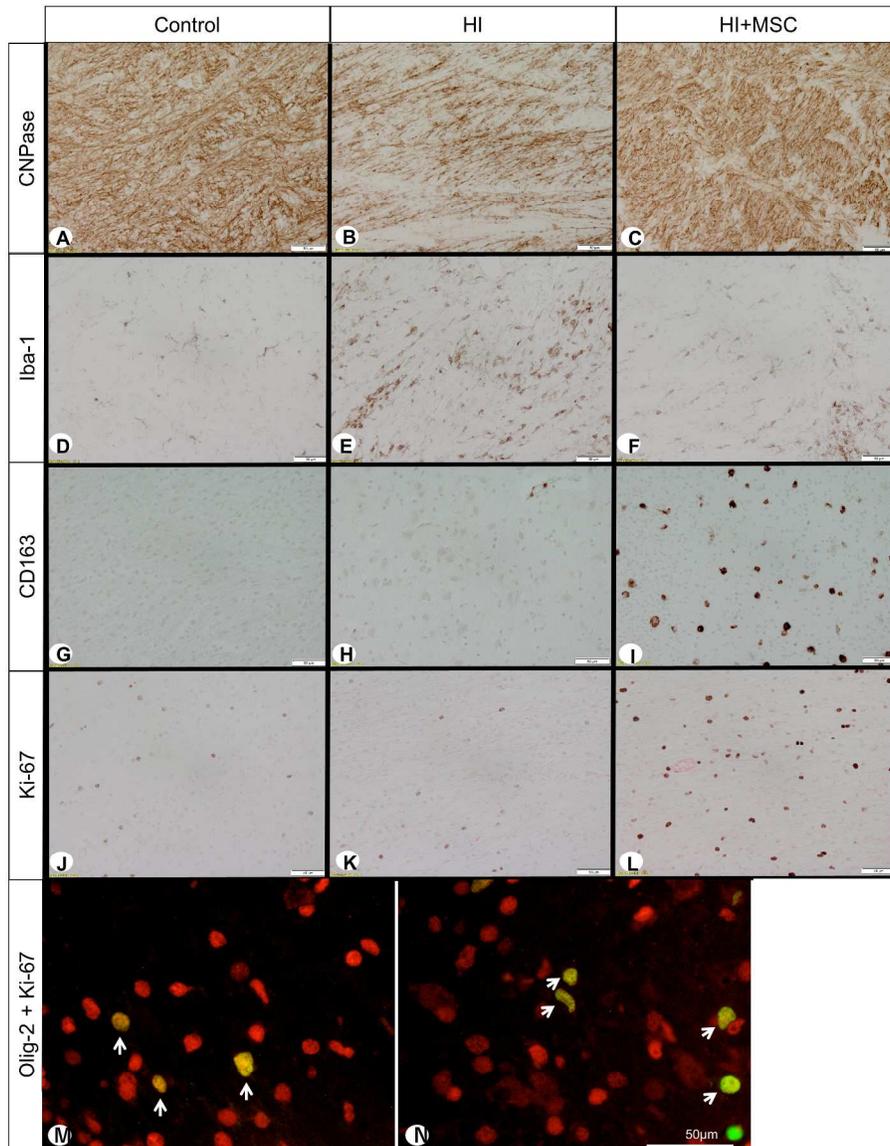
Figure 4. Representative photomicrographs showing CNPase (A-C), Iba-1 (D-F), and CD163 (G-I) positive staining in the periventricular white matter and Ki-67 positive staining (J-L) in the subventricular zone in the brain of control, HI and HI+MSC fetuses. Double-label immunohistochemistry in the SVZ (M) and PVWM (N) for Ki-67 and oligodendrocytes (Olig-2) in HI+MSCs brains. Cell types (Ki-67) were visualized with Alexa Fluor 488 (green) and Olig-2 with Alexa Fluor 594 (red). White arrows indicate yellow cells that are co-localized for the Olig-2 and for Ki-67. Scale bar = 50 μm.

250x309mm (300 x 300 DPI)
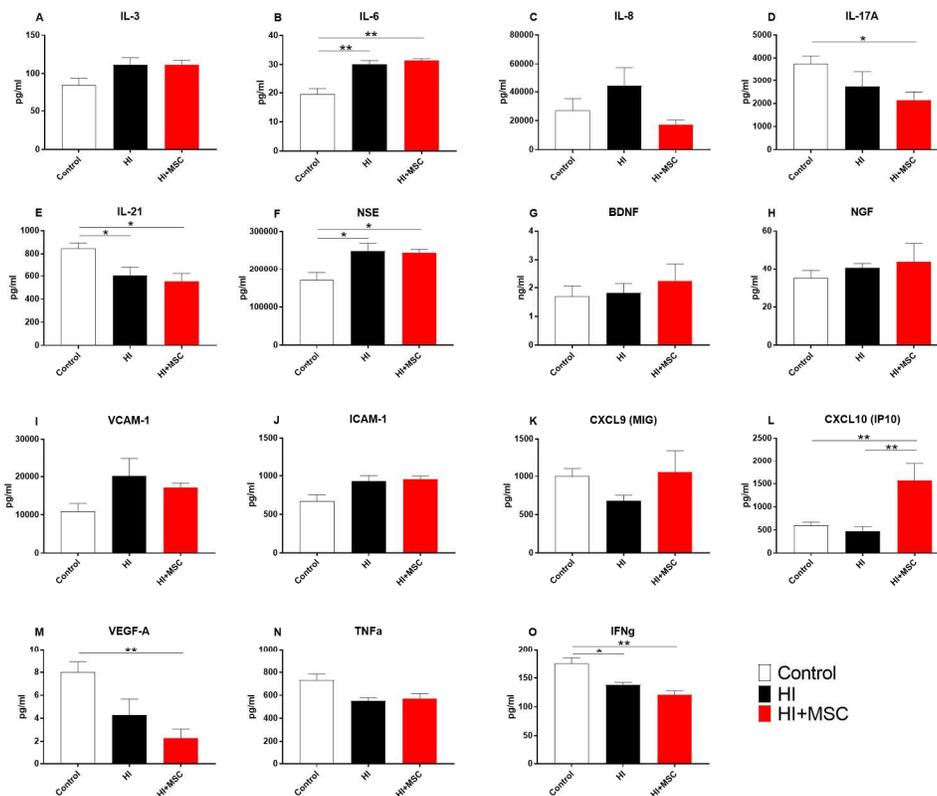
Figure 5. Cytokines, chemokines, and neurotrophic factors in white matter tissue
The concentrations of IL-3 (A), IL-6 (B), IL8 (C), IL-17A (D), IL-21 (E), neuron-specific enolase (NSE, F), brain derived neurotrophic factor (BDNF, G), nerve growth factor (NGF, H), VACM-1 (I), ICAM-1 (J), CXCL9 (MIG, K), CXCL10 (IP10, L), VEGF-A (M), TNFa (N), and IFNg (O) in white matter tissue lysate were measured. The concentration of CXCL10 (L) in white matter tissue lysate was significantly increased in HI+MSC animals compared with control and HI animals (p<0.05). While, IL-6 (B) and NSE (F) levels were significantly increased in HI and HI+MSC animals compared to controls (p<0.05), IL-21 (E) and IFNg (O) levels were significantly decreased in HI and HI+UCB animals compared to controls (p<0.05), with no differences between HI and HI+MSC animals. IL-17A (D) and VEGF-A (M) levels were significantly decreased in HI+MSC animals compared to controls (p<0.05). Data are mean ± SEM, and were analyzed using ANOVA followed by post hoc Tukey's tests. *P<0.05.

198x170mm (300 x 300 DPI)
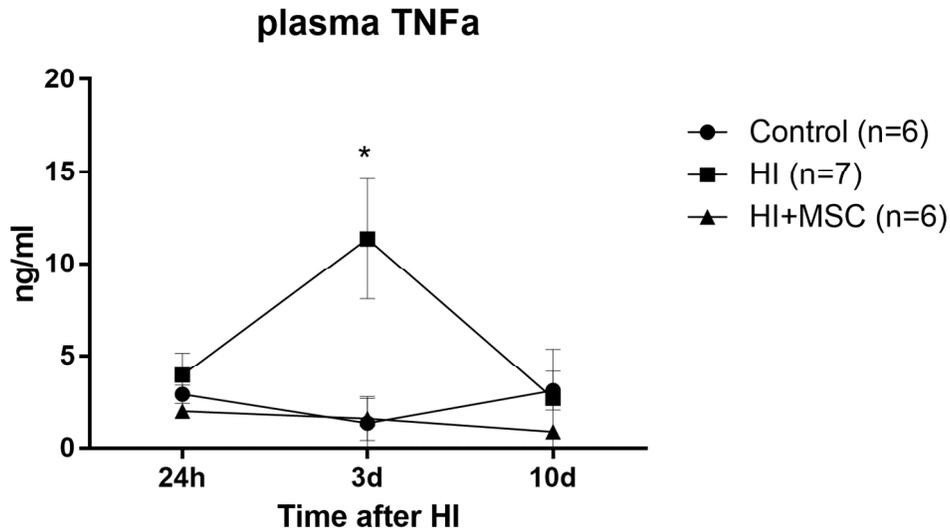
Figure 6. TNF-a concentration within fetal plasma in control, HI, HI+MSC groups. HI caused a significant increase (on day 3) in TNFa concentrations compared to the control group and this increase was attenuated in the HI+MSC group. *P<0.05.

136x80mm (300 x 300 DPI)

variables		Control	HI	HI+MSC
		n = 10	n = 7	n = 6
Female, n (%)		7 (70)	3 (43)	2 (33)
Twin, n (%)		6 (60)	2 (29)	0 (0)*
UCO duration, min		0	24.4 ± 0.3*	24.7 ± 0.3*
Weight	Brain weight, g	34.5 ± 0.7	30.5 ± 0.9*	30.5 ± 1.2*
	Body weight, kg	2.0 ± 0.1	2.4 ± 0.1	2.3 ± 0.1
	Brain/body weight, g/kg	17.6 ± 0.7	13.6 ± 0.9*	13.2 ± 0.9*

Mean (%) or mean ± standard error are presented for each group. One-way analysis of variance and post hoc Tukey tests were carried out on comparisons between groups. *P<.05 vs control. HI: hypoxia-ischemia; UCB: umbilical cord blood; UCO: umbilical cord occlusion; MSC: mesenchymal stem cells.
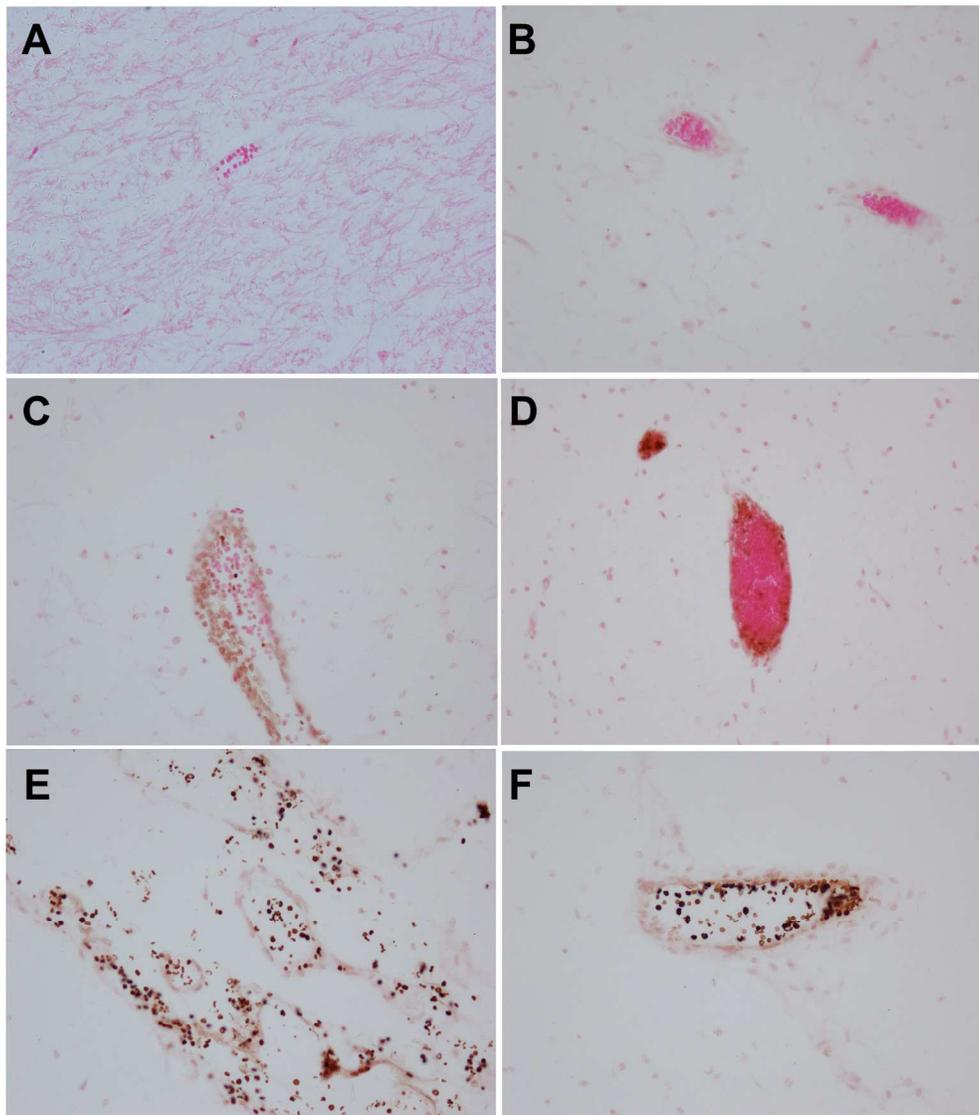
150x170mm (300 x 300 DPI)

Subventrivular zone (SVZ)
Red: Olig-2
Green: Ki-67
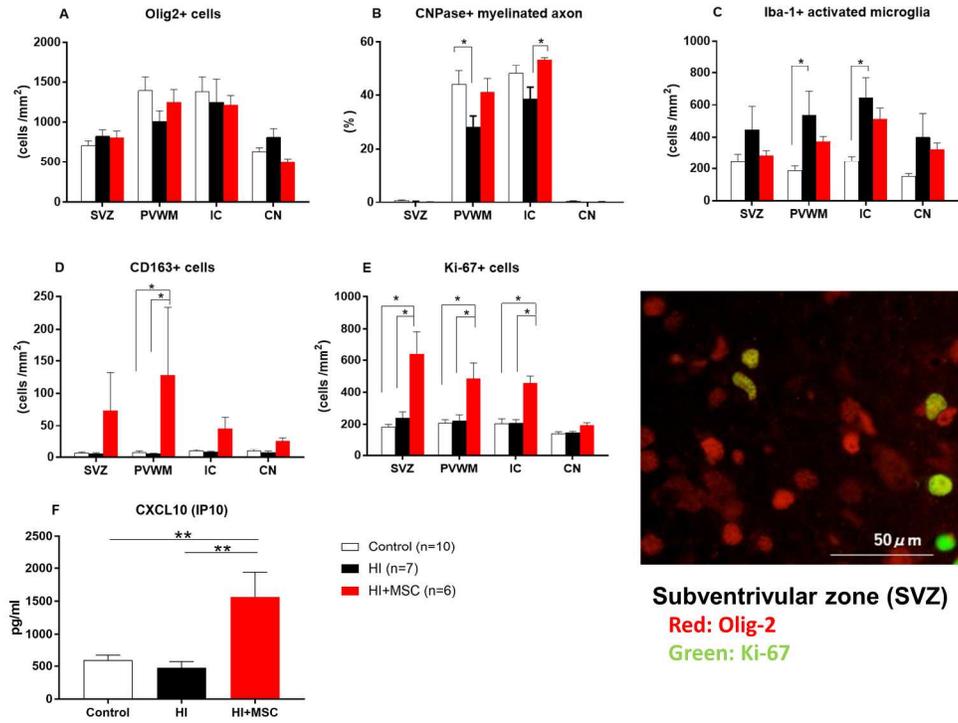
219x170mm (300 x 300 DPI)

Chapter 5

Thesis discussion and summary

5.1. Preface

The studies presented in this thesis were specifically aimed at examining whether allogeneic administration of UCB cells can reduce the progression of preterm brain injury caused by hypoxic-ischemic (HI) insult (Chapters 2, 3 and 4). This chapter brings together the studies, and results presented in this thesis, to assess the impact of UBC cells on preterm brain injury in the context of current literature. By way of overview, Chapter 1 reviewed the current knowledge and understanding of the brain injury that is evident in infants born preterm, mechanisms of injury and the potential for UCB therapy to protect against preterm brain damage, particularly white matter injury (published review, Li et al. (Li et al., 2014b)). Chapter 2 presented original research (published, Li et al. (Li et al., 2016)) that examined whether early, and/or delayed, UCB cell administration was neuroprotective for preterm brain injury induced by HI, and assessed mechanisms of UCB action. Chapter 3 compared the neuroprotective actions of cells derived from term UCB and preterm UCB for their capacity to protect against preterm brain injury (published, (Li et al., 2017)). Finally, Chapter 4 investigated whether a specific population of cells that are found in preterm UCB, specifically mesenchymal stem/stromal cells (MSCs), were therapeutic in protecting the preterm brain (Li et al, manuscript submitted to Stem Cells Translational Med).

5.2. The relevance of preterm brain injury induced by an acute HI insult in fetal sheep

As described in Chapter 1, preterm infants that are born less than 32 weeks of gestation are at a very high risk of suffering white matter injury (WMI) within the developing brain (Pharoah et al., 1996; Thorngren-Jerneck et al., 2006; Volpe, 2008). In order to examine the potential

neuroprotective properties of UCB cells, I utilised the established fetal sheep model of acute severe HI-induced brain injury, wherein the development of the ovine brain is equivalent to that of a preterm human infant, born at 28-32 weeks of gestation. This experimental animal model of preterm brain injury is well established and published (Bennet et al., 2007; Keogh et al., 2012), and was utilised for all original research studies in this thesis. In the first instance, fetal surgery was undertaken at 95-98 days gestation (term is ~147 days) for the implantation of an umbilical cord occluder and catheters to fetuses. At 101-104 days (0.7 gestation), acute HI was induced by up to 25 minutes of umbilical cord occlusion, the fetus was monitored, fetal blood taken at regular intervals and, 10 days after HI, the fetal brain collected for neuropathological analysis. My results show that white matter neuropathology was evident in response to acute severe HI, with reduced oligodendrocyte lineage (Olig2+ stained) cell numbers and decreased myelination density (CNPase+) in white matter areas of interest (SVZ, PVWM and IC) within the preterm brain. I also found that, principally, HI was more injurious to white matter areas of the brain than to gray matter (cortex and caudate nucleus neuronal cell populations).

The pattern of brain damage that I observed in preterm fetal sheep is consistent with clinical observations of diffuse periventricular leukomalacia (PVL), characterised by diffuse and pyknotic white matter injury related to HI events within the preterm brain (Gopagondanahalli et al., 2016; Logitharajah et al., 2009; Volpe, 2008). Barkovich, et al. showed that infants born at 28-34 weeks had periventricular gliosis and variably dilated ventricles after asphyxia (Barkovich et al., 1990). In the study by Logitharajah et al. (Logitharajah et al., 2009), white matter injury, which was mostly diffuse and mild, was noted in ~82% of preterm infants exposed to a HI insult before birth. In this study population, it was less frequently noted, but still evident, that many infants with white matter injury showed evidence of neuropathology in gray matter regions, particularly the basal ganglia and cortex. There is a complex interplay between white and gray matter injury in preterm infants, wherein neuronal injury could occur secondary to damage to white matter tracts (Volpe, 2008). In the studies described in this thesis,

our treatment induced profound prenatal acute hypoxia-ischemia and WMI via upregulation of the ischemia-reperfusion pathway. However in the clinical setting, preterm WMI is not only due to perinatal hypoxia, but also contributed by acute or chronic in utero hypoxia, postnatal episodes of ischemia-reperfusion, and/or infection/inflammation (Sie et al., 2000). While I only investigated an acute HI model of fetal sheep induced preterm WMI in the studies described in this thesis, I believe the benefits of this study design are threefold, i) I was able to produce a standardised HI insult which resulted in subsequent diffuse WMI; ii) the brain injury that I observed is consistent with the known neuropathology observed in preterm infants, incorporating principally white matter damage, but also with a lesser component of gray matter neuronal cell damage; and iii) I was able to examine the neuroprotective effects of UCB cells at 10 days after insult. Accordingly, this experimental paradigm allowed the examination of whether UCB stem cells would be neuroprotective for HI preterm brain injury, the effects of different timing of cell treatment, and potential mechanisms of action of cord blood stem/ progenitor cells on the preterm brain.

5.3. Multiple factors to cause preterm white matter injury

The etiology of cerebral white matter injury in infants born preterm is known to be heterogeneous, but the two leading mechanistic contributors are ischemia/reperfusion and infection/inflammation, as discussed in Chapter 1. Previous reports suggest that antenatal or perinatal ischemia/reperfusion may underlie around 50% of adverse events that result in white matter injury in preterm infants (Volpe, 2008). This thesis specifically focused on the neuroprotective effects of UCB derived cells on ischemia/reperfusion-induced preterm white matter brain injury, and demonstrated very promising findings. However, inflammation/infection is also a significant contributor to preterm WMI (Volpe, 2008). Cell based therapies have been shown to be effective for HI brain injury in many preclinical studies (Li et al., 2014b), the evidence of cell therapy for inflammation/infection-related perinatal brain injury is, however, sparse. Yawno et al. have shown that human amnion epithelial cells (AECs)

are effective in preventing preterm white matter brain injury caused by LPS-induced intrauterine inflammation in preterm fetal sheep (Yawno et al., 2017; Yawno et al., 2013). To my knowledge, there are currently no other published papers that have evaluated the effects of cell-based therapy using specific inflammation models. It is however important to note that neuroinflammation was significantly upregulated in response to acute HI in the preterm fetal sheep studied, as evidenced by an elevation in the density of activated microglia and pro-inflammatory cytokines. In turn, my results demonstrate that neuroinflammation is a key mediator of white matter brain injury in the developing brain (Chapter 2).

In the current studies, we showed that peripheral pro-inflammatory mediators were reduced by *preterm* UCB cell (Chapter 3) and MSC (Chapter 4) administration, and circulating levels of oxidative stress were reduced by *term* UCB cells (Chapters 2 & 3), which is particularly relevant for IUGR. Our results showed that UCB cells, as well as UCB-MSC, contributed to suppression of microglial activation in the white matter following HI. The microglia are the resident immune/ inflammatory modulatory cells within the brain, and I showed in Chapter 2 that increasing numbers of activated microglia within cerebral white matter is closely correlated to decreasing numbers of oligodendrocytes. This result confirms the work of others to show that neuroinflammation is a significant contributor to neuropathology in the preterm brain (as reviewed by Czeh et al. 2011). Clinically, it is well described that up to half of all preterm births may be complicated by chorioamnionitis (Paton et al., 2017), and therefore it would be most appropriate to examine whether administration of UCB cells or MSC are neuroprotective in this setting. While I did not study the myriad of potential contributors to preterm WMI (such as chorioamnionitis), I believe the benefits of undertaking this study in a preterm fetal sheep model of acute hypoxia-ischemia allowed me to examine what the effects of UCB cell therapies may have on such infants, and whether they could benefit from interventional strategies. Indeed my results to show that UCB derived stem/ progenitor cells have excellent anti-inflammatory benefits holds promise for WMI caused by multiple other factors.

5.4. Mechanisms of actions and therapeutic time window of UCB therapy

The results obtained in Chapter 2 and 3 showed that treatment using UCB cells obtained from term or preterm cord blood samples are associated with a variety of positive effects, protecting preterm white matter against HI insult, predominantly through suppressing cerebral microglial activation, along with prevention of cell death and systemic anti-inflammatory benefits (Chapter 2, 3). The administration of UCB cells from term samples was also associated with a systemic anti-oxidant effect (Chapter 2). However, as described in Chapter 2, it is important to consider that these neuroprotective benefits may be greatest when UCB cell administration is conducted early after insult. Indeed, my result show that the optimal therapeutic benefit of UCB therapy was observed mainly when the cells were administered at 12 hours after HI, when compared to administration at 5 days after acute HI (Chapter 2). It is thus apparent that it is critically important to explore the optimal timing of UCB therapy for translation to the clinic. Our study specifically examined time-points of 12 hours or 5 days after insult reflecting what might be feasible in the clinic for infants born preterm and then either identified as requiring therapeutic intervention soon after birth, or within the first days after preterm birth. Previously published studies, mostly undertaken in rodents, have also investigated the effects of timing of cell administration (Boltze et al., 2012; G. Yu et al., 2009). Our data strongly suggests that early treatment facilitates optimal recovery after acute preterm HI in fetal sheep. Many groups have administered cells 4 to 48 hours following acute injury in animal studies, and some benefits have been observed with cell delivery up to 10 days after cerebral HI (Li et al., 2014b). However, one recent study administered UCB cells at around 10 days after HI, and still observed beneficial effects via functional improvement in rabbits (Drobyshevsky et al., 2015). A further study has shown that delayed intranasal infusion of hAECs, again at 10 days after HI insult, improved white matter maturation after asphyxia in preterm fetal sheep (van den Heuij et al., 2017). It is important to note that in these studies, the days of assessment were set at more than 10 days after cell treatment (Drobyshevsky et al., 2015), which may allow the stem cells

adequate time to have a positive effect within the brain. The results described in this thesis were all obtained in post-mortem collection of brain tissue at 10 days after HI insult, which may not have allowed enough time for neuroregeneration to take place following treatment. Thus, when we compared results of cell administration at 12 hours versus at 5 days, the brains were all collected on day 10, the latter group of treated animals had a shorter time period in which cells could have exerted a positive effect. The mechanisms of actions of UCB cell treatment are related to not only neuroprotection, or reducing ongoing pathologies, but also neuroregeneration, such as enhancement of endogenous cerebral plasticity, which may mainly lead to functional improvement rather than anatomical improvement.

Interestingly, we noted that cellular proliferation (Ki67+ cell staining) was upregulated after UCB-MSC administration in Chapter 4. However, at 10 days after HI we did not observe a corresponding increase in myelinating oligodendrocytes (CNPase+ cells). This finding may also have been influenced by the short timecourse of the study and a longer duration prior to post-mortem may have resulted in a different outcome. Neuroprotective agents currently in use, or under examination, have a relatively narrow effective therapeutic time window, best characterised in human and animal trials for therapeutic hypothermia for term infants with hypoxic-ischemic encephalopathy, which must be initiated within 6 hours after birth (Bennet et al., 2007; Li et al., 2013; Li et al., 2014a). This is likely to be because established or potential treatments predominantly focus on the prevention of secondary cell death by attenuating inflammation, mitochondrial and excitotoxic activity (Bennet et al., 2007; Bennet et al., 2012). The timely evaluation and diagnosis of term neonatal encephalopathy allows clinicians to intervene with a therapy as soon as possible. However, unlike term HIE, it is widely accepted that preterm babies present a significantly more difficult cohort to appropriately address the timing of injury, that can occur any time before or after birth, often silently (Li et al., 2014b). Thus, although we showed that early administration of UCB cells is essential for significant neuroprotective effects after HI insult in Chapter 2, later histological or functional investigations

are needed to evaluate whether the transplanted cells have an ability to promote neuroregeneration, which will further increase our understanding of the potential of therapeutic treatments using UCB cells.

5.5. UCB-MSC (or whole UCB) vs individual expanded cells included in UCB for clinical application

Most studies to date investigating treatment of perinatal brain injury using UCB have utilized UCB–MNCs (Li et al., 2014b). UCB–MNCs are composed of three major types of stem and progenitor cell types, HSCs, EPCs, and MSCs, as well as containing lymphocytes and monocytes. Although the evidence from clinical trial is still sparse, UCB-MNCs have been shown to be neuroprotective in almost all animal models of neonatal HI previously published (Li et al., 2014b). Thus, the biggest advantage of using UCB-MNC or whole UCB cells for clinical application is the firm evidence for efficacy and safety over the last decade. Additionally, the current paradigm for allogeneic UCB transplantation is that HLA matching and immune suppression are strictly required to prevent GVHD. Thus, the advantage of autologous UCB-MNCs or whole UCB cells to avoid potential immunological side effects and the need for MHC matching makes autologous UCB treatment for preterm infants far more ethically acceptable. Two clinical trials giving autologous UCB to preterm infants in the first 5 or 14 days post-delivery, aiming to examine feasibility and efficacy for a variety of preterm complications, are currently underway [NCT02050971(Rudnicki et al., 2015) and NCT01121328]. Late clamping and milking of umbilical cord in preterm infants has also been studied in multiple clinical trials, where this technique is considered a form of early autologous UCB infusion after birth (Ghavam et al., 2014). Based on the findings from Chapter 2 and 3 in this thesis, the design of potential clinical trials that would use UCB for brain protection in preterm infants can be advanced in two ways. Firstly, the administration of autologous UCB cells to extremely preterm infants over a 3-day interval post-delivery, to examine whether short and long-term brain structure and function are improved. As discussed in Chapter 2, the

therapeutic window of UCB administration after HI is 2 to 3 days (Boltze et al., 2012; Li et al., 2016). Additionally, as discussed in Chapter 1, infants born extremely preterm are at the highest risk for cerebral palsy and neurocognitive impairment, but the time of onset of an insult in these preterm infants is very difficult to define (Li et al., 2014b). Therefore, it would be quite justified to administer autologous UCB cells prophylactically during the first to second week post-delivery to reduce preterm brain injury, as it is known that cystic PVL or diffuse WMI tend to develop over days to weeks after birth (Miller et al., 2005; Volpe, 1998). Preterm birth is however frequently associated with obstetric complications (e.g., chorioamnionitis or fetal growth restriction), which may alter the volume and cell composition of UCB (Hosono et al., 2015; Surbek et al., 2000), and may be a limitation to the routine collection and use of autologous UCB cell administration for preterm infants. In turn, allogeneic UCB cells are an alternative, but this would necessitate the need for MHC matching and co-administration of immunosuppressants. Therefore, another option for potential clinical trial is to administer allogeneic UCB cells within 12-48h after the onset of profound HI insult. Although it is not frequent to see the situation that preterm infants are exposed to a clearly defined HI, it would be appropriate to administer allogeneic UCB cells soon after the onset of acute profound HI insult such as severe birth asphyxia or intraventricular haemorrhage (IVH) complicated by haemorrhagic venous infarction in the periventricular white matter (Papile grade IV-IVH). A recent paper showed that 114 patients suffering from non-hematopoietic degenerative conditions were treated with non-matched, allogeneic UCB-MNCs, with 4-5 treatments both intrathecal and intravenously, and showed no profound hematological or immunological adverse effects (Yang et al., 2010). This suggests that the use of UCB for non-hematopoietic regenerative purposes, such as immune modulation, growth factor production, and stimulation of angiogenesis, may not require matching or immune suppression in immune competent recipients (Yang et al., 2010). This result may encourage the use of allogeneic UCB for non-hematopoietic regenerative purposes without matching or immune suppression. However, this issue is still to be addressed in preterm infants. Thus, I eventually reached the idea that

identifying a specific cell type in UCB that is neuroprotective, and then expanding the cells in vitro prior to administration, may be a reasonable strategy to best utilize UCB for the treatment of preterm brain injury. MSC treatment, especially allogeneic transplantation, can be used safely, and with good efficacy, as shown in many pre-clinical and clinical studies (Donega et al., 2013; E. S. Kim et al., 2012; J. S. Lee et al., 2010; Lim et al., 2011; Reinders et al., 2015; C. T. van Velthoven et al., 2012; van Velthoven et al., 2010a; Yasuhara et al., 2006b). This was the major reasons that I studied MSCs in Chapter 4.

Each stem cell type in UCB can be isolated and expanded in vitro. MSCs, CD34+ cells (EPCs+HSCs), monocytes, and lymphocytes have all been shown to be effective for brain protection in various brain injury models (Shahaduzzaman et al., 2013; Tsuji et al., 2014; Womble et al., 2014; Xia et al., 2010). However, the efficacy of each cell type, apart from MSCs, remains limited. In contrast, MSCs have been used in multiple animal studies for treatment of perinatal brain injury, and demonstrate promising results (Ahn et al., 2014; Ahn et al., 2013; Donega et al., 2015; Donega et al., 2013; Jellema et al., 2013; E. S. Kim et al., 2012; Y. E. Kim et al., 2016; J. S. Lee et al., 2010; Lim et al., 2011; C. T. van Velthoven et al., 2012; Van Velthoven et al., 2014; van Velthoven et al., 2010a, 2010b, 2011; Van Velthoven et al., 2013; C. T. J. Van Velthoven et al., 2012; Yasuhara et al., 2008; Yasuhara et al., 2006a; Yasuhara et al., 2006b). Moreover, MSCs are currently under clinical trial [NCT01929434], and the safety of allogeneic MSCs has been confirmed (Reinders et al., 2015). To date there has been no documented clinical advantage of the use of autologous over allogeneic MSCs in a clinical setting, since MSCs are shown to be immune evasive (Ankrum et al., 2014), which may allow allogeneic administration of the cells without MHC matching, and make the clinical application of this therapy feasible in preterm infants. Accordingly, in Chapter 4 we explored the collection and isolation of MSC from preterm ovine UCB, the expansion of these cells, and then whether allogeneic administration of ex-vivo expanded preterm UCB-MSC is effective for preterm brain neuroprotection.

5.6. Neuroprotective benefits of UCB-derived MSCs and other cell types

Results obtained in Chapter 4 demonstrate that preterm UCB-MSC protect white matter development within the preterm ovine brain following HI insult, mediated via inflammatory cell migration into cerebral white matter, systemic anti-inflammatory effects, along with inducing cell proliferation in the white matter. These findings support the use of ex-vivo expanded UCB-MSC as a potential therapeutic intervention in the treatment of preterm brain injury (Ahn et al., 2014; Ahn et al., 2013; Donega et al., 2015; Donega et al., 2013; Jellema et al., 2013; E. S. Kim et al., 2012; Y. E. Kim et al., 2016; J. S. Lee et al., 2010; Lim et al., 2011; C. T. van Velthoven et al., 2012; Van Velthoven et al., 2014; van Velthoven et al., 2010a, 2010b, 2011; Van Velthoven et al., 2013; C. T. J. Van Velthoven et al., 2012; Yasuhara et al., 2008; Yasuhara et al., 2006a; Yasuhara et al., 2006b). However, it is interesting to note that the mechanisms of actions of MSC in white matter protection appears to be different when compared with that of whole white blood cell fraction of UCB, as used in Chapters 2 and 3. In our findings, both MSC and UCB cells were shown to demonstrate anti-inflammatory benefits (Bennet et al., 2012; Li et al., 2014b; Li et al., 2017). However, our result showed that modulation of macrophage activation played a pivotal role in the neuroprotection that was attributed to MSC administration, along with an increase in cell proliferation in white matter and a partial suppression of cerebral microglial activation. In contrast, whole UCB cells mainly suppressed the activation of cerebral microglia, resulting in the protection of white matter brain architecture (Li et al., 2014b; Li et al., 2016).

MSC have been demonstrated to achieve immunomodulation by polarizing the M1 macrophages into a M2 phenotype (considered a switch towards a more reparative cell type), and to regulate injury/repair mechanisms (Mantovani et al., 2013; Selleri et al., 2016; Xu et al., 2017). Although UCB contains MSC, the total of these MSC cell counts is low, particularly in term UCB (Jain et al., 2013), while UCB includes many other cell types. This differential

outcome between MSC and UCB cell administration indicates that other cell types that are present in UCB are also very active and responsible for white matter protection in preterm infants. Indeed, a recent study demonstrated that monocytes within UCB are critical for the suppression of microglia in UCB therapy (Womble et al., 2014). A further recent report showed that human UC-MSC effectively suppressed microglial and T cell activation and reduced white matter injury following HI (Jellema et al., 2013). CD34+ haematopoietic cells have also been shown to reduce brain injury in neonatal hypoxic-ischemic mice, with a transient augmentation of cerebral blood flow in the peri-infarct area (Tsuji et al., 2014). The specific fraction or combination of the cells in UCB that are principally responsible for brain protection, thus, still remains to be determined. In order to construct a better treatment strategy in the most effective and efficient way, further investigation to elucidate the effects of each cells included in UCB is required.

5.6. Questions that remain unanswered

There are multiple ongoing preclinical studies and clinical trials that are testing UCB, or other stem cells, to reduce brain injury in infants with neonatal HIE around the world (Liao et al., 2013; Tsuji et al., 2015). However, there are still many questions that remain unanswered to bring this treatment to clinical application to reduce preterm brain injury.

(1) Are UCB cells, or specific cells derived from UCB, also effective for treatment of preterm brain injury caused by other factors, besides HI? Inflammation/ infection, chronic hypoxia and ischemia, and hyperoxia have been suggested to be major causes of white matter brain injury in preterm infants. Studies to examine the effects of stem cells in such animal models are currently underway by our group and others (Leaw et al., 2017). These studies will allow the examination of UCB-derived cells for different etiological causes of preterm brain injury.

(2) Autologous or allogeneic cell administration? In order to conduct UCB or MSC treatment in

an acute situation where autologous UCB may not be available, or of low volume, cryobanking may be a prerequisite. Great uncertainty exists around the therapeutic value of thawed and expanded cells. An in vitro study that compared the quality of UCB cells before and after cryopreservation showed that there is a slight decrease in the number of nucleated cells, and colony-forming activity after cryopreservation (Kobzeva et al., 2013). Another study also showed that bone marrow derived cryopreserved MSCs have the same growth characteristics as fresh bone marrow-derived MSCs (Kaplan et al., 2017), while almost all previous studies have compared fresh and thawed cells in vitro, but not in vivo. A recent study has also shown that MSCs appear to have compromised immunomodulatory activity directly after thawing when used for clinical application (Moll et al., 2016). Whether fresh or cryopreserved cells could be used for brain protection thus remains to be validated.

(3)   What are the functional benefits of UCB therapy? In all studies in this thesis, I assessed the beneficial effects of UCB cells or MSCs predominantly by histological analyses. However, some preclinical studies in rats and rabbits exposed to perinatal HI have demonstrated the neuroprotective effects of UCB cells via functional recovery (Drobyshevsky et al., 2015; Geissler et al., 2011; Meier et al., 2006). A clinical study has also shown that the beneficial effects of UCB cell administration are evident as a functional improvement in children with cerebral palsy (Min et al., 2013). The mechanisms of actions, and histological correlation, for the functional recovery are unknown. In order to further investigate and characterise the effects of UCB-derived cell therapy in preterm white matter injury, assessment of neuropathology via histology, MRI *and* functional outcomes should be considered.

(4)   What is the optimal dose of UCB cells? A dose–response effect of UCB therapy in a neonatal rat model of HI has been demonstrated (de Paula et al., 2012; Vendrame et al., 2004), and dose-dependent effects of human UCB-MNCs in a rat model of intracerebral hemorrhage also reported (Seghatoleslam et al., 2013). The optimal dose of UCB-MNCs as

well as MSCs for brain protection remains unestablished. A phase 1 dose-escalation clinical trial of MSCs for bronchopulmonary dysplasia has now been completed (Chang et al., 2014) but it is imperative that similar studies should be performed for UCB and MSC therapies in order to move UCB and MSC therapies forward towards a standardised clinical application.

(5) How do UCB cells compare with stem cells from other sources? Stem cells or stem cell-like cells from other source of fetal tissues such as umbilical cord, placenta, amnion and from amniotic fluid may also be neuroprotective for perinatal brain injury. Indeed, intravenous transplantation of human UC-MSCs at an early stage after HIE was shown to improve the behavior of hypoxic–ischemic rats and decrease gliosis (Zhang et al., 2014). Human amnion epithelial cells were also demonstrated to rescue neural cell death via immunomodulation of microglia in a mouse model of LPS/ hyperoxia-induced preterm brain injury (Leaw et al., 2017).

5.7. Safety

Throughout the studies in this thesis, no apparent side effects were observed after administration of allogeneic UCB-derived cells and UCB-MSCs. Although this series of studies examined the effects of the cell administration only on the brain and not on other organs, mortality of fetal sheep did not increase in cell-treated animals. Recent clinical trials have demonstrated that autologous UCB treatment is feasible and safe in term infants with HIE (Cotten et al., 2014) as well as children with cerebral palsy (Y. H. Lee et al., 2012). Another clinical study also showed the efficacy and safety of allogeneic UCB, with concomitant administration of erythropoietin and cyclosporine, in children with established cerebral palsy (Min et al., 2013). As discussed earlier, a recent paper even supports the safety of using non-matched, allogeneic cord blood cells to treat non-hematopoietic degenerative conditions (Yang et al., 2010). The longest follow-up with this protocol was 4 years with no evidence of immune reactivity or GVHD.

Evaluation of therapeutic benefit is currently in progress. With respect to MSCs, hUC–MSCs undergo successful cell expansion using animal serum-free culture medium, thereby removing safety concerns of animal-to-human viral transmission (Ding et al., 2013; Julavijitphong et al., 2014). However, due to their immunomodulatory, and multipotent, reparative properties, transplantation of MSCs potentially holds risks of eliciting an adverse immune response and tumor formation, which have cautioned their use in clinical trials. In general, the tumorigenicity of stem cells is predicted to increase proportionately with the length of time that they are cultured. However, MSCs appear to be maintained in culture for weeks without clear adverse consequences in karyotypic alterations (Knoepfler, 2009). A recent study of the safety of viral transduction of human HSCs and MSCs in which animals were followed for up to 18 months also found no evidence of tumorigenesis (Bauer et al., 2008). Although the safety of autologous and allogeneic MSCs has been confirmed in multiple renal transplant clinical studies (Reinders et al., 2015), these safety issues should be addressed before further studies are planned in children and infants that are followed for long term, preferably using allogeneic MSCs, as these cells offer the advantage of immediate availability for clinical use. Commercial allogeneic MSC products are now approved for the treatment of GVHD, and have been used in humans as an *off-the-shelf* product (Miyamura, 2016), which would allow early intervention for high risk extremely preterm infants in the future. MSCs are currently under clinical trial in children with cerebral palsy [NCT01929434], and infants with severe bronchopulmonary dysplasia [NCT01207869], and the results are awaited with interest.

5.8. Limitations

An important limitation of the studies described in this thesis is that I performed all experiments in utero. The fetuses underwent umbilical cord occlusion (UCO) and then recovered from the insult in utero and therefore in normoxic, well controlled, fetal conditions. By undertaking the UCO in utero, the fetus continues to maintain placental support and perfusion, which likely contributes to recovery. This is quite different to what would occur in human infants born

preterm, in which neonatal care could certainly influence outcome, and the administration of stem cells would occur postnatally. In the current study we administered fetal (UCB) cells into the fetal circulation, which is different to giving UCB cells into the neonatal circulation, as it has been described that white blood cell composition alters significantly within a few days after birth.

Administration of UCB-derived cells are demonstrated to be effective for preterm brain protection after HI in this thesis. However, investigation using other animal models such as rodents that have an immature developmental stage of brain at birth allows the initiation of cell therapy to preterm subjects after birth, which is similar to the situation in human preterm infants, may clear the confounding factor of our fetal experimental study. However, compared to rodents, sheep are one of the most widely accepted and reliable experimental models in preterm brain research, especially when focusing on WMI. Also, unlike immature rodent models, preterm fetal sheep have the advantage of being used for instrumentation to obtain blood samples regularly and physiological data such as blood pressure and heart rate continuously. Moreover, unlike human preterm infants, ovine fetuses are not viable at this stage of gestation outside the uterus. For these reasons, despite the limitations explained above, we chose to use the aforementioned preterm fetal sheep model.

There are currently no treatment options to protect or repair the brain of a baby born preterm with early signs of brain injury. With the knowledge that preterm babies are at great risk of developmental brain injury, it is imperative to extend the investigation of potential cell therapy from studies and clinical trials being performed on term infants and adolescents, through to preterm infants. The developing brain has excellent neuroplasticity and regenerative capacity, which is suggestive that preterm neonates may have a greater potential for therapeutic regeneration and restoration of neuronal and glial cell populations, compared to adults and even term newborn infants (Bennet et al., 2013).

UCB from preterm births is reported to be a rich source of stem and progenitor cells as described in Chapter 3 (Erices et al., 2000; Jain et al., 2013; Javed et al., 2008; M. Yu et al., 2004). The total number of MSC in cord blood decreases as gestation progresses, such that MSC in UCB at 24-28 weeks is 40-fold greater compared to term UCB (Jain et al., 2013; Javed et al., 2008). Not only UCB, but fetal tissues including umbilical cord tissue, amnion, and placenta, are well-known sources of MSCs. Fetuses are developing in an environment rich in stem cells while in the uterus. It might thus be possible that those stem and progenitor cells are playing crucial roles in growth and development of the fetus, especially at more premature stages of fetal development. A precipitous decrease in the circulating levels of stem and progenitor cells, and influence from the stem cells of fetal tissues in babies born preterm may therefore deprive them of their anti-inflammatory/immunomodulatory and nurturing effects. The results from our in utero experiments provided information on whether increasing circulating UCB cells or MSC results in altering the pathophysiological process of brain injury, and protecting the preterm brain following hypoxia-ischemia, and answered the question of whether systemic administration of UCB cells or MSC could be a therapeutic option for repair of preterm WMI. The findings in this thesis strongly suggest that it is possible that UCB cells and MSC play crucial roles in brain protection, repair or growth and development of the preterm brain. I believe that the results derived from this study will greatly enhance our fundamental scientific understanding of the cerebral actions of UCB stem cell treatment in preterm infants, and hope that this will lead to the clinical potential of revealing a new therapeutic intervention with the administration of cord blood derived cells to preterm infants, to reduce the severity and/or incidence of cerebral palsy all around the world in the future.

References

Ahn, S. Y., Chang, Y. S., & Park, W. S. (2014). Mesenchymal stem cells transplantation for neuroprotection in preterm infants with severe intraventricular hemorrhage. *Korean Journal of Pediatrics, 57*(6), 251-256. doi:10.3345/kjp.2014.57.6.251

Ahn, S. Y., Chang, Y. S., Sung, D. K., Sung, S. I., Yoo, H. S., Lee, J. H., Oh, W. I., & Park, W. S. (2013). Mesenchymal stem cells prevent hydrocephalus after severe intraventricular hemorrhage. *Stroke, 44*(2), 497-504.

Ankrum, J. A., Ong, J. F., & Karp, J. M. (2014). Mesenchymal stem cells: immune evasive, not immune privileged. *Nat Biotech, 32*(3), 252-260. doi:10.1038/nbt.2816

Barkovich, A. J., & Truwit, C. L. (1990). Brain damage from perinatal asphyxia: Correlation of MR findings with gestational age. *American Journal of Neuroradiology, 11*(6), 1087-1096.

Bauer, G., Dao, M. A., Case, S. S., Meyerrose, T., Wirthlin, L., Zhou, P., Wang, X., Herrbrich, P., Arevalo, J., Csik, S., Skelton, D. C., Walker, J., Pepper, K., Kohn, D. B., & Nolta, J. A. (2008). In vivo biosafety model to assess the risk of adverse events from retroviral and lentiviral vectors. *Mol Ther, 16*(7), 1308-1315. doi:10.1038/mt.2008.93

Bennet, L., Roelfsema, V., George, S., Dean, J. M., Emerald, B. S., & Gunn, A. J. (2007). The effect of cerebral hypothermia on white and grey matter injury induced by severe hypoxia in preterm fetal sheep. *Journal of Physiology, 578*(2), 491-506.

Bennet, L., Tan, S., Van Den Heuij, L., Derrick, M., Groenendaal, F., Van Bel, F., Juul, S., Back, S. A., Northington, F., Robertson, N. J., Mallard, C., & Gunn, A. J. (2012). Cell therapy for neonatal hypoxia-ischemia and cerebral palsy. *Annals of Neurology, 71*(5), 589-600.

Bennet, L., Van Den Heuij, L., Dean, J. M., Drury, P., Wassink, G., & Gunn, A. J. (2013). Neural plasticity and the Kennard principle: Does it work for the preterm brain? *Clin Exp Pharmacol Physiol, 40*(11), 774-784. doi:10.1111/1440-1681.12135

Boltze, J., Schmidt, U. R., Reich, D. M., Kranz, A., Reymann, K. G., Strassburger, M., Lobsien, D., Wagner, D. C., Förschler, A., & Schäbitz, W. (2012). Determination of the Therapeutic Time Window for Human Umbilical Cord Blood Mononuclear Cell Transplantation Following Experimental Stroke in Rats. *Cell Transplantation, 21*(6), 1199-1211.

Chang, Y. S., Ahn, S. Y., Yoo, H. S., Sung, S. I., Choi, S. J., Oh, W. I., & Park, W. S. (2014). Mesenchymal Stem Cells for Bronchopulmonary Dysplasia: Phase 1 Dose-Escalation Clinical Trial. *Journal of Pediatrics, 164*(5), 966-972.e966.

Cotten, C. M., Murtha, A. P., Goldberg, R. N., Grotegut, C. A., Smith, P. B., Goldstein, R. F., Fisher, K. A., Gustafson, K. E., Waters-Pick, B., Swamy, G. K., Rattray, B., Tan, S., & Kurtzberg, J. (2014). Feasibility of autologous cord blood cells for infants with hypoxic-ischemic encephalopathy. *Journal of Pediatrics, 164*(5), 973-979.e971.

de Paula, S., Greggio, S., Marinowic, D. R., Machado, D. C., & DaCosta, J. C. (2012). 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, 210*, 431-441.

Ding, Y., Yang, H., Feng, J. B., Qiu, Y., Li, D. S., & Zeng, Y. (2013). Human umbilical cord-derived MSC culture: The replacement of animal sera with human cord blood plasma. *In Vitro Cellular and Developmental Biology - Animal, 49*(10), 771-777.

Donega, V., Nijboer, C. H., Van Velthoven, C. T. J., Youssef, S. A., De Bruin, A., Van Bel, F., Kavelaars, A., & Heijnen, C. J. (2015). Assessment of long-term safety and efficacy of intranasal mesenchymal stem cell treatment for neonatal brain injury in the mouse. *Pediatric Research, 78*(5), 520-526. doi:10.1038/pr.2015.145

Donega, V., van Velthoven, C. T. J., Nijboer, C. H., van Bel, F., Kas, M. J. H., Kavelaars, A., & Heijnen, C. J. (2013). Intranasal Mesenchymal Stem Cell Treatment for Neonatal Brain Damage: Long-Term Cognitive and Sensorimotor Improvement. *PLoS ONE, 8*(1).

Drobyshevsky, A., Cotten, C. M., Shi, Z., Luo, K., Jiang, R., Derrick, M., Tracy, E. T., Gentry, T., Goldberg, R. N., Kurtzberg, J., & Tan, S. (2015). Human Umbilical Cord Blood Cells Ameliorate Motor Deficits in Rabbits in a Cerebral Palsy Model. *Developmental Neuroscience, 37*(4-5), 349-362. doi:10.1159/000374107

Erices, A., Conget, P., & Minguell, J. J. (2000). Mesenchymal progenitor cells in human umbilical cord blood. *British Journal of Haematology, 109*(1), 235-242.

Geissler, M., Dinse, H. R., Neuhoff, S., Kreikemeier, K., & Meier, C. (2011). Human umbilical cord blood cells restore brain damage induced changes in rat somatosensory cortex. *PLoS One, 6*(6), e20194. doi:10.1371/journal.pone.0020194

Ghavam, S., Batra, D., Mercer, J., Kugelman, A., Hosono, S., Oh, W., Rabe, H., & Kirpalani, H. (2014). Effects of placental transfusion in extremely low birthweight infants: Meta-analysis of long- and short-term outcomes. *Transfusion, 54*(4), 1192-1198.

Gopagondanahalli, K. R., Li, J., Fahey, M. C., Hunt, R. W., Jenkin, G., Miller, S. L., & Malhotra, A. (2016). Preterm Hypoxic–Ischemic Encephalopathy. *Front Pediatr, 4*(114). doi:10.3389/fped.2016.00114

Hosono, S., Hine, K., Nagano, N., Taguchi, Y., Yoshikawa, K., Okada, T., Mugishima, H., Takahashi, S., & Takahashi, S. (2015). Residual blood volume in the umbilical cord of

extremely premature infants. *Pediatrics International, 57*(1), 68-71. doi:10.1111/ped.12464

Jain, A., Mathur, N., Jeevashankar, M., Mukhopadhyay, A., Agarwal, R., Deorari, A. K., & Paul, V. K. (2013). Does mesenchymal stem cell population in umbilical cord blood vary at different gestational periods? *Indian Journal of Pediatrics, 80*(5), 375-379.

Javed, M. J., Mead, L. E., Prater, D., Bessler, W. K., Foster, D., Case, J., Goebel, W. S., Yoder, M. C., Haneline, L. S., & Ingram, D. A. (2008). Endothelial colony forming cells and mesenchymal stem cells are enriched at different gestational ages in human umbilical cord blood. *Pediatric Research, 64*(1), 68-73.

Jellema, R. K., Wolfs, T. G. A. M., Lima Passos, V., Zwanenburg, A., Ophelders, D. R. M. G., Kuypers, E., Hopman, A. H. N., Dudink, J., Steinbusch, H. W., Andriessen, P., Germeraad, W. T. V., Vanderlocht, J., & Kramer, B. W. (2013). Mesenchymal Stem Cells Induce T-Cell Tolerance and Protect the Preterm Brain after Global Hypoxia-Ischemia. *PLoS ONE, 8*(8). doi:10.1371/journal.pone.0073031

Julavijitphong, S., Wichitwiengrat, S., Tirawanchai, N., Ruangvutilert, P., Vantanasiri, C., & Phermthai, T. (2014). A xeno-free culture method that enhances Wharton's jelly mesenchymal stromal cell culture efficiency over traditional animal serum-supplemented cultures. *Cytotherapy, 16*(5), 683-691.

Kaplan, A., Sackett, K., Sumstad, D., Kadidlo, D., & McKenna, D. H. (2017). Impact of starting material (fresh versus cryopreserved marrow) on mesenchymal stem cell culture. *Transfusion, 57*(9), 2216-2219. doi:10.1111/trf.14192

Keogh, M. J., Drury, P. P., Bennet, L., Davidson, J. O., Mathai, S., Gunn, E. R., Booth, L. C., & Gunn, A. J. (2012). Limited predictive value of early changes in EEG spectral power for neural injury after asphyxia in preterm fetal sheep. *Pediatric Research, 71*(4), 345-353.

Kim, E. S., Ahn, S. Y., Im, G. H., Sung, D. K., Park, Y. R., Choi, S. H., Choi, S. J., Chang, Y. S., Oh, W., Lee, J. H., & Park, W. S. (2012). Human umbilical cord blood-derived mesenchymal stem cell transplantation attenuates severe brain injury by permanent middle cerebral artery occlusion in newborn rats. *Pediatric Research, 72*(3), 277-284.

Kim, Y. E., Park, W. S., Sung, D. K., Ahn, S. Y., Sung, S. I., Yoo, H. S., & Chang, Y. S. (2016). Intratracheal transplantation of mesenchymal stem cells simultaneously attenuates both lung and brain injuries in hyperoxic newborn rats. *Pediatric Research, 80*(3), 415-424. doi:10.1038/pr.2016.88

Knoepfler, P. S. (2009). Deconstructing stem cell tumorigenicity: A roadmap to safe regenerative medicine. *Stem Cells, 27*(5), 1050-1056.

Kobzeva, I. V., Astrelina, T. A., Yakovleva, M. V., Karpova, E. E., Kruglova, Y. A., Boyakova, E. V., Shachpazyan, N. R., & Gomzyakov, A. E. (2013). Evaluation of the quality of cryopreserved umbilical cord blood before and after cryostorage bioarchive® in stem cell bank of moscow. *Cellular Transplantation and Tissue Engineering, 8*(1), 19-23.

Leaw, B., Zhu, D., Tan, J., Muljadi, R., Saad, M. I., Mockler, J. C., Wallace, E. M., Lim, R., & Tolcos, M. (2017). Human amnion epithelial cells rescue cell death via immunomodulation of microglia in a mouse model of perinatal brain injury. *Stem Cell Res Ther, 8*(1), 46. doi:10.1186/s13287-017-0496-3

Lee, J. S., Hong, J. M., Moon, G. J., Lee, P. H., Ahn, Y. H., & Bang, O. Y. (2010). Autologous mesenchymal stem cell transplantation in stroke patients. *Stem Cells, 28*. doi:10.1002/stem.430

Lee, Y. H., Choi, K. V., Moon, J. H., Jun, H. J., Kang, H. R., Oh, S. I., Kim, H. S., Um, J. S., Kim, M. J., Choi, Y. Y., Lee, Y. J., Kim, H. J., Lee, J. H., Son, S. M., Choi, S. J., Oh, W., & Yang, Y. S. (2012). Safety and feasibility of countering neurological impairment by intravenous administration of autologous cord blood in cerebral palsy. *Journal of Translational Medicine, 10*(1).

Li, J., Funato, M., Tamai, H., Wada, H., Nishihara, M., Iwamoto, H., Okazaki, Y., & Shintaku, H. (2013). Predictors of neurological outcome in cooled neonates. *Pediatrics International, 55*(2), 169-176.

Li, J., Kobata, K., Kamei, Y., Okazaki, Y., Nishihara, M., Wada, H., Tamai, H., Funato, M., & Jenkin, G. (2014a). Nucleated red blood cell counts: An early predictor of brain injury and 2-year outcome in neonates with hypoxic-ischemic encephalopathy in the era of cooling-based treatment. *Brain and Development, 36*(6), 472-478.

Li, J., McDonald, C. A., Fahey, M. C., Jenkin, G., & Miller, S. L. (2014b). Could cord blood cell therapy reduce preterm brain injury? *Frontiers in Neurology, 5*(200), 1-17. doi:10.3389/fneur.2014.00200

Li, J., Yawno, T., Sutherland, A., Loose, J., Nitsos, I., Allison, B. J., Bischof, R., McDonald, C. A., Jenkin, G., & Miller, S. L. (2017). Term versus preterm cord blood cells for the prevention of preterm brain injury. *Pediatr Res*. doi:10.1038/pr.2017.170

Li, J., Yawno, T., Sutherland, A., Loose, J., Nitsos, I., Bischof, R., Castillo-Melendez, M., McDonald, C. A., Wong, F. Y., Jenkin, G., & Miller, S. L. (2016). Preterm white matter brain injury is prevented by early administration of umbilical cord blood cells. *Exp Neurol, 283*(Pt A), 179-187. doi:10.1016/j.expneurol.2016.06.017

Liao, Y., Cotten, M., Tan, S., Kurtzberg, J., & Cairo, M. S. (2013). Rescuing the neonatal brain from hypoxic injury with autologous cord blood. *Bone marrow transplantation, 48*(7), 890-900.

Lim, J. Y., Jeong, C. H., Jun, J. A., Kim, S. M., Ryu, C. H., Hou, Y., Oh, W., Chang, J. W., & Jeun, S.-S. (2011). Therapeutic effects of human umbilical cord blood-derived mesenchymal stem cells after intrathecal administration by lumbar puncture in a rat model of cerebral ischemia. *Stem Cell Research & Therapy, 2*(5), 38. doi:10.1186/scrt79

Logitharajah, P., Rutherford, M. A., & Cowan, F. M. (2009). Hypoxic-ischemic encephalopathy in preterm infants: Antecedent factors, brain imaging, and outcome. *Pediatric Research, 66*(2), 222-229. doi:10.1203/PDR.0b013e3181a9ef34

Mantovani, A., Biswas, S. K., Galdiero, M. R., Sica, A., & Locati, M. (2013). Macrophage plasticity and polarization in tissue repair and remodelling. *Journal of Pathology, 229*(2), 176-185. doi:10.1002/path.4133

Meier, C., Middelanis, J., Wasielewski, B., Neuhoff, S., Roth-Haerer, A., Gantert, M., Dinse, H. R., Dermietzel, R., & Jensen, A. (2006). Spastic paresis after perinatal brain damage in rats is reduced by human cord blood mononuclear cells. *Pediatric Research, 59*(2), 244-249.

Miller, S. P., Ferriero, D. M., Leonard, C., Piecuch, R., Glidden, D. V., Partridge, J. C., Perez, M., Mukherjee, P., Vigneron, D. B., & Barkovich, A. J. (2005). Early brain injury in premature newborns detected with magnetic resonance imaging is associated with adverse early neurodevelopmental outcome. *Journal of Pediatrics, 147*(5), 609-616.

Min, K., Song, J., Kang, J. Y., Ko, J., Ryu, J. S., Kang, M. S., Jang, S. J., Kim, S. H., Oh, D., Kim, M. K., Kim, S. S., & Kim, M. (2013). Umbilical cord blood therapy potentiated with erythropoietin for children with cerebral palsy: A double-blind, randomized, placebo-controlled trial. *Stem Cells, 31*(3), 581-591.

Miyamura, K. (2016). Insurance approval of mesenchymal stem cell for acute GVHD in Japan: need of follow up for some remaining concerns. *Int J Hematol, 103*(2), 155-164. doi:10.1007/s12185-015-1930-x

Moll, G., Geissler, S., Catar, R., Ignatowicz, L., Hoogduijn, M. J., Strunk, D., Bieback, K., & Ringden, O. (2016). Cryopreserved or Fresh Mesenchymal Stromal Cells: Only a Matter of Taste or Key to Unleash the Full Clinical Potential of MSC Therapy? *Adv Exp Med Biol, 951*, 77-98. doi:10.1007/978-3-319-45457-3_7

Paton, M. C. B., McDonald, C. A., Allison, B. J., Fahey, M. C., Jenkin, G., & Miller, S. L. (2017). Perinatal Brain Injury As a Consequence of Preterm Birth and Intrauterine Inflammation: Designing Targeted Stem Cell Therapies. *Frontiers in Neuroscience, 11*(200). doi:10.3389/fnins.2017.00200

Pharoah, P. O. D., Platt, M. J., & Cooke, T. (1996). The changing epidemiology of cerebral palsy. *Archives of Disease in Childhood: Fetal and Neonatal Edition, 75*(3), F169-F173.

Reinders, M. E., Dreyer, G. J., Bank, J. R., Roelofs, H., Heidt, S., Roelen, D. L., Zandvliet, M. L., Huurman, V. A., Fibbe, W. E., van Kooten, C., Claas, F. H., Rabelink, T. J., & de Fijter, J. W. (2015). Safety of allogeneic bone marrow derived mesenchymal stromal cell therapy in renal transplant recipients: the neptune study. *J Transl Med, 13*, 344. doi:10.1186/s12967-015-0700-0

Rudnicki, J., Kawa, M. P., Kotowski, M., Michalczyk, B., Ustianowski, P., Czajka, R., & Machalinski, B. (2015). Clinical Evaluation of the Safety and Feasibility of Whole Autologous Cord Blood Transplant as a Source of Stem and Progenitor Cells for Extremely Premature Neonates: Preliminary Report. *Exp Clin Transplant, 13*(6), 563-572.

Seghatoleslam, M., Jalali, M., Nikravesh, M. R., Hamidi Alamdari, D., Hosseini, M., & Fazel, A. (2013). Intravenous administration of human umbilical cord blood-mononuclear cells dose-dependently relieve neurologic deficits in rat intracerebral hemorrhage model. *Annals of Anatomy, 195*(1), 39-49. doi:10.1016/j.aanat.2012.05.002

Selleri, S., Bifsha, P., Civini, S., Pacelli, C., Dieng, M. M., Lemieux, W., Jin, P., Bazin, R., Patey, N., Marincola, F. M., Moldovan, F., Zaouter, C., Trudeau, L. E., Benabdhalla, B., Louis, I., Beausejour, C., Stroncek, D., Le Deist, F., & Haddad, E. (2016). Human mesenchymal stromal cell-secreted lactate induces M2-macrophage differentiation by metabolic reprogramming. *Oncotarget, 7*(21), 30193-30210. doi:10.18632/oncotarget.8623

Shahaduzzaman, M., Golden, J. E., Green, S., Gronda, A. E., Adrien, E., Ahmed, A., Sanberg, P. R., Bickford, P. C., Gemma, C., & Willing, A. E. (2013). A single administration of human umbilical cord blood T cells produces long-lasting effects in the aging hippocampus. *Age, 35*(6), 2071-2087.

Sie, L. T., van der Knaap, M. S., Oosting, J., de Vries, L. S., Lafeber, H. N., & Valk, J. (2000). MR patterns of hypoxic-ischemic brain damage after prenatal, perinatal or postnatal asphyxia. *Neuropediatrics, 31*(3), 128-136. doi:10.1055/s-2000-7496

Surbek, D. V., Glanzmann, R., Senn, H. P., Hoesli, I., & Holzgreve, W. (2000). Can cord blood be used for autologous transfusion in preterm neonates? *European Journal of Pediatrics, 159*(10), 790-791.

Thorngren-Jerneck, K., & Herbst, A. (2006). Perinatal factors associated with cerebral palsy in children born in Sweden. *Obstetrics and Gynecology, 108*(6), 1499-1505.

Tsuji, M., & Johnston, M. V. (2015). Cell-based therapies in neonatal stroke *Cell Therapy for Brain Injury* (pp. 321-349).

Tsuji, M., Taguchi, A., Ohshima, M., Kasahara, Y., Sato, Y., Tsuda, H., Otani, K., Yamahara, K., Ihara, M., Harada-Shiba, M., Ikeda, T., & Matsuyama, T. (2014). Effects of intravenous administration of umbilical cord blood CD34+ cells in a mouse model of neonatal stroke. *Neuroscience, 263*, 148-158.

van den Heuij, L. G., Fraser, M., Miller, S. L., Jenkin, G., Wallace, E. M., Davidson, J. O., Lear, C. A., Lim, R., Wassink, G., Gunn, A. J., & Bennet, L. (2017). Delayed intranasal infusion of human amnion epithelial cells improves white matter maturation after asphyxia in preterm fetal sheep. *J Cereb Blood Flow Metab*, 271678x17729954. doi:10.1177/0271678x17729954

van Velthoven, C. T., Kavelaars, A., & Heijnen, C. J. (2012). Mesenchymal stem cells as a treatment for neonatal ischemic brain damage. *Pediatric Research, 71*(4 Pt 2), 474-481.

Van Velthoven, C. T. J., Braccioli, L., Willemen, H. L. D. M., Kavelaars, A., & Heijnen, C. J. (2014). Therapeutic potential of genetically modified mesenchymal stem cells after neonatal hypoxic-ischemic brain damage. *Molecular Therapy, 22*(3), 645-654. doi:10.1038/mt.2013.260

van Velthoven, C. T. J., Kavelaars, A., van Bel, F., & Heijnen, C. J. (2010a). Mesenchymal stem cell treatment after neonatal hypoxic-ischemic brain injury improves behavioral outcome and induces neuronal and oligodendrocyte regeneration. *Brain, Behavior, and Immunity, 24*(3), 387-393. doi:10.1016/j.bbi.2009.10.017

Van Velthoven, C. T. J., Kavelaars, A., Van Bel, F., & Heijnen, C. J. (2010b). Repeated mesenchymal stem cell treatment after neonatal hypoxia-ischemia has distinct effects on formation and maturation of new neurons and oligodendrocytes leading to restoration of damage, corticospinal motor tract activity, and sensorimotor function. *Journal of Neuroscience, 30*(28), 9603-9611. doi:10.1523/JNEUROSCI.1835-10.2010

Van Velthoven, C. T. J., Kavelaars, A., Van Bel, F., & Heijnen, C. J. (2011). Mesenchymal stem cell transplantation changes the gene expression profile of the neonatal ischemic brain. *Brain, Behavior, and Immunity, 25*(7), 1342-1348. doi:10.1016/j.bbi.2011.03.021

Van Velthoven, C. T. J., Sheldon, R. A., Kavelaars, A., Derugin, N., Vexler, Z. S., Willemen, H. L. D. M., Maas, M., Heijnen, C. J., & Ferriero, D. M. (2013). Mesenchymal stem cell transplantation attenuates brain injury after neonatal stroke. *Stroke, 44*(5), 1426-1432.

Van Velthoven, C. T. J., Van De Looij, Y., Kavelaars, A., Zijlstra, J., Van Bel, F., Huppi, P. S., Sizonenko, S., & Heijnen, C. J. (2012). Mesenchymal stem cells restore cortical rewiring after neonatal ischemia in mice. *Annals of Neurology, 71*(6), 785-796.

Vendrame, M., Cassady, J., Newcomb, J., Butler, T., Pennypacker, K. R., Zigova, T., Sanberg, C. D., Sanberg, P. R., & Willing, A. E. (2004). Infusion of human umbilical cord blood cells in a rat model of stroke dose-dependently rescues behavioral deficits and reduces infarct volume. *Stroke, 35*(10), 2390-2395.

Volpe, J. J. (1998). Brain injury in the premature infant: Overview of clinical aspects, neuropathology, and pathogenesis. *Seminars in Pediatric Neurology, 5*(3), 135-151.

Volpe, J. J. (2008). Neurology of the Newborn: Elsevier Health Sciences.

Womble, T. A., Green, S., Shahaduzzaman, M., Grieco, J., Sanberg, P. R., Pennypacker, K. R., & Willing, A. E. (2014). Monocytes are essential for the neuroprotective effect of human cord blood cells following middle cerebral artery occlusion in rat. *Molecular and Cellular Neuroscience, 59*, 76-84.

Xia, G., Hong, X., Chen, X., Lan, F., Zhang, G., & Liao, L. (2010). Intracerebral transplantation of mesenchymal stem cells derived from human umbilical cord blood alleviates hypoxic ischemic brain injury in rat neonates. *Journal of Perinatal Medicine, 38*(2), 215-221.

Xu, C., Fu, F., Li, X., & Zhang, S. (2017). Mesenchymal stem cells maintain the microenvironment of central nervous system by regulating the polarization of macrophages/microglia after traumatic brain injury. *Int J Neurosci*, 1-12. doi:10.1080/00207454.2017.1325884

Yang, W. Z., Zhang, Y., Wu, F., Min, W. P., Minev, B., Zhang, M., Luo, X. L., Ramos, F., Ichim, T. E., Riordan, N. H., & Hu, X. (2010). Safety evaluation of allogeneic umbilical cord blood mononuclear cell therapy for degenerative conditions. *J Transl Med, 75*.

Yasuhara, T., Hara, K., Maki, M., Mays, R. W., Deans, R. J., Hess, D. C., Carroll, J. E., & Borlongan, C. V. (2008). Intravenous grafts recapitulate the neurorestoration afforded by intracerebrally delivered multipotent adult progenitor cells *Journal of Cerebral Blood Flow and Metabolism, 28*(11), 1804-1810.

Yasuhara, T., Matsukawa, N., Yu, G., Xu, L., Mays, R. W., Kovach, J., Deans, R., Hess, D. C., Carroll, J. E., & Borlongan, C. V. (2006a). Transplantation of cryopreserved human bone marrow-derived multipotent adult progenitor cells for neonatal hypoxic-ischemic injury: Targeting the hippocampus. *Reviews in the Neurosciences, 17*(1-2), 215-225.

Yasuhara, T., Matsukawa, N., Yu, G., Xu, L., Mays, R. W., Kovach, J., Deans, R. J., Hess, D. C., Carroll, J. E., & Borlongan, C. V. (2006b). Behavioral and histological characterization of intrahippocampal grafts of human bone marrow-derived multipotent progenitor cells in neonatal rats with hypoxic-ischemic injury. *Cell Transplantation, 15*(3), 231-238.

Yawno, T., Sabaretnam, T., Li, J., McDonald, C., Lim, R., Jenkin, G., Wallace, E. M., & Miller, S. L. (2017). Human Amnion Epithelial Cells Protect Against White Matter Brain

Injury After Repeated Endotoxin Exposure in the Preterm Ovine Fetus. *Cell Transplant, 26*(4), 541-553. doi:10.3727/096368916x693572

Yawno, T., Schuilwerve, J., Moss, T. J. M., Vosdoganes, P., Westover, A. J., Afandi, E., Jenkin, G., Wallace, E. M., & Miller, S. L. (2013). Human amnion epithelial cells reduce fetal brain injury in response to intrauterine inflammation. *Developmental Neuroscience, 35*(2-3), 272-282.

Yu, G., Borlongan, C. V., Stahl, C. E., Hess, D. C., Ou, Y., Kaneko, Y., Yu, S. J., Yang, T., Fang, L., & Xie, X. (2009). Systemic delivery of umbilical cord blood cells for stroke therapy: A review. *Restorative Neurology and Neuroscience, 27*(1), 41-54. doi:10.3233/RNN-2009-0460

Yu, M., Xiao, Z., Shen, L., & Li, L. (2004). Mid-trimester fetal blood-derived adherent cells share characteristics similar to mesenchymal stem cells but full-term umbilical cord blood does not. *British Journal of Haematology, 124*(5), 666-675.

Zhang, X., Zhang, Q., Li, W., Nie, D., Chen, W., Xu, C., Yi, X., Shi, J., Tian, M., Qin, J., Jin, G., & Tu, W. (2014). Therapeutic effect of human umbilical cord mesenchymal stem cells on neonatal rat hypoxic-ischemic encephalopathy. *Journal of Neuroscience Research, 92*(1), 35-45.