Novel strategies for early detection and treatment of fetal growth restriction related brain injury

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Acknowledgements

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and meetings. Many of my successful collaborations have been as a result of this networking mantra. He is also a top-notch physiologist and stem cell scientist, and has been instrumental in translating some of my science work to the clinic. I hope you are not planning to completely retire anytime soon, Jenks!

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I hope my son, Avi who used to think I am a baby sitter (neonatologists do look after babies!), can now qualify what his dad does just a little bit better when someone asks him next time!

My biggest inspiration is my wife, Dr Arunaz Kumar. Arunaz is an obstetrician and gynaecologist, and till recently was also the coordinator of student teaching for Women's Health at Monash University. She completed her PhD last year, writing up 8 papers during that time, mostly starting her scientific writing after 11pm at night. If I can match 50% of Arunaz's energy and capacity for academic work despite a crazy clinical workload, I am sure I will be on track to be a very successful clinician scientist.

I dedicate this thesis to my late father, Mr K.K Malhotra and my late grandfather, Mr R.N. Malhotra who taught me two things: Work really hard, especially while you are young and able; and Always be a good human being! I hope I can continue to make them proud. Lastly, I say this to my mother, Mrs Shashi Malhotra – your courage and prayers are the reason I have achieved what I have so far! Love you, mum!

Declarations

In accordance with Monash University Doctorate Regulation, 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 review papers and three original science papers either published or under review in peer reviewed journals. The core theme of the thesis is "early detection and early treatment of FGR related 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 Department of Paediatrics, Monash University, under the supervision of Suzanne Miller, Graham Jenkin and Margie Castillo-Melendez.

Co-authors are included in all published work to acknowledge active collaboration between researchers and the nature of team-based research. Sections are renumbered and collected to generate a consistent presentation within this thesis.

Thesis Chapter	**Publication Title**	**Status**	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution***	**Co-authors Monash student Y/N***
1	Neonatal morbidities of fetal growth restriction: pathophysiology and impact.	Published: Front Endocrinol 2019; 10: 55.	Concept, literature review, analysis and manuscript preparation, 70%	Allison BJ, literature review, manuscript, 20% Castillo-Melendez M, manuscript, 2% Jenkin G, manuscript, 2% Polglase GR, manuscript, 2% Miller SL, concept, manuscript, 4%	N

2	Neuropathology as a consequence of neonatal ventilation in premature growth restricted lambs.	Published: Am J Physiol Regul Integr Comp Physiol 2018 Dec; 315(6): R1183-1194.	Concept, experiment, analysis and manuscript preparation, 64%	Castillo-Melendez M, histology, manuscript, 10% Allison BJ, analysis, 2% Sutherland AE, animal surgery, 2% Nitsos I, animal surgery, 2% Pham Y, manuscript, 2% Alves de Alencar Rocha AK, manuscript, 2% Fahey MC, manuscript, 2% Polglase GR, manuscript, 2% Jenkin G, manuscript, 2% Miller SL, concept, experiment, manuscript, 10%	N
3	Neurovascular effects of umbilical cord blood cell therapy in growth restricted newborn lambs.	Under peer review: Stem Cell Research and Therapy	Concept, experiment, analysis and manuscript preparation, 64%	Castillo-Melendez M, histology, manuscript, 10% Allison BJ, analysis, 2% Sutherland AE, animal surgery, 2% Nitsos I, animal surgery, 2% Pham Y, manuscript, 2% McDonald CA, manuscript, 2% Fahey MC, manuscript, 2% Polglase GR, manuscript, 2% Jenkin G, manuscript, 2% Miller SL, manuscript, 10%	N
4	Detection and assessment of brain injury in the growth restricted fetus and neonate	Published: Pediatr Res. 2017 Aug; 82(2): 184-193.	Concept, literature review, analysis and manuscript preparation, 74%	Ditchfield M, manuscript, 2% Fahey MC, manuscript, 2% Castillo-Melendez M, manuscript, 2% Polglase GR, manuscript, 2% Allison BJ, manuscript, 2% Wallace EM, manuscript,	N

				2% Hodges R, manuscript, 2% Jenkin G, manuscript, 2% Miller SL, manuscript, 10%	
5	Advanced MRI analysis to detect white matter brain injury in growth restricted newborn lambs.	Published: Neuroimage Clin. 2019 Aug 23;24:101991	Concept, experiment, analysis and manuscript preparation, 63%	Sepehrizadeh T, MRI analysis, manuscript, 10% Dhollander T, MRI analysis, 2% Wright D, MRI analysis, 2% Castillo-Melendez M, histology, 2% Ditchfield M, MRI analysis, 2% Sutherland AE, animal surgery, 2% Pham Y, animal surgery, 2% Polglase GR, experiment, 2% De Veer M, experiment, 2% Jenkin G, animal surgery, 2% Pannek K, MRI analysis, 2% Shishegar R, MRI analysis, 2% Miller SL, concept, experiment, manuscript, 5%	N

**If no co-authors, leave fields blank*

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Thesis summary

Fetal growth restriction (FGR) is an important and relatively common complication of pregnancy, describing a fetus that does not grow to full potential due to suboptimal placental function. FGR affects up to 10% of pregnancies, and is a leading cause of perinatal mortality and morbidity. FGR is also highly associated with long-term risk of neurodevelopmental deficits, which may be influenced by the timing of onset of placental insufficiency, severity of FGR, and gestation at birth. The detection and assessment of FGR related brain injury is complex and is not always amenable to conventional imaging. For my original science research contribution to this PhD thesis, I investigated the interaction of neonatal ventilation and FGR on brain injury using a well-established large animal (sheep) model of placental insufficiency and FGR. Next, I evaluated the effects of early umbilical cord blood cell therapy on brain injury in preterm FGR lambs. Finally, I evaluated the feasibility and role of novel, advanced brain imaging analysis techniques for detection of brain injury in newborn FGR lambs.

Chapter 1 comprises a published review article describing common neonatal morbidities of FGR, which sets the scene for experimental studies to improve outcomes in these infants. This is a comprehensive review of the perinatal morbidities caused by placental insufficiency, and the underlying pathophysiology associated with the in-utero and postnatal consequences of FGR. I have also focused on the clinical presentation and complications associated with specific neonatal organ morbidities, and discussed their long-term impact. Finally, I discussed targeted novel therapies and interventions, and their mode(s) of actions in specific organ systems in FGR offspring.

Chapter 2 is a published, original science research article that examines whether neonatal ventilation contributes to neuropathology in preterm growth restricted offspring (lambs). We used an established early-onset placental insufficiency and FGR animal model, undertaken in one of twins in pregnant sheep. Lambs were delivered at mid-to-late preterm age and ventilated for 24 hours before euthanasia for brain collection. Brain pathology was examined using standard histology and immunohistochemical techniques. We found that neonatal ventilation exacerbated neuropathology in FGR lambs, as evidenced by increased brain bleeds and infiltration of inflammatory cells adjacent to the blood vessels, most notable in the white matter region of the brain. We noted that the likely mechanisms of brain injury in ventilated FGR lambs included an increase in microglial activation, astrogliosis, oxidative stress and increased blood brain barrier permeability. In turn, this led to region-specific upregulation of programmed cell death in FGR brains. This provides critical evidence that infants born small due to FGR are a specific and particularly vulnerable cohort of preterm babies who might have increased care needs than infants born preterm but appropriately-grown.

Chapter 3 is a published, original science research article that examines the effects of umbilical cord blood (UCB) stem cell therapy on neuropathology in the ventilated preterm FGR lamb, following on from the results of experiments included in chapter 2. I focused, particularly, on the neurovascular unit of the brain in this paper, given most of the effects seen in chapter 2 involved the constellation of cells and structures that form the neurovascular unit in the FGR brain. First, I described the haemodynamic effects of UCB cell therapy on systemic blood pressure, carotid blood flow and cerebrovascular resistance. UCB cell administration at 1 hour after birth was associated with decreased cerebrovascular resistance in FGR lambs. I hypothesised that the mechanisms underpinning this could be related to stabilisation of the neurovascular unit. UCB cells decreased cerebral microglial activation, oxidative stress and stabilised the neurovascular

unit by acting on its various component cells. These positive effects of UCB within the preterm neonatal FGR brain may provide longer term neuroprotective benefit to developing white and grey matter.

Given the spectrum of neuropathology associated with FGR, from subtle differences in white matter development through to more profound brain bleeds and lesions, I next explored the detection of neuropathology in FGR infants. Chapter 4 is a published review article describing the current status and utility of tools and techniques for the detection and assessment of fetal growth restriction related brain injury in the fetus and newborn. I divided the review into methods available for application in the fetal/ antenatal period, and those available in the postnatal/ neonatal period. Antenatal tools for detection of abnormal brain development include fetal ultrasound (including Dopplers), and MRI, while postnatal tools include cranial ultrasound, MRI, and general movements assessment. An appraisal of each available tool is provided and the current status of each technique is discussed.

The final chapter (Chapter 5) is a proof of concept, original research article describing the role of advanced MRI techniques in the detection of FGR related brain injury in preterm newborn lambs, recently submitted for peer-review. Using our ovine model of FGR, we delivered lambs preterm, and newborn lambs had brain imaging performed soon after birth in a 3T MRI scanner to evaluate the use of conventional MRI, MR spectroscopy, voxel based analysis and fixel-based analysis for detection of neonatal brain injury. The rationale was that early detection of brain injury, whether subtle or severe, would provide the basis for early administration of neuroreparative treatments, such as UCB stem cells. Results show that there were no significant differences between groups with respect to conventional MRI, spectroscopy or voxel based imaging. There were, however, significant fixel-based analysis differences seen in cerebral white

matter, hippocampus and cerebellar regions of the brain in FGR versus appropriately-grown preterm lambs, indicative that the developmental profile of specific tracts within the brains of the FGR cohort are altered during in-utero development. For the first time, the subtle brain injury seen on MRI was confirmed on histological analysis.

In summary, using a lamb model of FGR I studied the consequences of neonatal ventilation on neuropathology, and then evaluated the neurovascular effects of umbilical cord blood cell therapy. I also explored the feasibility and role of advanced MRI analysis techniques to study FGR related brain injury in the newborn. I believe the body of work included in this thesis provides new knowledge based on strong pre-clinical scientific evidence to show that preterm FGR infants are a specific cohort with increased vulnerability to brain injury, that is contributed by suboptimal antenatal brain development, together with postnatal ventilation. Preterm FGR infants are therefore a cohort of babies who would likely benefit from targeted neuroprotective/ neuroregenerative treatment after birth.

Publications arising from this thesis

1. **Malhotra A**, Ditchfield M, Fahey MC, Castillo-Melendez M, Allison BJ, Polglase GR, Wallace EM, Hodges R, Jenkin G, Miller SL. Detection and assessment of brain injury in the growth-restricted fetus and neonate. Pediatr Res. 2017Aug;82(2):184-193.

2. **Malhotra A**, Castillo-Melendez M, Allison BJ, Sutherland AE, Nitsos I, Pham Y, Alves de Alencar Rocha AK, Fahey MC, Polglase GR, Jenkin G, Miller SL. Neuropathology as a consequence of neonatal ventilation in premature growth-restricted lambs. Am J Physiol Regul Integr Comp Physiol. 2018 Dec 1;315(6):R1183-R1194.

3. **Malhotra A**, Allison BJ, Castillo-Melendez M, Jenkin G, Polglase GR, Miller SL. Neonatal Morbidities of Fetal Growth Restriction: Pathophysiology and Impact. Front Endocrinol (Lausanne). 2019 Feb 7;10:55.

4. **Malhotra A**, Castillo-Melendez M, Allison BJ, Sutherland AE, Nitsos I, Pham Y, McDonald CA, Fahey MC, Polglase GR, Jenkin G, Miller SL. Neurovascular effects of umbilical cord blood therapy in growth restricted preterm lambs. *(Under peer review: Stem Cell Res Ther)*

5. **Malhotra A**, Sepehrizadeh T, Dhollander T, Wright D, Castillo-Melendez M, Sutherland AE, Nitsos I, Pham Y, Ditchfield M, Polglase GR, de Veer M, Jenkin G, Pannek K, Shishegar R, Miller SL. Advanced MRI analysis techniques to study brain injury in growth restricted newborn lambs. Neuroimage Clin. 2019 Aug 23;24:101991.

Publications associated with this thesis

1. **Malhotra A**, Sasi A, Miller SL, Jenkin, G, Polglase GR. The efficacy of surfactant replacement therapy in the growth restricted preterm infant: what is the evidence? Front Pediatr 2014; 2:118.

2. **Malhotra A**, Yahya Z, Sasi A, Jenkin G, Ditchfield M, Polglase GR, Miller SL. Does fetal growth restriction lead to increased brain injury as detected by neonatal cranial ultrasound in premature infants? J Paediatr Child Health. 2015 Nov; 51(11):1103-8.

3. Sasi A, Abraham V, Davies-Tuck M, Polglase GR, Jenkin G, Miller SL, **Malhotra A**. Impact of intrauterine growth restriction on preterm lung disease. Acta Paediatr 2015; 104(12): e552–e556.

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5. Polglase GR, Allison BJ, Coia E, Li A, Jenkin G, **Malhotra A**, Sehgal A, Kluckow M, Gill AW, Hooper SB, Miller SL. Altered cardiovascular function at birth in growth-restricted preterm lambs. Pediatr Res. 2016 Oct; 80(4):538-46.

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7. Allison BJ, Hooper SB, Coia E, Jenkin G, **Malhotra A**, Zahra VA, Sehgal A, Kluckow M, Gill AW, Yawno T, Polglase, GR, Castillo-Melendez M, Miller SL. Does fetal growth restriction increase the vulnerability to acute ventilation induced brain injury in newborn lambs? Implications for future health and disease. J Dev Orig Health Dis. 2017 Oct; 8(5):556-565.

8. Polglase GR, Barbuto J, Allison BJ, Yawno T, Sutherland AE, **Malhotra A**, Schulze KE, Wallace EM, Jenkin G, Ricardo SD, Miller SL. Effects of antenatal melatonin therapy on lung structure in growth-restricted newborn lambs. J Appl Physiol (1985). 2017 Nov 1; 123(5):1195-1203.

9. **Malhotra A**, Miller SL, Jenkin G, Hooper SB, Allison BJ, Souzo F, Zahra V, Sehgal A, Polglase GR. Fetal growth restriction is associated with an altered cardiopulmonary and cerebral hemodynamic response to surfactant therapy in preterm lambs. Pediatr Res 2019 April.

Manuscripts currently under review

1. Allison BJ, Youn H, **Malhotra A**, McDonald C, Castillo-Melendez M, Pham Y, Sutherland AE, Jenkin G, Polglase GR, Miller SL. Is umbilical cord blood therapy an effective treatment for early lung injury in growth-restricted newborn lambs? *Submitted to Front Endocrinol, Nov 2018*

2. Krishnamurthy MB, Pharande P, Whiteley G, Hodges RJ, **Malhotra A**. Postnatal middle cerebral artery Dopplers in growth restricted neonates. *Submitted to Eur J Pediatr, June 2019*

In addition, I have published 21 other papers, which are not directly related to my PhD studies during my PhD candidature (2014-19).

Presentations arising from this thesis

Conference abstracts

1. **Malhotra A**, Jenkin G, Polglase G, Allison B, Castillo-Melendez M, Wallace E, Miller S. Cell therapy and fetal growth restriction. 11th Annual Ritchie Centre Colloquium 2014, Melbourne.

2. **Malhotra A**, Allison B, Castillo-Melendez M, Polglase G, Wallace E, Jenkin G, Miller S. Neonatal ventilation exacerbates brain injury in prematurely delivered fetal growth restricted lambs. PSANZ 2015, Melbourne. J Paediatr Child Health 2015; 51(S1): 29.

3. **Malhotra A**, Allison B, Castillo-Melendez M, Polglase G, Wallace E, Jenkin G, Miller S. Effects of neonatal care on preterm brain development in an ovine fetal growth restriction model. 12th Annual Ritchie Centre Colloquium 2015, Melbourne.

4. **Malhotra A**, Castillo-Melendez M, Allison BJ, Jenkin G, Miller SL. Effects of neonatal ventilation on preterm brain development in an ovine fetal growth restriction model. Student of Brain research Symposium 2015, Melbourne.

5. **Malhotra A**, Castillo-Melendez M, Allison BJ, Polglase GR, Jenkin G, Miller SL. Impact of neonatal ventilation on white matter development in an early onset ovine model of fetal growth restriction. PAS 2016, Baltimore.

6. **Malhotra A**, Castillo-Melendez M, Allison BJ, Polglase GR, Jenkin G, Miller SL. Neonatal ventilation exacerbates brain injury in premature growth restricted lambs. FNPS 2017, Osaka, Japan

7. **Malhotra A**, Castillo-Melendez M, Allison BJ, Sutherland AE, Pham Y, Polglase GR, Jenkin G, Miller SL. Umbilical Cord Blood Therapy Mitigates Brain Injury Associated with Ventilation in Premature Growth Restricted Lambs. PSANZ 2018, Auckland. J Paediatr Child Health, 54: 88-88.

8. **Malhotra A**, Castillo-Melendez M, Allison BJ, Pham Y, Polglase GR, Jenkin G, Miller SL. Umbilical Cord Blood Therapy Mitigates Brain Injury Associated with Ventilation in Premature Growth Restricted Lambs. PSANZ 2019, Gold Coast. J Paediatr Child Health.

Invited talks arising from this thesis

2016

1. Advanced MRI for neonatal brain injury, MIME Medtech Industry Innovators Showcase Day, Monash University, Melbourne

2. Brain amyloid – simultaneous PET & MRI, MBI Pre-Clinical Symposium, Monash University, Melbourne

2018

1. Umbilical cord blood stem cell therapy for fetal growth restriction, European Workshop on fetal growth restriction, Prato, Italy

2. Reducing perinatal brain injury, Mercy Perinatal Australian Reproduction Update 2018, Melbourne

Abbreviations

AD	Axial diffusivity
AGA	Appropriate for gestational age
ANOVA	Analysis of variance
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
BM	Betamethasone
BNP	Brain natriuretic peptide
BP	Blood pressure
BPD	Bronchopulmonary dysplasia
BSA	Bovine serum albumin
CBF	Cerebral blood flow
CGM	Cortical grey matter
Chol	Choline
CPR	Cerebroplacental ratio
Cr	Creatine
CRI	Cerebral resistance index
CSF	Cerebrospinal fluid
CSD	Constrained spherical deconvolution
DOHAD	Developmental origins of health and disease
dMRI	Diffusion MRI
DTI	Diffusion tensor imaging
DV	Ductus venosus
EC	External capsule
FA	Fractional anisotropy
FBA	Fixel-based analysis
FC	Fibre cross-section
FD	Fibre density
FDC	Fibre density and cross-section
FGR	Fetal growth restriction
GM	General movement
Hippo	Hippocampus
H&E	Haematoxylin and Eosin
IGFBP	Insulin like growth factor binding protein
IGF	Insulin like growth factor
IL	Interleukin
IUGR	Intrauterine growth restriction
IVH	Intraventricular haemorrhage
LFB	Luxol fast blue

MCA	Middle cerebral artery
MD	Mean diffusivity
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
NAA	N-acetylaspartate
NGF	Neuronal growth factor
NIRS	Near infrared spectroscopy
NEC	Necrotising enterocolitis
NPV	Negative predictive value
NO	Nitric oxide
NSE	Neuron specific enolase
NVU	Neurovascular unit
PDE	Phosphodiesterase
PPV	Positive predictive value
PVWM	Periventricular white matter
RDS	Respiratory distress syndrome
RD	Radial diffusivity
ROI	Region of interest
SBP	Systemic blood pressure
SCWM	Subcortical white matter
SGA	Small for gestational age
SUAL	Single umbilical artery ligation
SVZ	Subventricular zone
TDI	Tissue Doppler imaging
TNF	Tumor necrosis factor
TOI	Tissue oxygenation index
UA	Umbilical artery
UCB	Umbilical cord blood
UCBC	Umbilical cord blood cell
VCAM	Vascular cell adhesion molecule

Chapter 1: Literature Review A

Fetal growth restriction is a common pregnancy complication that leads to a number of perinatal and neonatal morbidities and long-term consequences. Placental insufficiency is the principal cause of FGR and its associated complications. Whilst a number of studies and review articles exist on individual morbidities and complications associated with FGR, there is a lack of a comprehensive combined review of the various neonatal morbidities.

In this literature review chapter, I reviewed the causes, pathophysiology and impact of the common neonatal morbidities associated with FGR and their long-term consequences. I focused on respiratory, neurological and cardiovascular morbidities, as these are most often the critical complications in the neonatal period, and beyond. Accordingly, I conducted a focused review and narrative synthesis on the pathophysiology and impact of common neonatal morbidities of FGR. I also discussed the targeted therapies that are being evaluated in preclinical and clinical models of FGR. This chapter sets the scene for the thesis and pertains directly to the research reported in Chapters 2 and 3, which focuses on understanding the pathophysiology of brain injury associated with FGR and neonatal ventilation (Chapter 2), and targeted therapy (in this case umbilical cord blood derived stem cells) for ventilation associated FGR brain injury (Chapter 3). This is the unaltered version of the review paper published in Frontiers in Endocrinology (Lausanne).

1.1. Neonatal Morbidities Of Fetal Growth Restriction: Pathophysiology And Impact.

Atul Malhotra[1,2,3], Beth J Allison[2,4], Margie Castillo-Melendez[2,4], Graham Jenkin[2,4], Graeme R Polglase[2,4], Suzanne L Miller[2,4] (2019) **Neonatal Morbidities Of Fetal Growth Restriction: Pathophysiology And Impact**. Feb 7: 10:55. doi: 10.3389/fendo.2019.00055

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REVIEW
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Neonatal Morbidities of Fetal Growth Restriction: Pathophysiology and Impact

Atul Malhotra[1,2,3*†], *Beth J. Allison*[2,4†], *Margie Castillo-Melendez*[2,4], *Graham Jenkin*[2,4], *Graeme R. Polglase*[2,4] *and Suzanne L. Miller*[2,4]

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Being born small lays the foundation for short-term and long-term implications for life. Intrauterine or fetal growth restriction describes the pregnancy complication of pathological reduced fetal growth, leading to significant perinatal mortality and morbidity, and subsequent long-term deficits. Placental insufficiency is the principal cause of FGR, which in turn underlies a chronic undersupply of oxygen and nutrients to the fetus. The neonatal morbidities associated with FGR depend on the timing of onset of placental dysfunction and growth restriction, its severity, and the gestation at birth of the infant. In this review, we explore the pathophysiological mechanisms involved in the development of major neonatal morbidities in FGR, and their impact on the health of the infant. Fetal cardiovascular adaptation and altered organ development during gestation are principal contributors to postnatal consequences of FGR. Clinical presentation, diagnostic tools and management strategies of neonatal morbidities are presented. We also present information on the current status of targeted therapies. A better understanding of neonatal morbidities associated with FGR will enable early neonatal detection, monitoring and management of potential adverse outcomes in the newborn period and beyond.

Keywords: IUGR, FGR, bronchopulmonary dysplasia, cardiac, brain injury, necrotizing enterocolitis

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OVERVIEW AND DESCRIPTION

Fetal growth restriction (FGR) describes the fetus that does not grow to its expected biological potential *in utero*, and is a relatively common complication of pregnancy. True FGR, as compared to constitutional smallness, is a pathological condition wherein the placental fails to deliver an adequate supply of oxygen and nutrients to the developing fetus, termed placental insufficiency. As a consequence, fetal growth becomes stunted. It is only in the last several years that consensus definitions for pathological FGR have been developed (1), but it remains that many cases of FGR *in utero* remain undetected, and therefore the neonatal description of small for gestational age (SGA) continues to be a useful and necessary proxy for FGR (2). Traditionally, an estimated fetal weight or abdominal circumference of less than the 10th centile for the population at a given gestational age was considered highly suggestive of FGR. However this broad description of SGA includes the many infants (~20%) that are born small, but are otherwise healthy (2). Accordingly, consensus definitions for FGR now incorporate Doppler indices of placental function/ dysfunction during pregnancy (1), to provide a more robust assessment of pathological fetal growth restriction.

Clear and well-defined guidelines for description of FGR subsequent to placental insufficiency are important for two broad reasons, (i) early identification of FGR flags infants who are at significantly elevated risk for neonatal complications, and (ii) early identification of infants with FGR who would benefit from intervention(s) to improve outcomes. The etiology of many adverse consequences of FGR arise *in utero* from fetal hypoxia and nutrient deprivation secondary to placental dysfunction, with fetal hemodynamic adaptations *in utero* laying the foundation for altered organ structure and function in the neonatal period and beyond.

ETIOLOGY AND UTEROPLACENTAL FACTORS

The basic determinants of fetal growth are the individual's genetic makeup, nutrient availability from the mother, and environmental factors, coupled with the capacity of the placenta to adequately transfer nutrients and oxygen to the fetus, and endocrine modulation of these interactions (3, 4). Reduced fetal growth, and subsequent pathological FGR, can be caused by maternal factors (e.g., under nutrition, hypertension, preeclampsia), fetal (chromosomal abnormalities, multiple fetuses) or placental factors (5), however in the majority of cases, FGR results from placental dysfunction (6). Here, the term *placental insufficiency* is broadly used to describe reduced transfer of oxygen and nutrients to the fetus, with adverse effects on fetal development. Antecedents of placental insufficiency can include maternal malnutrition and hypertension, but in up to 60% of cases the placental insufficiency is idiopathic, wherein there is a physiological deficiency in the remodeling of uterine and placental spiral arteries resulting in restricted uteroplacental perfusion (7).

Abnormalities in placental function provide a primary clinical indicator that transfer of oxygen and nutrients is suboptimal, and fetal growth may be adversely affected. In the fetus, placental insufficiency is characterized by preferential blood flow redistribution to the vital organs (brain, myocardium, and adrenal glands), while other organs, including the gastrointestinal tract, skin, and others may be deprived of sufficient blood flow. This fetal redistribution of blood flow occurs as a direct result of hypoxia, and can be detected as altered umbilical, uterine and/or middle cerebral artery Doppler flows (8). Large population studies of small but otherwise healthy infants at birth (Apgar $\geq$ 7 at 5 min of life) demonstrates that severely growth restricted infants at the third birth weight centile are indeed chronically hypoxic; umbilical vein median pO_2 13 mmHg (FGR) versus 26 mmHg (normally grown infants), and median SaO_2 16 vs. 55% respectively (9, 10).

In addition to the fundamental roles of oxygen and glucose for development, fetal growth is dependent on a number of key anabolic hormones—placental, pancreatic, thyroid, adrenal and pituitary hormones—any disruption in these can also lead to FGR (11, 12). The insulin-like growth factors -I and -II (IGF-I and IGF-II) are both proposed to play central roles in normal fetal growth, stimulating fetal cell proliferation, differentiation, protein and glycogen synthesis, where these actions are mediated via their receptors and the IGF-binding proteins (IGFBPs). The two IGFs are detected in the fetal circulation in early gestation, and in particular it is noted that decreased serum IGF-1 is correlated with reduced fetal growth (3, 13). IGF-1 also has a central role in brain growth, white matter development and brain connectivity (14). Pregnancy-associated plasma protein-A (PAPP-A), secreted by the placental decidua, cleaves IGFBP-4, which in turn is a potent inhibitor of IGF bioactivity. Accordingly, low levels of PAPP-A in early pregnancy are linked with an increased risk for FGR, although the predictive value of this biomarker still remains poor (15). A recent study has investigated whether administration of IGF-1 into the amniotic fluid can improve postnatal growth and metabolism in a sheep model of FGR, and results from this study look promising (16) (see Interventions for Improved Outcomes section). Glucocorticoid hormones play a central role in the development and maturation of fetal organs, while growth hormone, which is the major hormonal regulator of postnatal growth, has no demonstrable effect on fetal growth *per se* (17). Exogenous glucocorticoids are administered to pregnant women at imminent risk of preterm birth to mature the fetal lungs, and preterm birth is a common complication of FGR. Preclinical and clinical evidence demonstrates that antenatal steroids may exacerbate growth restriction (particularly repeat doses) (18) and that the FGR fetus differentially responds to antenatal steroids compared to appropriately-grown fetuses, likely mediated via altered placental response to steroids (19). Antenatal glucocorticoids may not significantly improve neonatal outcomes in FGR preterm infants (20), and indeed, may have adverse effects on brain development (21, 22). Further research is clearly needed in this area.

The fetus mounts a critical hemodynamic response to hypoxia, aimed at ensuring the most important fetal organs maximize their oxygen supply. This adaptive response redistributes blood flow away from peripheral vascular beds which is preferentially shunted toward essential organs, termed *brain sparing* (23). This results in preferential supply of blood flow to favor the brain, heart, and adrenals, at the expense of the gut, kidney, hematologic organs, and peripheral vascular beds. When fetal hypoxia is chronic in nature, as occurs with placental insufficiency, the persistent fetal hemodynamic shift has significant consequences for the fetus and neonate. Characteristically, prolonged fetal hypoxia reduces fetal weight overall, but also does so in an asymmetric manner, with relatively spared head size and a thin and/or shorter body length. While hemodynamic redistribution may be an attempt to protect vital organs from hypoxic injury, an adverse impact on fetal organ development and vascular remodeling is increasingly being recognized (23, 24). For example, the shunting of blood flow away from the kidneys is now recognized as contributing to suboptimal renal development with reduced nephron endowment (25). Further, sustained vasoconstriction of peripheral vascular beds alters local arterial wall properties including endothelial vasodilator dysfunction and sympathetic hyperinnervation, and consequently contributes to cardiac remodeling (26). The short and long-term consequences of sustained redistribution of cardiac output are profound, for

both spared and non-spared organs, and these will be discussed in more detail below.

The overall incidence of FGR depends on the diagnostic criteria used, and the population being examined. It is estimated that between 3 and 9% of pregnancies in the developed world, and up to 25% of pregnancies in low-middle income countries are affected by FGR (27, 28). Factors that influence FGR rates in communities include maternal nutrition, maternal and paternal smoking rates, alcohol and drug addiction, socio-economic status, maternal activity, stress during pregnancy and genetic make-up (29). The incidence of FGR is significantly higher in low- and middle- income countries, compared to high-income countries, and this is notably contributed by a large number of FGR infants born in the Asian continent, which accounts for approximately 75% of all affected infants in the world, followed by Africa and South America (30).

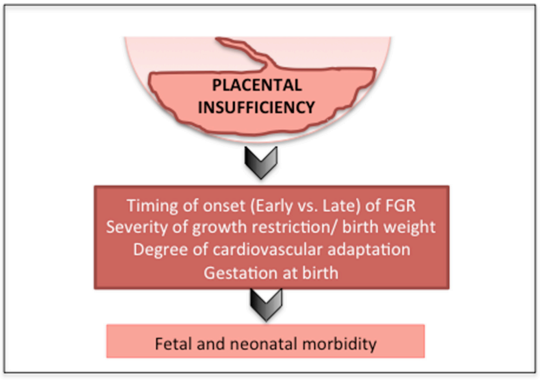

FIGURE 1 | Factors that affect outcomes in FGR.

CLASSIFICATION TYPES OF FGR

FGR can be classified as early- or late-onset, reflecting the gestational age when growth restriction is diagnosed. Early onset FGR (<32 weeks gestation) is the more severe phenotype, associated with significant disruption to placental perfusion leading to chronic fetal hypoxia, and with subsequent fetal cardiovascular adaptation *in utero* (31). Fetuses with early-onset placental insufficiency are more likely to be born preterm, to deteriorate over weeks, and have a high risk of morbidity or mortality. Late onset FGR (≥32 weeks gestation) is the more common presentation of growth restriction (up to 80% of FGR cases), and is generally linked with a milder placental deficit, together with a lesser degree of fetal hemodynamic adaptation. Although placental dysfunction is mild, this group has a high risk of deteriorating rapidly, such that they have an elevated risk of stillbirth (31). This broad distinction between early- and late-onset FGR demonstrates that the timing when placental function becomes rate limiting for the fetus is a principal factor affecting outcome.

Advances in obstetric monitoring mean that it is increasingly likely that placental insufficiency and fetal growth restriction are detected during pregnancy. However, a significant proportion (up to 50%) of FGR fetuses remain undiagnosed, and are first recognized only very late in pregnancy or at birth (32–34). Furthermore, debate continues around the utility of third trimester ultrasound for the detection of late-onset FGR (35), with a recent study reporting that undiagnosed FGR does not lead to increased incidence of morbidity in neonates (36). These data likely reflect that it is predominantly the early-onset FGR infants with severe placental insufficiency, and worse neonatal outcomes, who are more straightforward to detect during pregnancy. Currently, no effective antenatal therapy exists for FGR, hence, delivery of the fetus remains the only viable option for a severely affected pregnancy; this often occurs preterm, introducing further risk of morbidity and mortality (37, 38). Together these data are indicative that the timing of the onset of placental insufficiency (early vs. late), gestation at birth, and severity of compromise/birth weight are the most predictive factors for neonatal outcomes (39) (**Figure 1**).

PERINATAL MORBIDITIES

A typical FGR infant at term age and an appropriately grown infant at term are shown in **Figure 2**. Key pathophysiological mechanisms driving fetal growth restriction and the resulting *in-utero* and postnatal consequences are highlighted in **Figure 3**. Placental pathology and FGR are strongly associated with fetal demise *in utero*, and stillbirth (40–42). FGR is the greatest risk factor for stillbirth; overall it is shown that up to 50% of infants who are stillborn were small for gestational age or growth restricted (43). The detection, early diagnosis, surveillance and delivery of the severely growth restricted fetus are paramount to decrease stillbirth, but it remains that 40% of severe FGR infants (<3rd centile for birth weight) remain undetected *in utero* (44).

After birth, FGR infants are more likely to spend a significantly longer time in NICU compared to gestation age-matched infants (45). Accordingly, financial costs associated with the care of FGR infants are high, given that many of them will remain in NICU for prolonged periods (46, 47). FGR infants demonstrate elevated rates of intolerance to feeds/ milk, feeding difficulties and necrotizing enterocolitis (NEC). NEC is predominantly seen in infants who are born preterm, but late preterm infants are more likely to develop NEC if they were growth restricted (48). It is likely that i*n utero* chronic fetal hypoxia and subsequent cardiovascular redistribution of blood flow away from the gastrointestinal tract contribute to immature gut development (49). FGR newborns, especially with abnormal flows in the umbilical artery prior to birth, are shown to have more feed intolerance when compared to their well-grown preterm counterparts (50). Superior mesenteric artery blood flows have been used as a marker for splanchnic perfusion in neonates and decreased flows correlate with feed intolerance (51). Application of near infra-red spectroscopy in the neonatal period as an assessment tool for monitoring gut perfusion can detect changes in splanchnic oxygen delivery, which may be reduced in FGR

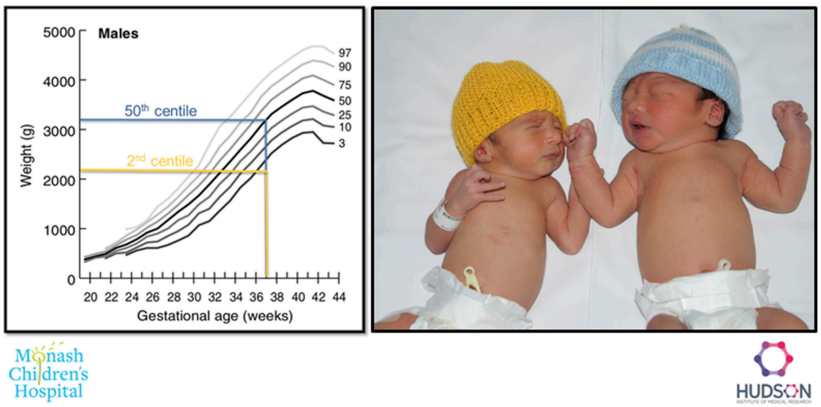

FIGURE 2 | Example of a FGR (2nd centile weight for age, yellow), and an appropriately grown (50th centile weight for age, blue) infant born at 37 weeks gestation.

infants and may predict feeding intolerance and development of NEC (52). Studies have shown that preterm FGR infants do not tolerate enteral feeds in the first few days of life (53) but conversely there is evidence that delaying enteral feeds in preterm FGR infants does not confer any protection against feed intolerance or NEC (54). In fact, it may delay establishment of feeds and increase length of stay in the neonatal unit (55).

Malnutrition and low birth weight puts FGR infants at an increased risk of a number of transient neonatal morbidities including hypothermia, altered glucose metabolism (hypoglycemia, hyperglycemia), hypocalcemia, polycythemia, jaundice and sepsis (5). Increased risk of infection is also common, potentially related to depressed immunological state and competence (56). FGR infants born preterm also have an increased risk of retinopathy of prematurity (57). FGR is linked to altered nephrogenesis, due to suboptimal tubular development caused by intrauterine hypoxia (58), and in turn, urinary Cystatin-C excretion is increased in FGR infants compared to appropriately-grown infants which is seen to reflect reduced renal volume (59). It is therefore suggested that increased secretion of Cystatin-C signifies nephron loss as a result of the negative impact of FGR on kidney development. Factors involved in nephron loss may include intrauterine hypoxia, decreased antioxidant capacity, and altered levels of growth factors.

SPECIFIC NEONATAL MORBIDITIES (TABLE 1)

Cardiovascular Morbidity

Clinical Features

In addition to chronic hypoxia, placental insufficiency imposes other important stressors for the developing fetus, such as oxidative stress, inflammation and increased hemodynamic stress. This leads to elevated cardiac afterload due to high placental vascular resistance, which in turn directly and indirectly impacts on the developing cardiovascular system. It is now accepted that the fetal adaptations to these combined stressors sets the fetus, and future offspring, on a path of predetermined increased risk of cardiovascular disease (60, 61). It is also now apparent that subclinical or subtle evidence of cardiovascular dysfunction is present in fetal and/or early neonatal life, well before the onset of significant cardiovascular or metabolic disease in adulthood, supporting the notion of perinatal programming (60).

Advances in Doppler ultrasonography of the placental and fetal circulations provide a window of opportunity to observe and quantify fetal cardiovascular function, and early dysfunction. In early-onset FGR, severe placental insufficiency is characterized by high vascular resistance within placental vascular beds, resulting in absent or reversed diastolic umbilical artery flow, as well as high pulsatility index in the ductus venosus and increased dilation of cerebral vessels evident of fetal brain sparing (31). In late-onset FGR, umbilical artery flow may be normal, representing a milder placental insufficiency. Despite this, brain sparing is still evident, with increased cerebral to placental blood flow driven primarily by vasodilation within the middle cerebral artery in response to hypoxia (62). In both early- and late-onset FGR, increasing vasodilation within cerebral vascular beds is indicative of a worsening fetal state (63). Increased myocardial performance index, an index incorporating both diastolic and systolic function to assess global cardiac function/dysfunction, is evident from 24 weeks gestation in early onset-FGR fetuses (64). An increase in myocardial performance index is not indicative of improved performance, but rather demonstrates an increased time of systolic relaxation evident in early-onset FGR. Increased myocardial dysfunction is also present in late-onset FGR from >35 weeks gestation. In this population, late-onset placental insufficiency and FGR results in fetuses with larger, more globular hearts and early indices of functional deficits with impaired relaxation (65). This study is the first to show that late-onset placental insufficiency and FGR induces cardiac dysfunction that is detectable in the third trimester of pregnancy (~35 weeks gestation), indicating the presence of cardiac programming prior to birth. It has also been shown that

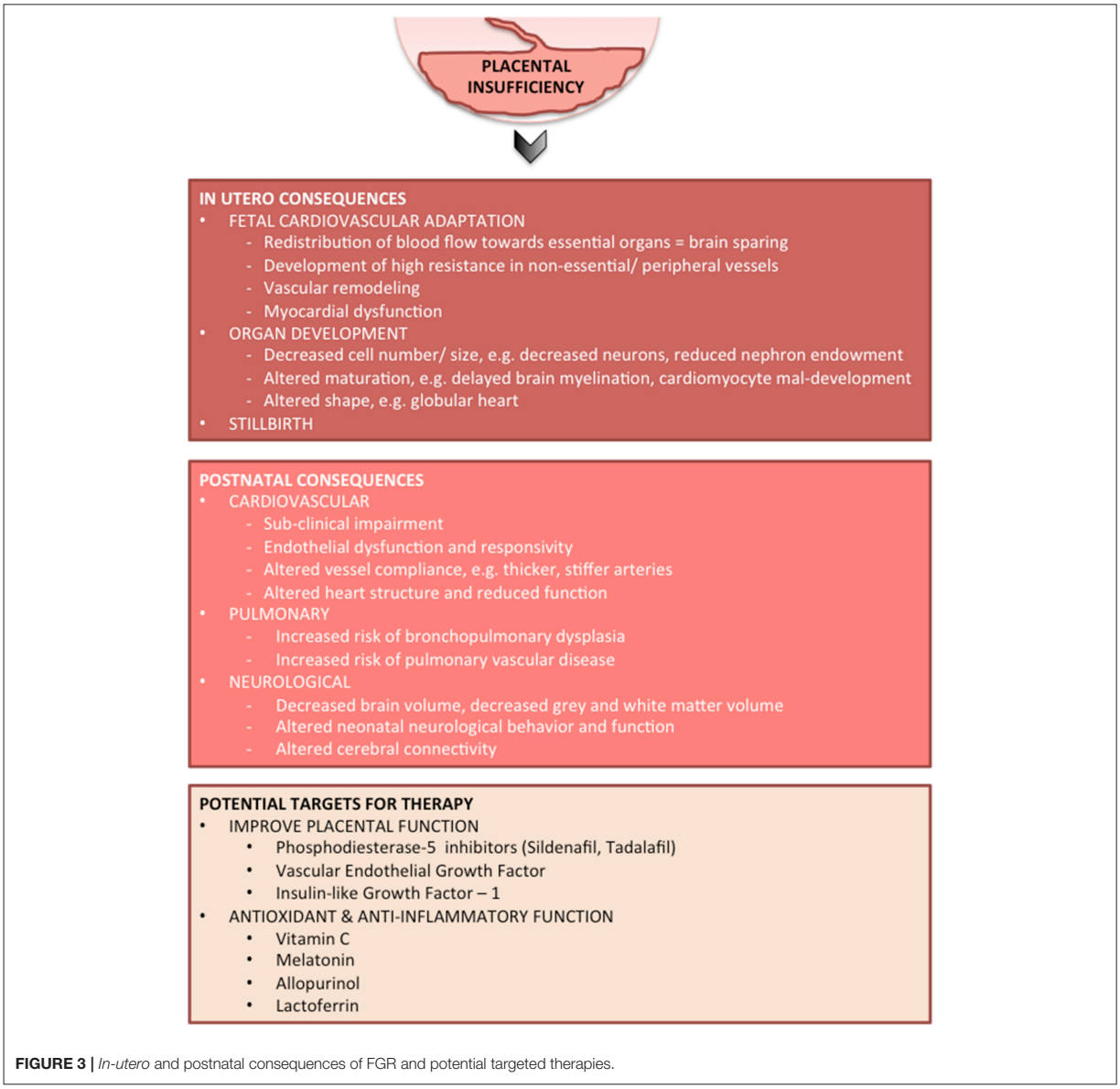

FIGURE 3 | *In-utero* and postnatal consequences of FGR and potential targeted therapies.

cardiac dysfunction and markers of cardiac injury such as BNP and H-FABP become increasingly worse as the severity of fetal compromise progresses (66).

Pathophysiology

In the presence of very high placental resistance associated with a sub-optimal pregnancy, the fetal heart contracts against an increased afterload, thereby increasing the work required to contract with each beat, resulting in increased heart wall stress and hypertrophy (65). Over a sustained period, hypertrophy increases wall thickness altering ventricular compliance. Increased afterload is evidenced by the presence of increased serum B-natriuretic peptide in infants born growth restricted (67).

Where placental insufficiency is present, the fetal heart must also adapt to a reduced supply of glucose, with the fetal heart producing ATP from glycolysis and oxidation of lactate. Despite this, cardiac glucose consumption is not altered in growth restriction due to increase in insulin receptor GLUT4 in the heart, which increases insulin transport to maintain glucose consumption (68). Thus, glucose availability is not considered a primary limiting factor for fetal cardiac function. More in-depth analysis of the effects of suboptimal oxygen and glucose supply to the developing heart can be examined

TABLE 1 | Neonatal morbidities in fetal growth restriction.

	Cardiovascular morbidity	Respiratory morbidity	Neurological morbidity	Others
Neonatal period	Early hypotension Persistent fetal circulation/PPHN Structural heart changes Vessel wall rigidity Cardiac function issues Late systemic hypertension Secondary pulmonary hypertension	Increased need for respiratory/ventilator support Meconium aspiration syndrome Pulmonary hemorrhage Bronchopulmonary dysplasia	Perinatal asphyxia Microcephaly Cranial ultrasound abnormalities (IVH, PVL) White matter and gray matter changes on MRI Functional and DTI MRI changes General movement assessment abnormalities EEG abnormalities	Poor transition Hypoglycemia Hypocalcemia Hypothermia Sepsis Jaundice Polycythemia Prolonged NICU stay Feed intolerance Delay in establishment of feeds Necrotizing enterocolitis Renal tubular injury Retinopathy of prematurity
Long term impact	Hypertension Ischemic heart disease Stroke Atherosclerosis	Chronic respiratory insufficiency Reactive airway disease	Neurodevelopmental issues Behavioral problems Learning difficulties Cerebral palsy Dementia Mental health issues	Failure to thrive Obesity Immune dysfunction Osteoporosis Metabolic syndrome Renal issues Hormonal issues Cancer Shortened life span

PPHN, persistent pulmonary hypertension; IVH, intraventricular hemorrhage; PVL, periventricular leukomalacia; MRI, magnetic resonance imaging; DTI, diffusion tensor imaging; EEG, electroencephalography; NICU, neonatal intensive care unit.

in animal studies of FGR (69). These studies show that the fetal heart is remodeled in a manner similar to that seen in dilated cardiomyopathy. Cardiomyocyte development is adversely affected and programmed cell death is increased in growth restricted fetal guinea pigs and sheep, with a persistence of the mononucleated, primitive cell type (70, 71). Permanent alterations in heart morphology are detected into adulthood, as evidenced by persistence in the deficits in cardiomyocyte number and cardiac hypertrophy (70).

The transition to ex utero represents a particularly critical period where the heart must rapidly adapt to new pressure and flow demands. After birth, the external pressures around the heart are reduced, due to alleviation of the liquid-filled lungs and amniotic fluid. Concurrently, the low resistance placental circulation is removed, temporarily decreasing cardiac output and increasing afterload, and thus heart rate, end-diastolic pressure and stroke volume must all be increased to maintain adequate cardiac output. In response to these altered pressure demands throughout the transition to ex-utero life, the myocardium undergoes rapid changes in cardiac muscle protein expression (72). One critical change precipitated by such pressure changes at birth is a shift in the fibrous component of sarcomeres toward smaller isoforms, which increase the passive tension within with postnatal heart (72). It is postulated that the growth-restricted fetus undergoes these changes *in utero*, due to the presence of increased afterload secondary to high placental resistance, and resulting in altered cardiac compliance (73). In human infants and in experimental animal models of FGR, the heart is shown to have shorter sarcomeres, which likely contributes to decreased contractile strength (73, 74). Further, changes in the large sarcomere protein titin are described in the FGR heart, reflecting a shift from a large compliant isoform toward a small and stiff isoform (73). As titin is a major determinant of sarcomere length, this change in isoform is consistent with overall reduction in sarcomere length in the hearts of growth-restricted fetuses, and has consequences for cardiac development and function.

Changes in the hearts of growth-restricted fetuses are directly coupled with changes in the wider cardiovascular system, notably the vasculature. It is now well described that vascular responses to placental insufficiency and chronic hypoxia are vascular bed-dependent. In peripheral vascular beds, human and animal data show that sustained vasoconstriction and peripheral vascular resistance in response to chronic hypoxia induces arterial stiffness and elevated central pulse pressure (75–77). Growth restriction induced via chronic hypoxia increases peripheral vascular tone via numerous methods, including endothelial dysfunction (76), increased sympathetic nervous system activation (69) and oxidative stress (78). Oxidative stress, induced via increased reactive oxygen species generation, quenches nitric oxide (NO), thereby reducing its bioavailability and increasing peripheral vascular tone. We have previously described an increase in plasma urate levels arising from chronic fetal hypoxia (78) suggesting activation of a potent oxidative enzyme, xanthine oxidase. Importantly, it is this altered vascular tone in fetal life that sets up developmental programming for future hypertension, as evidenced in both FGR animals (79) and human cohorts (61). Central vessels, such as the aorta and carotid arteries, have increased wall thickness (80) and increased stiffness (81) in FGR humans and animals. The vascular changes described above persist into adulthood, however, they are more

pronounced in peripheral vascular beds compared to central vascular bed (82).

Vascular compensation is observed in FGR offspring, wherein remodeling of the arterial wall, collagen and elastin content contribute to altered vascular mechanics (83). Rodent and guinea pig studies show that interruption of fetal growth in mid gestation coincides with a crucial period of elastin production within vasculature, attenuating elastin deposition and subsequently content, such that elastin is reduced and collagen increased (84, 85). This remodeling greatly impacts on vascular mechanics, as collagen is 100-times stiffer than elastin and, as a consequence, vascular stiffness is significantly increased (85). These changes in vessel biomechanics are most notably in the lower body arteries of growth-restricted offspring (83). Following low protein diet restriction, the aorta from adolescent rodents are not only stiffer, they also have increased fibrotic tendency, despite being normotensive (67). However, a more profound effect on vascular extracellular matrix remodeling is seen with placental dysfunction-induced growth restriction, compared to other factors such as diet (high fat) or fetal sex. These data are suggestive that vascular remodeling occurs primarily in response to changes in pressure and flow caused by chronic hypoxia and adaptive hemodynamic redistribution, rather than metabolic or hormone alterations.

Impact

The evidence presented above all indicates that exposure to placental insufficiency and chronic hypoxia significantly alters *fetal* development of the cardiovascular system. Unsurprisingly, the fetal cardiovascular alterations subsequent to placental insufficiency persist into clearly detectable structural and functional changes in the early postnatal period. After birth, tissue Doppler imaging (TDI) has allowed detection of persistent sub-clinical changes in movement and timing of the myocardium throughout the cardiac cycle, in particular during myocardial relaxation (86). In the first days of life, infants who were growth restricted show altered cardiac structure detectable on ultrasound with decreased sphericity index (a more globular shape), together with increased interventricular septum and left ventricle wall thickness (77). Further, load-dependent diastolic function is impaired (77, 87) this often represents impaired cardiac relaxation resulting in the transition from contraction to relaxation occurring prior to aortic valve opening, a situation which is common in hearts exposed to chronically high afterload. Frequently, FGR does not alter overall cardiac output, however components of cardiac output are altered with decreased stroke volume and increased heart rate often presenting in the FGR newborn (86). With increasing severity of FGR there is increased biomarkers of myocardiac cell damage, such as heart fatty acid binding protein (H-FABP), and incremental worsening of both systolic and diastolic dysfunction and in particular heart relaxation is altered (66). These alterations are indicative of hemodynamic compromise and are linked to worsening outcomes including fetal demise (88). Early signs of alteration in blood pressure in association with FGR remains contentious—we have documented increased blood pressure in the early postnatal period (80), whilst others show no change in blood pressure (82); these differences may reflect the difference between clinical and pre-clinical studies or the severity of the growth restriction induced.

In turn, *in utero* cardiac and vascular remodeling in FGR neonates programs for cardiovascular disease into adulthood. Indeed, the consequences of growth restriction on adult cardiovascular function are now well studied, and are central to the Developmental Origins of Health and Disease (DOHAD) hypothesis. These findings are apparent from both human epidemiological and experimental paradigms in growth-restricted offspring (76, 85) and adults (60). Long-term evidence of the link between low birth weight and developmental programming is available in the infants born in famine conditions in Europe in the 1900s wherein SGA is linked with significantly higher blood pressure in later life (89), and with increased risk of ischemic heart disease and cerebrovascular disease (90). Precursors of long-term suboptimal outcomes such as stroke and hypertension (91) have been proposed to be evident in growth restriction offspring as pre-atherosclerotic vascular damage in both newborns (92) and 18 month old FGR offspring (93).

Despite excellent evidence of the link between FGR and adult cardiovascular disease, there is some difficulty in dissociating the potentially separate effects of placental insufficiency/FGR and preterm birth. Growth restricted fetuses are often born preterm, particularly early-onset severe FGR infants, and preterm birth is also associated with adverse effects on the developing cardiovascular system (94). A recent study by Cohen et al. (87) followed both preterm and preterm FGR infants to 6 months of age to determine cardiac morphology. They found that changes in cardiac structure and function associated with preterm birth alone were sub-clinical, and normalized in childhood, while only thickened ventricular walls persisted into 6 months of age in FGR infants (87). This study goes some way to delineate the separate effects of prematurity and growth restriction and suggests a possible persistence of structural changes in FGR over and above the effects of prematurity.

Respiratory Morbidity

Clinical Features

There is heterogeneity in descriptions of pulmonary complications associated with FGR, which probably reflect the heterogeneity in growth restriction itself. There is however good evidence that chronic hypoxia associated with FGR interrupts normal pulmonary development, and increases susceptibility to both short- and long-term respiratory compromise. Preterm FGR newborns are 45% more likely to have bronchopulmonary dysplasia (BPD) or die from respiratory complications after birth as compared to well-grown infants (45). Further, even FGR infants born at term have worse respiratory outcomes than appropriately grown infants (95). FGR infants spend a significantly increased time in NICU and on mechanical ventilation compared to age-matched control infants, and rates of respiratory distress syndrome (70) and BPD are increased with FGR (45, 96, 97). Indeed, large multicenter trials for early-onset FGR describe that BPD is the most common morbidity for this population. The risk of BPD is greater when FGR and preterm

birth are co-morbidities. Growth restriction is also associated with pulmonary hypertension of the newborn (96). FGR is associated with impaired lung function in children (98) that can persist to adulthood (99).

Pathophysiology

Human FGR cohort studies and preclinical animal studies describe that FGR can result in altered lung development; in some cases these are subtle structural and/or biochemical changes, wherein the timing and severity of compromise modulates effect. In animal studies, an early onset placental or hypoxic compromise mediates a more pronounced adverse outcome. Chronic hypoxia in fetal sheep resulting in FGR induces an adaptive response within the developing lung, where genes regulating hypoxic signaling, lung liquid reabsorption and surfactant maturation are increased (100). A 2-week exposure to hypoxia alone in rats disrupts alveolarization, reducing alveolar number via reduced septation (101). In fetal sheep we have induced late-onset placental insufficiency and FGR to examine lung morphology in preterm and term-born lambs. Lambs born naturally at term have simplified lung architecture with decreased secondary crest abundance and increased elastin deposition (102). Lambs that are delivered preterm and exposed to 2 h of mechanical ventilation do not demonstrate a difference in lung structure between FGR and appropriately-grown lambs, with no difference in the ratio of lung tissue to airspace or septal crest density, however the early tissue injury marker cyr61 is significantly increased in FGR lambs (103). Further, we observed that both FGR and appropriately grown lambs had similar ventilation requirements in the first hours of life. These findings extend previous results in FGR animal experiments from our group (104) and others (105, 106), which find no overt difference in pulmonary structure of FGR offspring. When we compare results between our preterm and term lamb cohorts, it is evident that the timing and duration of placental insufficiency is a critical determinant of lung dysfunction. We have recently examined the effects of early-onset placental insufficiency on lung structure and function, finding that lung cellular morphological changes are present (unpublished results). Accordingly, we propose that altered lung structural development is dependent on the timing of compromise, rather than the severity of growth restriction. Further, in early-onset FGR, the severity of fetal hypoxia has an inverse relationship with pulmonary surfactant production leading to decreased surfactant, a relationship not maintained in late-onset FGR (107). It is well accepted that without adequate surfactant, the newborn is at increased risk of pulmonary complications after birth, particularly when the infant is born preterm.

As discussed above, chronic hypoxia results in the fetal adaptive response of redistribution of cardiac output. MRI studies have confirmed that in late gestation of human fetuses, growth restriction is associated with an increased superior vena cava flow and, consequently, decreased pulmonary artery flow (108). This hemodynamic response is contributed by increased pulmonary vascular resistance (97). Increased pulmonary vascular resistance also reduces venous return to the left heart (109) and enhances right ventricular afterload. Combined ventricular output is thus maintained (108). Postnatally, FGR does not alter pulmonary blood flow during the transition to ex utero life, but left ventricular output is lower (110). Thus it is apparent that the hemodynamic adaptation to chronic hypoxia also has important implications for pulmonary vascular development, and accordingly, lung structure and function in FGR offspring.

A handful of studies have also examined fetal breathing movements in the developing fetus, and undertaken comparison in FGR versus appropriately grown fetuses. Fetal breathing movements are an important component of normal lung development, as they provide a stretch stimulus for growth throughout gestation (111). In FGR sheep, fetal breathing movements are significantly reduced in late gestation, although it is noted that not all experimental models of placental insufficiency show such changes (69). The cessation of fetal breathing movements in response to placental insufficiency is thought to occur by way of physiological response to reduce metabolic rate and thus conservation of oxygen, and is associated with disrupted alveolarization (112).

Deficits in pulmonary development subsequent to placental compromise are not confined to lung alveolar morphology. There is a growing understanding of the link between poor alveolar development and poor lung vascular development (113–115), called the *Vascular Hypothesis*. Growth restriction, induced via hyperthermia in pregnant sheep, impairs both lung alveolar and vascular development in the developing fetus (116). In complimentary experiments, lung alveolar cells isolated from the same growth-restricted fetuses demonstrate reduced cell growth, migration and branching, which are key components of normal lung development (116). These findings are confirmed *in vivo* in which growth-restricted offspring demonstrate diminished pulmonary vascular function and density, together with decreased pulmonary alveolarization (116, 117). Abnormal pulmonary vascular development in growth restricted fetuses is likely to be a key mechanism increasing the risk of BPD, pulmonary hypertension and life-long reduction in respiratory capacity, such as seen in chronic obstructive disease (116).

Impact

BPD is a chronic lung disease characterized by arrested airway and parenchymal development and resulting in long-term respiratory complications, with a high susceptibility in preterm and growth restricted infants. BPD is a multifactorial condition, however it is primarily thought to result from chronic ventilation-induced injury in preterm infants, contributed by lung exposure to excess oxygen and inflammation. FGR is an independent risk factor for BPD in human infants (118–120). Being born subsequent to placental insufficiency and growth restricted is associated with a 3.6-fold higher risk of developing BPD than age-matched control infants (120), despite FGR infants having similar RDS rates as appropriately-grown counterparts. Lio et al. (121) have also recently shown that FGR infants with placental dysfunction have a 6-fold increased risk of developing BPD compared to low birth weight/ SGA infants. Further, they noted that birth weight *per se* and not ventilation duration, or other neonatal morbidities, contributed to the presence of BPD.

Maternal vascular unit deficiency, a marker for pre-eclampsia, is a common placental pathology associated with FGR, and it has also been shown that maternal vascular unit dysfunction doubles the risk of BPD in preterm human infants (118). Thus, human and animal data strongly support that the foundations of postnatal lung deficits and BPD are laid down *in utero* in FGR infants with placental insufficiency, and that vascular pathology is likely to be a contributing factor.

Neonatal pulmonary hypertension is highly associated with decreasing gestational age and low birth weight, and is a common complication of BPD (96). Pulmonary hypertension is characterized by hypoxemia of the newborn and right-to left shunting through the ductus arteriosus, due to maintenance of high pressures within the pulmonary circulation. Accordingly, neonatal pulmonary hypertension occurs via a failure of structural cardiovascular remodeling after birth, and is likely developmentally programmed *in utero* (122). In post mortem tissue analysis it is shown that newborns with pulmonary hypertension displayed reduced pulmonary vascular surface area with increased muscularization of distal pulmonary vasculature (123). These data suggest a strong association with FGR induced by vascular remodeling in chronically hypoxic fetuses, resulting in impaired control of vascular tone within the pulmonary circulation after birth. Altered pulmonary vascular composition has been more closely examined in growth restricted rats, demonstrating increased pulmonary vasoconstriction caused by local endothelial dysfunction and excessive collagen and reduced elastin in the pulmonary vasculature (124). Animal models of FGR also provide strong evidence that the hallmarks of pulmonary hypertension are already present in the growth-restricted fetus and offspring soon after birth. Our group and others have shown vascular changes, including decreased vascular density and dysfunction in fetal sheep (116) and in 2-h-old lambs (110). Thus, even prior to birth, FGR is associated with pulmonary hypertension.

The long-term effects of low birth weight have been examined in adult offspring conceived during the 1940s Dutch famine, who show an increased risk of obstructive airway disease (89). Further analysis of this cohort determined that neither serum immunoglobulin E concentration nor mean lung volumes were different (125). The authors speculate that bronchial reactivity must be the cause of the airway disease following growth restriction.

Neurological Morbidity

Clinical Features

FGR is strongly linked to suboptimal brain development, and long-term neurological dysfunctions in motor ability, cognition and learning, and behavior. We have recently reviewed the consequences of placental insufficiency and FGR on the developing brain (28), and describe that the age of onset and severity of FGR, together with gestational age at birth, play important modulatory roles in altered brain structure and function. The first indication of structural anomalies of the FGR brain can be derived from magnetic resonance imaging (MRI) during fetal development. MRI of the fetal brain during development demonstrates reduced brain volume, and altered cortical folding and brain morphology in FGR fetuses (126, 127). Arthurs et al. (128) showed lower diffusion weighted imaging values in parts of the brain in severe FGR fetuses as compared to normal age-matched controls, which were suggestive of an abnormal maturational profile. Postnatally, at term-equivalent age, MRI detects reduced intracranial volume, particularly contributed by decreased cortical gray matter volume in FGR infants (129), and altered developmental profile of white matter myelination (130), the hippocampus (131) and the basal ganglia (132) of growth-restricted infants, compared to appropriately grown infants. Functional MRI is also an upcoming tool to study whole brain functional networks in newborn infants for the assessment of altered organization and prediction of long-term neurodevelopment (133). Diffusion tensor imaging (DTI) and connectivity-based analysis of the FGR brain in the neonatal period is also being increasingly investigated (134).

MRI has the ability to detect even relatively small volume, structural, and organizational differences within the brain of FGR and appropriately-grown infants (135) but MRI capability and expertise in analysis is not readily available at all birth centers. In contrast, neonatal cranial US is widely used, but shows less sensitivity for detection of these subtle, but important neurological changes associated with neuropathology in FGR infants (136). Cranial ultrasound is frequently used as an assessment tool in premature infants, and term infants with severe FGR, to identify significant neuropathology in the neonatal period. There remains uncertainty as to whether cranial ultrasound can adequately detect neuropathology associated with FGR when compared to age-matched appropriately grown preterm infants (135, 136). Certainly in older preterm and term FGR infants, the benefit of routine cranial ultrasound screening in the neonatal period is questionable (137). We did not find evidence of altered cerebral ventricular volume using ultrasound imaging in FGR infants <10th centile, however we did observe a correlation between increasing ventricular volume and a decrease in functional motor scores (138). Cruz-Martinez et al. (139) have suggested that FGR infants with signs of middle cerebral artery and other Doppler abnormalities (indicative of significant brain sparing) are more likely to have neuropathology that can be detected on neonatal cranial ultrasound. This is interesting, as it further supports that the term *brain sparing* is a misnomer, and while it represents an appropriate survival response in the fetus, it is actually associated with worsening fetal condition and greater brain injury (28).

FGR infants frequently have a reduced head circumference compared to age-matched appropriately-grown infants, which is likely due to reduced brain volume (129), and reduced brain volume persists to 12 months of age (140). Cerebellar and hippocampal volumes may also be reduced (130). Brain myelination and connectivity have been shown to be adversely affected in FGR infants in the first 12 months of life, representative of white matter injury (141). Diffusion MRI of the human brain shows that the overall neuronal network complexity and connectivity of the FGR brain is reduced, with reduced global and local axonal circuits (142). Long-range cortical-basal ganglia (thalamocortical) connections are decreased in children born preterm with FGR, compared to children born preterm

but appropriately-grown (142), indicating that brain connectivity is significantly worse in children who were FGR compared to children who were preterm but well-grown. Deficits in brain connectivity correlate with neurobehavioral impairments including hyperactivity and poor cognition at school in children who were born FGR (143).

Neonatal functional assessment may detect early problems with neurological processing and behavior in infants who were born growth restricted. Tolsa et al. (129) showed that FGR newborns had specific alterations of brain structure as studied by volumetric MRI at preterm and term age, with reduced cortical gray matter volume correlating with deficits in attention and responsivity at term-equivalent age. General movement assessments (Prechtl movements) provide an early motor analysis, wherein abnormalities are predictive for cerebral palsy, and general movements may be adversely affected in some FGR infants (144). Similarly, electroencephalography performed early in the neonatal period has been shown to be affected and may correlate with adverse neurodevelopment in studies of FGR infants (145, 146). There is however limited data on early detection of functional deficits in growth-restricted infants, reflecting challenges in detecting delayed neurodevelopment in the neonatal period.

Pathophysiology

It is now well established that the traditional brain sparing physiology does not necessarily mean normal cerebral development *in utero* (28). In fact, fetuses with the most severe brain sparing are at the highest risk of adverse neurodevelopment in childhood. Prenatal loss of vasoreactivity in FGR has been suggested as a mechanism for poor outcomes, in which fetuses who do not adjust their cerebral circulatory control in response to hypoxic challenge may be more at risk of impaired cerebrovascular regulation (147). There are also reports of preferential perfusion and cerebral redistribution of brain blood flow in FGR fetuses, leading to some brain regions being at higher risk of injury (148). This is supported by work in fetal sheep to demonstrate that FGR is associated with regional cerebral blood flow redistribution, with the most notable differences between FGR and appropriately grown fetuses seen in the cerebral cortex and periventricular white matter (21).

Cerebral blood flow frequently continues to be abnormal for the first few days after birth in FGR human infants, but whether this puts infants at an increased risk of acute brain injury is not known. It has been reported the cerebral blood flow remains elevated after birth in FGR infants (149), even when the neonate is no longer exposed to a hypoxic environment and is no longer in need of a compensatory change in cardiac output. Postnatally, elevated cerebral blood flow might potentiate hyperoxia and oxidative stress within the fragile brain, which could also contribute to further neurological damage.

Animal models of chronic hypoxia and growth restriction have helped us to understand the development of neuropathology associated with placental insufficiency and FGR (28, 150–152). Adverse effects on brain gray matter development, white matter, and cerebellum have been described both in sheep, rabbit and rat models of FGR (153–155). In fetal sheep, we showed that early-onset placental dysfunction is associated with more widespread and severe white matter brain injury and neuroinflammation compared with late-onset, however both early- and late-onset FGR demonstrate complex patterns of gray and white matter neuropathology (154). Animal studies also show that the severity of brain injury, and the resultant neurodevelopment deficits, depends on the extent and severity of brain involvement in FGR (156). Hypomyelination and delayed myelination due to oligodendrocyte maturational deficits have been identified as possible mechanisms causing the white matter injury seen in FGR infants (157). Deficits in neuronal connectivity have also been described in animal models (158). Our group, and others, has observed that deficits in various components of the neurovascular unit play a significant role in the brain injury seen in animal models of FGR (159). Prematurity is a confounder in human FGR, but studies in FGR animals allow the separation of growth restriction and preterm birth. The individual contributions of preterm birth and/or neonatal ventilation of the FGR newborn on the progression of brain injury are now being examined (160, 161). These studies have determined that preterm birth and ventilation synergistically predispose the vulnerable FGR brain to neuropathology.

Impact

FGR infants are at increased risk of adverse neurodevelopmental outcomes in childhood. Neurological morbidities related to motor deficits, including cerebral palsy, behavioral issues, and cognitive impairment is significantly increased in young children and adolescents who were diagnosed as growth restricted at birth (28, 162–164). The risk of cerebral palsy is 30-fold greater in FGR infants, compared to those that are well grown (165), and increases with worsening growth restriction. Overall, >40% of children who have cerebral palsy had a low birth weight; that is, they were growth restricted, born preterm, or both (166). This is important, as FGR and preterm birth are frequent co-morbidities. In addition to motor deficits, preterm FGR infants followed-up at 1, 2, and 3 years of age showed deficits in cognition and behavioral outcomes compared to preterm age-matched appropriately-grown infants (167). Further, a longitudinal study observing FGR offspring with evidence of brain sparing from birth to middle school age (9–10 years old) found a complex set of neurodevelopmental deficits, such as a significant reduction in IQ, compared to age-matched appropriately-grown children (168). Multiple follow-up studies of FGR infants into school age describe diminished gross and fine motor skills, cognition, memory, and academic ability, as well as neuropsychological dysfunctions encompassing poor attention, hyperactivity and altered mood (143, 169–171). FGR infants born preterm and those with fetal circulatory redistribution are at the greatest risk for the worst outcomes (172). These adverse outcomes can continue into adolescence and young adulthood (173). It is apparent that determining the neurodevelopmental consequences of FGR is complicated by the severity of FGR, early- or late-onset, and the gestational age at delivery (28). However, in both early- and late-onset FGR, the presence of

cardiovascular redistribution and brain-sparing is associated with abnormal neurodevelopmental outcomes (28).

Interventions for Improved Outcomes

Management of pregnancies complicated by FGR represents a balance between antenatal compromise, often with worsening chronic hypoxia that contributes to subpotimal organ development, and the risks associated with preterm delivery and postnatal intensive care, which may also contribute to morbidities. In high-income countries, about half of fetuses with moderate- to severe-growth restriction are detected antenatally and are therefore amenable to treatment during pregnancy, but it remains that nearly 40% of infants born at the 3rd centile for weight are not detected *in utero* (44). With this in mind, both antenatal and postnatal therapies must be considered. Currently, no specific treatment is available for FGR. Potential treatments should target maldevelopment of multiple organs, various injurious pathways, cell types, and structural deficits that manifest over different developmental stages. Here we will provide an overview of the current state of understanding for a handful of treatments for FGR (**Figure 3**).

Antenatal

Antenatal treatments are principally aimed at improving placental function and thereby increasing fetal growth *in utero*. To date, the best studied of these has been sildenafil citrate. Sildenafil is a potent phosphodiesterase type 5 (PDE5) inhibitor that is an effective smooth muscle relaxant where the PDE5 enzyme is present in an organ or tissue, as is the case for the human placenta (174). The effects of sildenafil on smooth muscle are mediated via an enhanced and prolonged nitric oxide release leading to vasodilatation. Both *in vitro* and *in vivo* studies demonstrate that sildenafil vasodilates human myometrium vessels from normal (175, 176) and growth restricted placenta. Most experimental studies to date support that sildenafil increases fetal weight in compromised rat, sheep and human pregnancies (177). In contrast, we have shown that antenatal sildenafil administration to pregnant sheep with placental insufficiency decreases fetal weight and worsens fetal hypoxia (178). Although initial preclinical evidence for the multinational STRIDER trial suggested improved outcomes for FGR infants, this trial has now been aborted due to unexpected baby deaths (179), leading to a call for increased preclinical studies underpinning clinical trials (180), and improved understanding of the effects of sildenafil on the fetus given that it crosses the placenta (181). The longer acting tadalafil remains an active clinical experimental treatment of interest as an antenatal therapy for FGR and, given that tadalafil does not cross placenta (174), it may be more favorable as a targeted placental treatment.

The EVERREST Project is also investigating a targeted approach to improve placental function in pregnancies complicated by FGR using gene therapy to inject vascular endothelial growth factor (VEGF) into uterine arteries (182). VEGF is known for its role in inducing angiogenesis and in the EVERREST Project it is hypothesized that application of adenovirus VEGF in, or near placental arteries will induce a local and acute increase in VEGF expression, and subsequent angiogenesis of the placental vasculature. Preclinical studies have shown promise with improved blood flow (183) and fetal weight gain (184) in animal models of growth restriction, resulting from the improved vascularization of the placenta. The clinical trial is ongoing.

A recent large animal (sheep) study examined intra-amniotic administration of the growth-promoting protein insulin-like growth factor-1 (IGF-1) (16). This work showed that increasing the bioavailability of IGF-1 in pregnancies complicated by placental insufficiency and FGR improved birth weight in female lambs, but not males, and modified postnatal catch-up growth in both females and males. Intrauterine IGF-1 also mediated expression of key somatotrophic and metabolic genes, indicative that antenatal treatment could be utilized to positively affect postnatal growth and wellbeing.

A number of antenatal treatments have been explored preclinically that aim to restore fetal oxidative tone via maternal antioxidant administration, using agents such as allopurinol, melatonin and vitamin C (75, 79, 185). Antioxidant treatment has principally targeted improved cardiovascular and neurological outcomes in growth-restricted offspring. To date, melatonin has been the most widely studied, given melatonin's established safety profile, ease of administration, and strong antioxidant benefits. In sheep, we have shown that maternal melatonin administration to ewes carrying a growth restricted fetus results in a significant improvement in vascular function and reduced arterial stiffness, two vital pathologies evident in FGR offspring, which predispose to cardiovascular disease (75). Melatonin administration also resulted in improved cardiac function in the right ventricle. Further, this study showed that maternal melatonin improved fetal oxygenation and increased birth weight (75), however other ovine studies show either no improvement in birth weight (186) or exacerbation of growth restriction with melatonin (187). In cultured human umbilical vein endothelial cells (HUVECs), melatonin improves vascular endothelial integrity, likely via combined anti-oxidant and anti-inflammatory mechanisms (188). Exposure to antenatal melatonin does not reverse alveolar simplification in FGR newborn lambs (102), but does improve pulmonary vascular structure and function (189), and pulmonary tone may be maintained long term via alteration to receptor populations (190). As our understanding of perturbations to lung growth in FGR offspring continues to be explored, so too does the opportunity for targeting novel pathways. For example, recent work has shown that NPY is down regulated in FGR, where NPY is a sympathetic neurotransmitter that is critical for normal lung growth (191).

The effects of maternal melatonin administration on brain development have also been examined. Antenatal melatonin crosses the placental and the blood brain barrier, and melatonin is a strong antioxidant and also demonstrates anti-inflammatory benefits in the developing brain (186, 192–194). In pregnancies complicated by placental insufficiency and FGR, maternal melatonin improves white matter brain development via increased myelination and decreased axonopathy in the fetal brain, and subsequently, neurobehavior of FGR+MLT lambs is significantly improved after birth (186). Melatonin has also been shown to have beneficial effects on cerebral vasculature

by preventing FGR-related apoptosis and disruption of blood brain barrier instability via improved vascular interactions with astrocytes and pericytes (195). Antenatal melatonin has been examined in pilot studies to treat FGR (186) and preeclampsia (188) with results supporting that melatonin is an effective anti-oxidant that is safe for the mother and baby, and may extend pregnancy (196).

Emerging evidence supports that the glycoprotein lactoferrin shows potential as an antenatal treatment for pregnancies complicated by FGR, particularly for the developing brain (197). Lactoferrin is a glycoprotein that demonstrates strong antioxidant, anti-inflammatory and anti-microbial effects—important factors that could mediate neuroprotective benefits. In rats, lactoferrin supplementation during pregnancy shows positive benefits for dexamethasone-induced fetal growth restriction (197). Maternal lactoferrin significantly increased birth weight of control rat pups, and FGR offspring exposed to lactoferrin showed a normalized weight at postnatal day 21. Lactoferrin supplementation also improved brain hippocampal structure and stimulated brain derived neurotrophic factor (BDNF) (197), important observations in light of the neuropathology associated with human FGR. Nutritional supplementation (glucose, amino acids and electrolytes) into the amniotic sac of FGR rabbits has also recently been explored, with some promising results suggesting that survival rate for FGR offspring was improved with treatment, although birth weight and cardiac function deficits were not improved (198).

Postnatal

As mentioned above, nearly 40% of human infants with severe FGR are not detected antenatally (44), and therefore not amenable to antenatal treatments. In light of this we must continue to investigate therapies to improve multi-organ dysfunctions in growth restricted infants. We have highlighted in this paper that deficits in cardiovascular, pulmonary and cerebral development are already present at birth in FGR infants, principally caused by chronic hypoxia *in utero*. Therefore any potential postnatal therapy would aim to be reparative and to prevent progression of ongoing multi-organ damage.

Lactoferrin shows great potential as a postnatal therapy, in addition to positive effects antenatally. Lactoferrin is highly abundant in human colostrum and milk, and it reaches the brain after oral administration (197, 199). In this regard, breastfed infants show higher total anti-oxidant capacity and a lower oxidative stress index compared to non-breastfed infants. Importantly, randomized controlled trials with nutritional lactoferrin supplementation in premature neonates demonstrate a promising reduction in late onset sepsis and necrotizing enterocolitis (200). Lactoferrin supplementation during lactation is protective for neonatal rats exposed to either hypoxia-ischemia or lipopolysaccharide-induced systemic inflammation (201, 202). Analysis of the neonatal rat brain using a combination of advanced MRI analysis and histology demonstrates that gray and white matter microstructure is normalized with lactoferrin supplementation, myelination is protected and measures of axonal integrity and brain organization are restored in rats with lactoferrin supplementation (201). Early environment enrichment or postnatal stimulation has also been shown to have some benefits in brain connectivity in a rabbit model of FGR (203). While there are no current published studies on stem cell therapy for FGR related brain injury, our lab and others are working on testing their applications in FGR and other pregnancy complications (204). The application of postnatal therapies to improve multi-organ deficits associated with FGR should remain a foremost preclinical research area.

CONCLUSIONS

Understanding the pathophysiological mechanisms that underlie neonatal morbidities that are particularly associated with FGR provide the fundamental basis for improving short- and long-term outcomes in growth restricted offspring. It is clear that placental compromise and chronic fetal hypoxia program the fetus for suboptimal growth and development, with fetal cardiovascular dysfunctions and altered organ development already apparent in the FGR fetus during pregnancy. The timing of the onset of placental insufficiency, the severity of growth restriction, the degree of cardiovascular adaptation, and gestational age at birth are all critical factors that modify outcome for FGR infants. In the neonatal period, FGR infants demonstrate early evidence of cardiac, vascular, pulmonary, neurological and other deficits, which can lead to long durations in neonatal intensive care, and long-term health problems. Improved antenatal detection, and both antenatal and postnatal therapies that target the key pathophysiological mechanisms underlying altered multi organ structure and function must be considered critical research areas.

AUTHOR CONTRIBUTIONS

AM and BA critically researched literature, co-wrote the first draft of the manuscript and approved the final version. MC-M, GJ, GP, and SM reviewed the manuscript, contributed to various sections and approved the final version.

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1.2. Overall PhD Aims and Hypothesis

1.2.1. Rationale and Global Aim

The literature review in Chapter 1 brings together strong evidence that neonatal morbidities associated with fetal growth restriction are contributed by both antenatal and postnatal compromise or complications. The rates of respiratory morbidity, including bronchopulmonary dysplasia (BPD), are increased in FGR infants and relate to the number of days spent on neonatal ventilation. FGR infants also have elevated rates of neuropathology evident on imaging and neurodevelopmental follow up, however it is not known whether brain injury is only sustained in utero or exacerbated by preterm birth and neonatal ventilation; this was the rationale for the study conducted in Chapter 2.

The results obtained in Chapter 2 demonstrate that FGR related white matter brain injury is present at preterm birth and is made worse with ventilation, compared to control lambs and unventilated FGR lambs. Brain injury in FGR lambs is mediated by upregulation of neuroinflammation and oxidative stress pathways, leading to neurovascular compromise, breakdown of the blood brain barrier and apoptotic cell death. These findings indicated that a potential targeted therapy (with stem cells) for FGR related brain injury in ventilated premature lambs may be feasible; this was next evaluated in Chapter 3. Stem cell therapies have received great interest in the past decade and show excellent potential as neuroprotective intervention because of their anti-inflammatory, antioxidant, anti-apoptotic, neurogenic and angiogenic properties. I chose umbilical cord blood derived stem cells as the stem cells of choice for these experiments given their ease of availability, and versatility arising from multiple stem cell lines that constitute umbilical cord blood. Further, currently there are no studies investigating the use of stem cell therapies as an early treatment option for FGR related brain injury.

The literature review in Chapter 4 presents current evidence on the tools available for the detection and assessment of FGR related brain injury in the fetus and newborn. It is well recognised that FGR brain injury may not always be evident on conventional imaging, which in most centres is limited to neonatal cranial ultrasound and conventional MRI brain scans. Advanced MRI analysis techniques include post processing of diffusion MRI data to delineate brain region and fibre specific characteristics of structural brain changes. In Chapter 5, I evaluated whether advanced MRI analysis techniques may aid in the detection of brain injury associated with FGR. This injury is principally in the white matter regions of the brain, and hence histological analysis of white matter brain regions was also conducted to correlate with the changes seen on advanced MRI analysis.

The global aim for my thesis was to study the pathophysiology, early detection and early treatment of FGR related brain injury in preterm lambs.

1.2.2. Specific Aims

1. To study the impact of neonatal ventilation on FGR related brain injury in preterm lambs.
2. To study the effect of umbilical cord blood derived stem cells in FGR related brain injury in preterm lambs.
3. To study the use of advanced MRI analysis techniques to detect FGR related brain injury in preterm lambs.

1.2.3. Hypotheses

1. Neonatal ventilation may exacerbate FGR related brain injury in preterm lambs.
2. Umbilical cord blood stem cells, when given early in the neonatal period may mitigate FGR related brain injury in preterm lambs.
3. Advanced MRI analysis techniques may detect subtle FGR related brain injury in preterm lambs.

Chapter 2

Through my clinical work, I was aware that infants born growth restricted and/or preterm had increased risks for neonatal morbidities associated with pulmonary, cardiovascular and neurological function. There were a good number of individual preclinical and clinical studies to describe these morbidities, however there was no single reference or review to bring these pieces of information together. Accordingly, in Chapter 1, I performed a literature review on neonatal morbidities of FGR, focusing primarily on the pathophysiology of the neurological, cardiac and respiratory complications of FGR fetuses and neonates. A notable gap in literature I identified was the paucity of literature on the brain injury associated with premature birth and whether or how neonatal ventilation might contribute to altered brain structure and function in FGR neonates.

A significant number of FGR infants are delivered preterm and are exposed to the potential "double-hit" of FGR and neonatal ventilation. In this chapter, I therefore focused on the pathophysiology of brain injury resulting from neonatal ventilation in our preterm lamb model of FGR.

I induced early-onset placental insufficiency and subsequent FGR and delivered lambs preterm. Lambs were then ventilated according to clinical guidelines, and I evaluated the impact of neonatal ventilation and FGR on brain development. This study is directly related to the overall aims of the thesis to examine "pathophysiology", "early detection" and "early treatment" of FGR brain injury. Understanding the pathophysiology of neonatal ventilation-associated FGR brain injury will provide us with the fundamental causes of neuropathology in this population of infants and, in turn, help us to design targeted neuroprotective therapies for this high risk cohort of infants. This is the unaltered version of the paper published in American Journal of Physiology: Regulatory, Integrative and Comparative Physiology.

2.1. Neuropathology as a consequence of neonatal ventilation in premature growth restricted lambs

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RESEARCH ARTICLE | *Neural Control*

Neuropathology as a consequence of neonatal ventilation in premature growth-restricted lambs

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Malhotra A, Castillo-Melendez M, Allison BJ, Sutherland AE, Nitsos I, Pham Y, Alves de Alencar Rocha AK, Fahey MC, Polglase GR, Jenkin G, Miller SL. Neuropathology as a consequence of neonatal ventilation in premature growth-restricted lambs. *Am J Physiol Regul Integr Comp Physiol* 315: R1183–R1194, 2018. First published September 19, 2018; doi:10.1152/ajpregu.00171.2018.—Fetal growth restriction (FGR) and prematurity are associated with high risk of brain injury and long-term neurological deficits. FGR infants born preterm are commonly exposed to mechanical ventilation, but it is not known whether ventilation differentially induces brain pathology in FGR infants compared with appropriate for gestational age (AGA) infants. We investigated markers of neuropathology in moderate- to late-preterm FGR lambs, compared with AGA lambs, delivered by caesarean birth and ventilated under standard neonatal conditions for 24 h. FGR was induced by single umbilical artery ligation in fetal sheep at 88-day gestation (term, 150 days). At 125-day gestation, FGR and AGA lambs were delivered, dried, intubated, and commenced on noninjurious ventilation, with surfactant administration at 10 min. A group of unventilated FGR and AGA lambs at the same gestation was also examined. Over 24 h, circulating pH, Po_2, and lactate levels were similar between groups. Ventilated FGR lambs had lower cerebral blood flow compared with AGA lambs ($P = 0.01$). The brain of ventilated FGR lambs showed neuropathology compared with unventilated FGR, and unventilated and ventilated AGA lambs, with increased apoptosis (caspase-3), blood-brain barrier dysfunction (albumin extravasation), activated microglia (Iba-1), and increased expression of cellular oxidative stress (4-hydroxynonenal). The neuropathologies seen in the ventilated FGR brain were most pronounced in the periventricular and subcortical white matter but also evident in the subventricular zone, cortical gray matter, and hippocampus. Ventilation of preterm FGR lambs increased brain injury compared with AGA preterm lambs and unventilated FGR lambs, mediated via increased vascular permeability, neuroinflammation and oxidative stress.

blood-brain barrier; FGR; IUGR; neuroinflammation; oxidative stress

INTRODUCTION

Fetal growth restriction (FGR) describes the pregnancy condition in which a fetus does not grow to its genetic potential, affecting up to 9% of all pregnancies (21). FGR is associated with stillbirth, and in survivors is strongly linked to prematurity, neonatal mortality, and morbidities (4). Past infancy, FGR is acknowledged as a significant contributor toward deficits in neurological, cardiovascular, and metabolic functions (8, 53).

Placental insufficiency is the principal cause of FGR resulting in progressive reduction in transfer of oxygen and nutrients to the developing fetus (21). Chronic hypoxia induces redistribution of fetal cardiac output resulting in body growth restriction with "brain sparing" in an attempt to ensure adequate oxygenation of essential organs (particularly brain and heart), but brain sparing is not totally neuroprotective (18, 35). FGR is associated with specific impairments in brain structure and function, where neurodevelopmental deficits depend on the fetal age at onset of poor placental function (early or late) detected by ultrasound imaging, severity of growth restriction, and gestation at birth (35). Early onset FGR, generally diagnosed in midpregnancy, is more likely to be linked with severe placental dysfunction and a greater degree of growth restriction (20). Brain development is also profoundly compromised in early onset FGR, as demonstrated in both human imaging studies and experimental animal studies (2, 13). Assessment of the neuropathology associated with early onset FGR is often confounded by the strong link between the presence of early placental dysfunction and FGR, with preterm birth (22). When FGR infants are delivered prematurely, they are likely to require neonatal intensive care, including resuscitation at birth and invasive mechanical ventilator support over the first days of life. Mechanical ventilation, particularly when poorly controlled, is associated with inflammation and lung and brain injury in appropriately grown preterm infants (12). The onset of ventilation increases early biomarkers of neuropathology in late-onset FGR lambs (1), but the interactions between chronic in utero compromise arising from early onset placental dysfunction and neonatal ventilation on the brain have not been well studied.

In this study we examined the short-term (24 h) effects of ventilation in preterm lambs with early onset FGR. There is an association between intensity and duration of mechanical ventilation and brain injury, and we hypothesized that mechanical ventilation in the neonatal period may exacerbate neuropathology associated with early onset FGR. To test this hypothesis, we used our early onset (0.6 gestation) placental insufficiency and FGR sheep model (2), with lambs delivered preterm and

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ventilated using standard clinical care conditions for 24 h. We then examined the effects of neonatal ventilation on brain injury in the premature growth-restricted lamb using established physiological, histological, biochemical, and tissue immunohistochemistry methods. This would assist in designing targeted therapies for brain injury in vulnerable infants.

METHODS

Ethics Approval

Experiments complied with the National Health and Medical Research Council of Australia guidelines for the care and use of animals for scientific purposes and were approved by Monash Medical Centre Animal Ethics Committee A.

Surgery to Induce FGR

Single umbilical artery ligation (SUAL) was the experimental procedure used to induce FGR, as reported by us previously (38, 48). At 88 days gestation (term is 150 days), pregnant twin-bearing Border-Leicester Merino crossbred ewes underwent sterile surgery induced with sodium thiopentone (Pentothal; Bomac Laboratories, Auckland, New Zealand) and maintained under 1–2.5% isoflurane (Isoflo; Abbott Australasia, Botany, NSW, Australia). The first fetus exposed from the uterus was assigned as the control fetus [appropriate for gestational age (AGA)] in which the umbilical cord was manipulated (the outer sheath was opened and umbilical artery identified) but not ligated. In the second fetus, two silk sutures were tied tightly around one of the umbilical arteries (SUAL), ~1 cm apart, 3–4 cm from the fetal body. A single lumen polyethlyene catheter (inner diameter: 0.5 mm; outer diameter: 1.0 mm; Critchley Electrical, Kingsgrove, NSW, Australia) was inserted into the femoral artery of each of the AGA and FGR fetus, and catheters were exteriorized through an incision in the right flank of the ewe. A maternal jugular vein catheter was also implanted via an incision in the jugular groove of the right side of the neck. The ewe was recovered from anesthesia and surgery and provided analgesia (paracetamol suppository; Panadol; GSK).

Fetal Monitoring

For 3 consecutive days after surgery, antibiotics (ampicillin and Engemycin; Austrapen; CSL) were administered intravenously to the ewe. Fetal blood samples were taken daily for assessment of fetal well-being, until 10 days postsurgery. The partial pressures of arterial oxygen (Pa_{O_2}), carbon dioxide (Pa_{CO_2}), oxygen saturation (Sa_{O_2}), pH, hematocrit, glucose, HCO_3^-, and lactate were measured (ABL 700 blood gas analyzer; Radiometer, Copenhagen, Denmark). At fetal gestational age 123 and 124 days, betamethasone (Celestone Chronodose; Schering Plough) was administered to the ewe to mature the lungs before planned preterm delivery, as previously described (49); this is equivalent to that used in human preterm pregnancies (2 doses of 11.4 mg im 24 h apart).

Lamb Delivery and Ventilation

At 125 days gestation, ewes were placed under general anesthesia, as above, and a caesarean delivery performed to first access the head and chest of each fetus, in turn. A transonic flow probe (size 4; ADInstruments) was placed and secured around the carotid artery of the fetus to measure carotid blood flow as an index of cerebral blood flow (CBF). The umbilical cord was clamped and cut, and the lamb was delivered. The lamb was dried, weighed, and transferred to an infant warmer (Fisher and Paykel) where each lamb was intubated (4.0-mm cuffed endotracheal tube), lung liquid was passively drained, and ventilation commenced. Umbilical venous and artery single lumen polyurethane catheters (size 5 mm) were inserted and secured using silk sutures. A pulse oximeter probe (Masimo, Irvine, CA) was placed around the tail for measurement of transcutaneous oxyhemoglobin saturation levels (Sp_{O_2}). Near infrared spectroscopy (FORE-SIGHT Tissue Oximeter; CAS Medical Systems, Branford, CT) was used for continuous recording of cerebral oxygenation using a small sensor, which was placed over the head (the frontoparietal brain region) and covered with a lightproof (aluminum foil) dressing. Cerebral oxygenation was expressed as tissue oxygenation index (%) at 0.5 Hz. Cerebral oxygen extraction was then calculated using CBF, Sp_{O_2}, and tissue oxygenation index as previously described (40).

Ventilation of FGR and AGA lambs (vent FGR and vent AGA) was initiated using assisted control ventilation (Babylog 8000 plus; Dräger, Lüberk, Germany) with an initial peak inspiratory pressure of 30 cmH_2O and positive end-expiratory pressure of 5 cmH_2O for the first 10 min. The inspired oxygen fraction (FI_{O_2}) commenced at 0.3 but was adjusted to maintain Sp_{O_2} between 85 and 95% after initial resuscitation. All lambs received prophylactic surfactant (100 mg/kg; Curosurf, Chiesi Pharma, Italy) via the endotracheal tube at 10 min after birth. Lambs were subsequently ventilated for 24 h using volume guarantee mode with a tidal volume (V_T) set at 5–7 ml/kg. The settings for lamb ventilation were based on previous studies of preterm lambs from our group, which are known not to result in significant lung injury (10, 46). Throughout ventilation, rectal temperature of the lamb was monitored and maintained within normal range (38.5–39.5°C), and lambs were kept lightly sedated by continuous infusion of Alfaxan (2–4 $mg \cdot kg^{-1} \cdot min^{-1}$; Jurox, Rutherford, NSW, Australia) via the umbilical vein catheter. Lamb well-being was assessed by regular arterial blood gas measurements via samples collected from the umbilical arterial catheter. At the completion of the experiment, lambs were humanely killed by intravenous pentobarbital sodium overdose (100 mg/kg iv; Valabarb, Rutherford). All ventilation and physiological parameters were digitally acquired using Powerlab (1 kHz) and Laboratory Chart 8 software (ADInstruments, Castle Hill, Australia).

A separate cohort of animals was euthanized without ventilation (unvent AGA and FGR) at the time of delivery (at 125 days) immediately after the cord was cut.

Brain Processing

After euthanasia at around 24 h after delivery, cerebrospinal fluid (CSF) was collected using a 3-ml syringe and 18-gauge needle, and the brain was then removed and weighed. The left brain hemisphere was divided into four sections (anterior to posterior) and frozen for analysis. The right brain hemisphere was coronally cut into 5-mm slices/blocks and fixed in formalin for 48–72 h and then embedded in paraffin (ProSci Tech; Thuringowa, QLD, Australia) for histological and immunohistochemistry analysis.

Histological Staining

Hematoxylin and eosin (H&E; cat. no. HH-1NPR and EOA1-1L; Amber Scientific, WA, Australia) staining was performed to examine for the presence of gross neuropathology. Thioflavin T (cat. no. T3516-25G; Sigma, St. Louis, MO) staining was performed to assess for the deposition of amyloid within the brain. After serial hydrations, sections were immersed in 1% thioflavin for 30–60 min, dehydrated, coverslipped, and assessed under fluorescent microscopy. Congo red stain was also used to confirm the presence of amyloid presence in tissue sections. With this technique, amyloid deposits stain red and cell nuclei blue. Briefly, sections were deparaffinized, rehydrated, and stained in Congo red solution for 15–20 min at room temperature. After being rinsed in distilled water, the sections were quickly differentiated in alkaline alcohol solution and counterstained with Gill's hematoxylin for 30 s. After being rinsed in tap water for 2 min, the sections were dehydrated, cleared, and coverslipped.

Molecular Assessment and Cytokine Assays

Concentrations of intercellular adhesion molecule, vascular cell adhesion molecule, interleukin (IL)-3, IL-6, neuron-specific enolase, Decorin, interferon-γ, IL-17A, IL-21, IL-8, IP-10, monokine induced by gamma, secreted frizzled-related protein, tumor necrosis factor-α, and vascular endothelial growth factor A were assessed in lamb serum and CSF samples using a human 5-plex and an ovine 10-plex quantibody array following the manufacturer's instructions (Crux Biolab, Scoresby, VIC, Australia). Ovine brain-derived neurotrophic factor, nerve growth factor, and amyloid precursor protein (26) ELISAs were also conducted on homogenized brain tissue.

Immunohistochemistry of Brain Tissue Sections

Single label immunohistochemistry. Immunohistochemistry (IHC) utilizing selective antibodies for imaging-specific antigens in brain tissue was conducted. Cellular apoptosis was assessed using activated caspase-3 (cat. no. AF835; R&D Systems, Minneapolis, MN), astrocytes were assessed using glial fibrillary acidic protein (GFAP; cat. no. G3893; Sigma-Aldrich), cerebral inflammatory cells were evaluated using ionized calcium-binding adapter molecule 1 (Iba-1; cat. no. 019-19741; Wako Pure Chemical Industries, Osaka, Japan), blood-brain barrier (BB) integrity was assessed by staining with albumin (cat. no. S4265-2ML; Sigma-Aldrich), and oxidative stress was assessed using 4-hydroxynonenal (4HNE, 100 μl; cat. no. 393207; Merck). Briefly, for IHC, brain blocks containing cortical gray matter (CGM), subcortical white matter (SCWM), periventricular white matter (PVWM), subventricular zone (SVZ), external capsule, hippocampus, and corpus callosum (7) were sectioned at 10 μm at the level of the striatum. Serial sections were placed on SuperFrost glass slides (Menzel Glaser) and dewaxed in xylene followed by rehydration in serial ethanol solutions. Antigen retrieval was carried out by heating in citric acid buffer (pH 6) for 15 min (3 × 5 min) and allowing the sections to remain in the hot buffer at room temperature for a further 20 min, followed by incubation in 0.3% hydrogen peroxide buffer in 50% methanol. Nonspecific binding was blocked by animal serum (goat or rabbit serum in BSA). Respective primary antibody was then added and sections incubated at 4°C overnight. The following day, sections were incubated in the appropriate secondary antibody followed by streptavidin horseradish peroxidase (2 ml, 1:200; cat. no. GERPN1051; Amersham Bioscience). Staining was visualized using 3,3′-diaminobenzidine (Pierce Biotechnology, Rockford, IL), and sections were coverslipped using mounting medium (DEPX; Merck, Kilsyth, Australia).

Vascular and astrocyte double label fluorescent IHC. First, endogenous peroxidases were blocked with 0.3% hydrogen peroxide in 50% methanol, and sections were then washed with sodium borohydride (10 mg/ml) in PBS to reduce autofluorescence. Sections were subsequently treated with a serum-free protein blocker (DAKO, Campbellfield, VIC, Australia) and incubated with monoclonal anti-GFAP (1:200; Sigma) and laminin (1:200; Sigma-Aldrich) to identify astrocyte processes (end-feet) associated with blood vessels. Fluorescent secondary antibodies were used to study the astrocyte blood vessel interface.

Quantitative Analysis of Brain Injury

Sections were viewed at a magnification of ×400 using light microscopy (Olympus BX-41) and examined in a blinded fashion by two investigators (A. Malhotra and M. Castillo-Melendez). Immunoreactive cell counts and/or density of stain were assessed in three fields of view within regions of interest on two slides per animal to give six fields of view per region per animal, for which an average was then calculated. Manual counts of GFAP-positive astrocytes, Iba-1 (activated or amoeboid microglia), caspase-3 (cell death), and 4-HNE (oxidative stress) were undertaken. Albumin IHC, GFAP-laminin double label, and H&E staining for neuropathological features were assessed descriptively and semiquantitatively. The percentage of end-feet perivascular astrocyte coverage of blood vessels was determined using GFAP and laminin; total number of laminin-positive blood vessels was first manually counted, after which only the blood vessels showing association with astrocyte end-feet (coverage) were counted. The percentage of blood vessels in close contact with GFAP-positive astrocytes was then calculated from the total number of blood vessels counted per field of view and expressed as percent astrocyte end-feet blood vessel coverage as previously described (16).

Statistics

Data are presented as means ± SE. Statistical comparisons were carried out using GraphPad Prism (version 5.0a; GraphPad Software, San Diego, CA). Body and organ weights, cell counts, and density staining were all analyzed by two-way ANOVA and Bonferroni post hoc tests for multiple comparisons between groups. Blood gas data werre analyzed by two-way repeated measures ANOVA and Tukey posttest using SigmaPlot (version 12; Systat Software, San Jose, CA). Significance was accepted at $P < 0.05$.

RESULTS

Baseline Characteristics

A total of 24 lambs ($n = 6$ per group for unvent AGA, unvent FGR, vent AGA, and vent FGR) were studied. There were five fetal losses (3 AGA and 2 FGR) and one neonatal death (FGR), and data are not included from these animals. Body weight and organ weight-to-body weight ratios are shown in Table 1. SUAL induced placental insufficiency and FGR; body weight at birth in FGR lambs was reduced by ~15%

Table 1. *Body and organ weight data of lamb groups*

	Unvent AGA	Unvent FGR	Vent AGA	Vent FGR
Male:female	3:3	2:4	3:3	3:3
Body weight, kg	2.8 ± 0.1	2.4 ± 0.2*	3.0 ± 0.2	2.5 ± 0.2*
Brain weight, g	45.9 ± 1.7	45.1 ± 0.5	43.9 ± 0.9	43.9 ± 1.9
Brain/body weight, g/kg	16.4 ± 0.7	19.5 ± 1.6*	14.8 ± 0.7	17.9 ± 1.0*
Liver weight, g	86.3 ± 9.4	70.4 ± 8.2	127.3 ± 10.7*	94.4 ± 12.5*
Liver/body weight, g/kg	30.2 ± 1.9	29.2 ± 2.1	42.1 ± 1.5*	37.4 ± 3.1*
Lung weight, g	88.7 ± 7.9	78.0 ± 6.1	93.6 ± 4.6	80.0 ± 15.2
Lung/body weight, g/kg	31.4 ± 2.0	33.0 ± 2.0	31.6 ± 2.2	31.4 ± 4.2
Heart weight, g	22.3 ± 1.4	19.6 ± 1.4	26.7 ± 1.8	20.4 ± 2.8
Heart/body weight, g/kg	7.9 ± 0.1	8.2 ± 0.2	8.9 ± 0.6	8.1 ± 0.8

Data are expressed as ratio or means ± SE. AGA, appropriate for gestational age; FGR, fetal growth restriction; unvent, unventilated; vent, ventilated. *Significant differences (two-way ANOVA) between FGR and AGA lamb group in body weights ($P = 0.02$) and brain-to-body weight ratios ($P = 0.008$) and between ventilated and unventilated lamb groups in liver-to-body weight ratios ($P = 0.0002$).

Table 2. *Blood gas measurements in the first 24 h after birth*

	1 h	6 h	12 h	24 h
pH^T				
Vent AGA	7.30 ± 0.01	7.45 ± 0.02	7.35 ± 0.04	7.36 ± 0.05
Vent FGR	7.27 ± 0.03	7.47 ± 0.04	7.37 ± 0.05	7.33 ± 0.06
P_{O2}, mmHg				
Vent AGA	38.0 ± 5.9	47.3 ± 5.9	34.6 ± 3.1	39.0 ± 3.2
Vent FGR	35.3 ± 3.2	34.9 ± 2.0	38.2 ± 4.2	41.8 ± 3.6
$P_{CO_2}{}^T$, mmHg				
Vent AGA	51.3 ± 4.4	39.8 ± 2.7	58.7 ± 7.1	45.6 ± 6.4
Vent FGR	52.2 ± 2.9	35.1 ± 4.3	49.9 ± 3.5	37.6 ± 9.6
$Lactate^{T/G}$, mmol/l				
Vent AGA	3.5 ± 0.3	2.1 ± 0.3	1.6 ± 0.1	1.4 ± 0.2
Vent FGR	4.2 ± 0.5	3.0 ± 0.2	1.8 ± 0.1	1.8 ± 0.3
$HCO_3^{-T/G}$, mmol/l				
Vent AGA	27.9 ± 2.0	27.5 ± 0.7	31.2 ± 1.2	24.3 ± 6.0
Vent FGR	23.6 ± 2.3	25.5 ± 1.5	27.4 ± 1.6	15.9 ± 0.8

All values are displayed as means ± SE. AGA, appropriate for gestational age; FGR, fetal growth restriction; vent, ventilated. Two-way ANOVA showed significant ($P < 0.05$) time-related influences (T) on pH and P_{CO_2} and time-related and FGR-related (T/G) influence on lactate and HCO_3^-.

as compared with AGA lambs [2.4 vs. 2.8 kg (unvent); 3.0 vs. 2.5 kg (31); $P = 0.02$, two-way ANOVA]. Brain-to-body weight ratios were also significantly increased in FGR lamb groups as compared with AGA animals ($P = 0.008$, two-way ANOVA). Ventilation caused a significant increase in liver-to-body weight ratios in both AGA and FGR lambs ($P = 0.0002$, two-way ANOVA).

Physiological Parameters and Ventilation Requirements

Fetal blood gas parameters following SUAL induction have previously been reported for the unvent AGA and FGR groups (2), and the fetal blood gases for the ventilated groups were comparable to previously reported data. In brief, while there were no significant differences in pH, Pa_{CO_2}, Pa_{O_2}, or Sa_{O_2} in the first 7 days after SUAL-induced FGR onset in either AGA or FGR lambs, there was a significant difference ($P < 0.05$) in Pa_{O_2}, and Sa_{O_2} on *day 10* after the onset of FGR. Blood gas parameters after birth are shown in Table 2, for the vent AGA vs. vent FGR lambs. Overall, there was a significant time-related influence on pH and P_{CO2}; and time and growth-related influence on lactate and HCO_3^- (two-way ANOVA, $P < 0.05$). There were no significant differences in the ventilation requirements of the FGR lambs as compared with AGA lambs throughout the experiment (Table 3).

Figure 1 summarizes the changes in cerebral tissue oxygenation index, cerebral oxygen extraction, and CBF in vent AGA and vent FGR lambs. There was a significant difference in the mean cerebral blood flow measured as carotid blood flow, wherein CBF was lower in the vent FGR lambs over the duration of the study ($P = 0.01$) compared with vent AGA lambs. Tissue oxygenation index also tended to be lower in the FGR lambs over the duration of the experiment and cerebral oxygen extraction higher in the FGR group ($P = 0.12$ and $P = 0.06$, respectively).

Neuropathology

We first stained all brains with H&E to assess whether there were areas of gross neuropathology associated with FGR and/or ventilation. Our baseline group of animals, the unvent AGA lambs, demonstrated normal structural integrity and white matter organization, with no evidence of red blood cell (RBC) infiltration (hemorrhage) in any brain region examined (Fig. 2, *A* and *E*, shows normal white matter within PVWM). In the vent AGA group, we observed accumulation of (RBCs and inflammatory cell cuffs around some capillaries within the SCWM and PVWM (Fig. 2, *C* and *G*); however, these were confined to the capillary lumen. Unvent FGR brains showed a mild degree of infiltration of RBCs, accompanied by perivascular infiltrates of inflammatory cells within the white matter (SCWM and PVWM) consistent with microbleeds (Fig. 2, *B* and *F*). In the vent FGR group, we observed large numbers of RBCs (Fig. 2, *D* and *H*) within the vascular lumen (consistent with vascular congestion) as well as RBC infiltration into the brain parenchyma within the SCWM and PVWM, which was accompanied by a loss of the capillary wall (Fig. 2*J*) and inflammatory cell infiltrates (Fig. 2, *K* and *L*). Vent FGR animals also displayed WM disorganization, hypocellularity, and cavity formation in the PVWM (Fig. 2*I*) consistent with periventricular leukomalacia (Fig. 2*I*).

We examined for the presence of axonal injury using two standard staining techniques. Axonal injury was not observed in any AGA brains, whether vent or unvent (Fig. 3, *A–C*). In contrast, axonal injury (positive for thioflavin staining) was present in four out of six unvent FGR brains and five out of six vent FGR brains (Fig. 3). This axonal injury was observed across a number of brain regions but was quite irregular in both

Table 3. *Ventilation requirements in the first 24 h after birth*

	1 h	6 h	12 h	24 h
Compliance, $ml \cdot g^{-1} \cdot cmH_2O^{-1}$				
Vent AGA	0.016 ± 0.02	0.016 ± 0.02	0.018 ± 0.008	0.02 ± 0.013
Vent FGR	0.011 ± 0.003	0.016 ± 0.0006	0.011 ± 0.001	0.008 ± 0.001
Peak pressure, cmH_2O				
Vent AGA	16.8 ± 1.6	21 ± 1.6	21 ± 2.6	25 ± 3.4
Vent FGR	18.6 ± 6.4	20 ± 3.6	19.6 ± 3.4	26 ± 6.4
Tidal volume, ml/kg				
Vent AGA	5.2 ± 0.3	4.9 ± 0.3	5.0 ± 0.3	5.6 ± 0.5
Vent FGR	5.2 ± 0.4	5.0 ± 0.1	4.6 ± 0.3	4.8 ± 1.0
FI_{O_2}, %				
Vent AGA	40.0 ± 11.2	22.6 ± 1.0	26.0 ± 5	31.0 ± 10
Vent FGR	36.5 ± 10.5	22 ± 1.2	27.6 ± 4.0	52.8 ± 15

All values are displayed as means ± SE. No significant differences seen. AGA, appropriate for gestational age; FGR, fetal growth restriction; vent, ventilated.

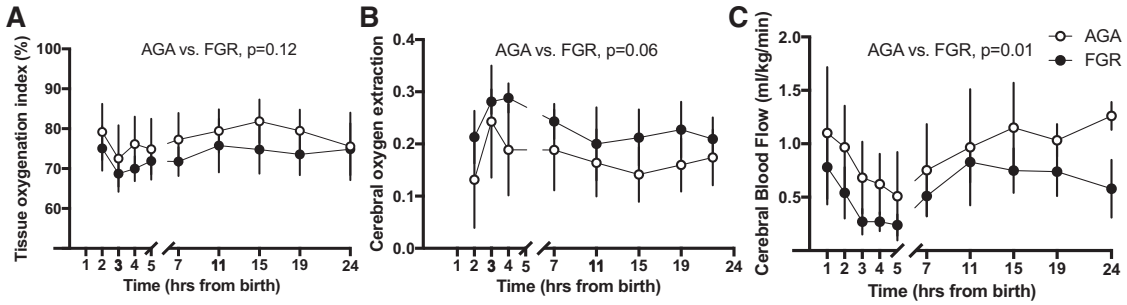


Fig. 1. Graphs showing cerebral tissue oxygenation index (*A*), oxygen extraction (*B*), and blood flow (*C*) measurements of ventilated lambs in the first 24 h after birth. Open circles: Appropriate for gestational age (AGA) lambs; solid circles: fetal growth restriction (FGR) lambs ($n = 6$ each group). All values are means ± SE. There was significant overall difference in cerebral blood flow between AGA and FGR lambs ($P = 0.01$, two-way ANOVA).

size and position and predominantly within white matter regions (Fig. 3 shows staining within the SCWM). Thioflavin staining of amyloid deposits were identified by the presence of apple green fluorescence (Fig. 3, *F* and *I*), showing compact plaques with homogeneous material radiating from the center of the plaques (Fig. 3*I*). We confirmed the presence of axonal injury within FGR brains using Congo red staining (Fig. 3, *D*, *E*, *G*, and *H*) under light and fluorescent microscopy, in which dense masses of axonal retraction were observed.

Immunohistochemistry

Neuroinflammation was assessed by the presence of astrogliosis (GFAP-positive staining) and activated microglia (Iba-1-positive staining). Astrocyte cell counts were similar across the unvent AGA, unvent FGR, and vent AGA groups for all brain regions examined, with the exception of a significant astrogliosis within cortical gray matter in vent AGA brains, compared with unvent AGA brains ($P < 0.05$; Fig. 4). The brains of vent FGR lambs demonstrated profound astrogliosis, with increased GFAP-positive cell density compared with all other groups, in the respective brain regions examined ($P < 0.05$) (Fig. 4). A similar finding was also seen for activated microglia within the PVWM, SCWM, and SVZ, wherein vent FGR brains showed significantly higher numbers of activated microglia compared with all other groups (Fig. 5).

During our quantification of microglia and astrocytes, we observed that the morphology of these glial cells appeared different across groups (Fig. 4*A*). While microglia cell counts were unchanged, microglia in unvent FGR, vent AGA, and vent FGR brains were shifted toward an activated (amoeboid) state, characterized by swollen ramified cells with a larger cell body and shorter, thick processes. In contrast, unvent AGA

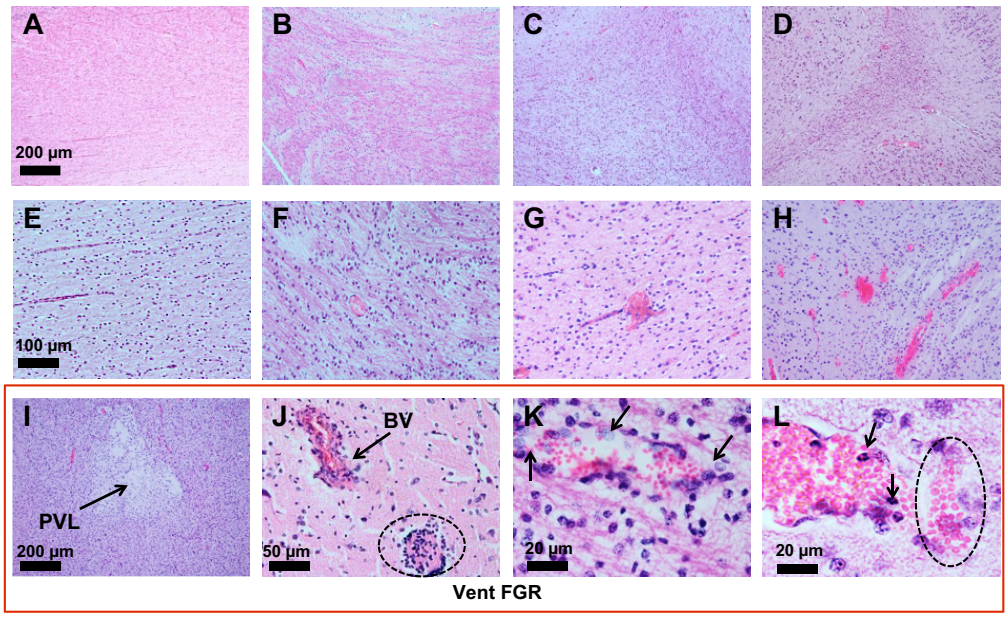


Fig. 2. Representative photomicrographs of hematoxylin and eosin staining of preterm lamb brains. *A*: unventilated (unvent) appropriate for gestational age (AGA). *B*: ventilated (vent) appropriate for gestational age (AGA). *C*: unvent fetal growth restriction (FGR). *D*: vent FGR, $n = 6$ each group. *E–H*: corresponding higher magnification images for *A–D*. *I–L*: vent FGR lamb brains showing periventricular leukomalacia (PVL), loss of vascular integrity, perivascular inflammatory cuffs, macrophage infiltration, neutrophil infiltration, and infiltration of red blood cells near blood vessels (BV).

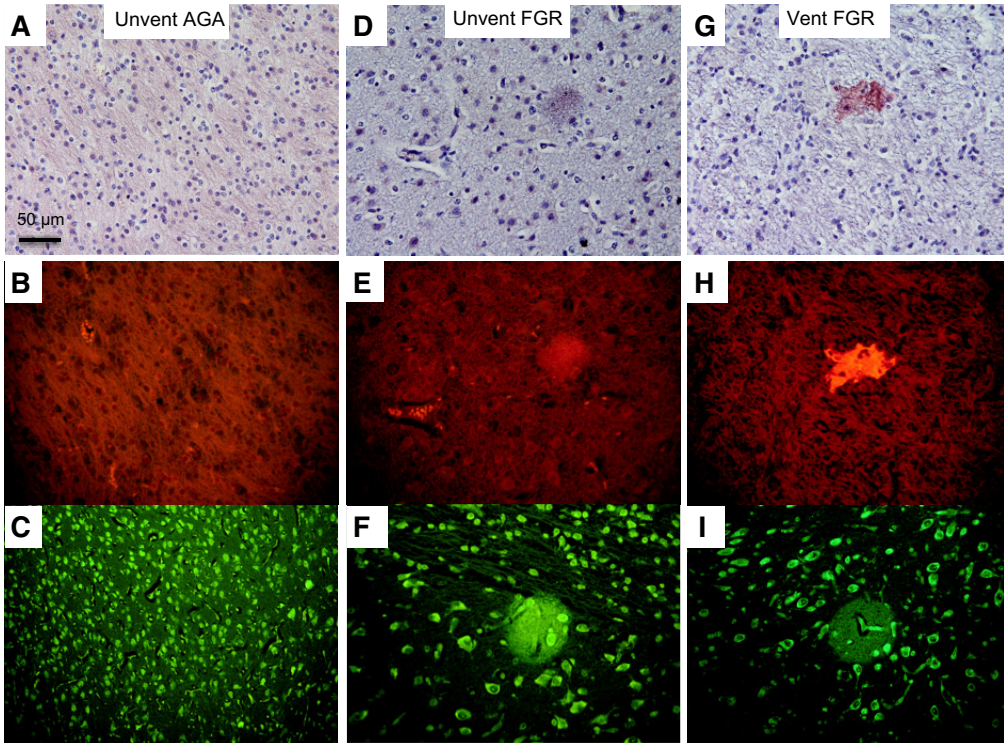


Fig. 3. Representative photomicrographs of Congo red staining under light microscopy (*A*, *D*, and *G*), Congo red staining under fluorescent microscopy (*B*, *E*, and *H*), and thioflavin staining (*C*, *F*, and *I*). *A–C*: unventilated (unvent) appropriate for gestational age (AGA). *D–F*: unvent fetal growth restriction (FGR). *G–I*: vent FGR lambs.

brains showed resting microglia cells with small cell bodies and many branching processes in all regions examined. Similarly, the astrocytes within the unvent FGR, and vent AGA brains were more likely to appear reactive as evidenced by cell body hypertrophy, loss of astrocyte domain, and overlapping astrocytic processes (Fig. 4*A*).

This neuropathology was accompanied by a disrupted interaction of astrocyte end-feet with cerebral blood vessels, which was only evident in FGR brains; thus we consequently studied this association using GFAP-laminin double label immunofluorescence (Fig. 6). Although it was apparent that FGR led to dissociation of the astrocyte foot processes from the blood vessels in response to ventilation in a number of FGR animals (as represented in Fig. 6*B*), quantification of this association did not demonstrate a significant difference in the percent coverage of blood vessels overall (Fig. 6*C*).

Disruption of the astrocyte barrier associated with compromise of the BBB was examined using albumin staining. We did not observe albumin extravasation into brain parenchyma surrounding blood vessels in any of the unvent AGA brains; however, semiquantitative analyses showed that there was BBB disruption (albumin extravasation) within the SCWM and

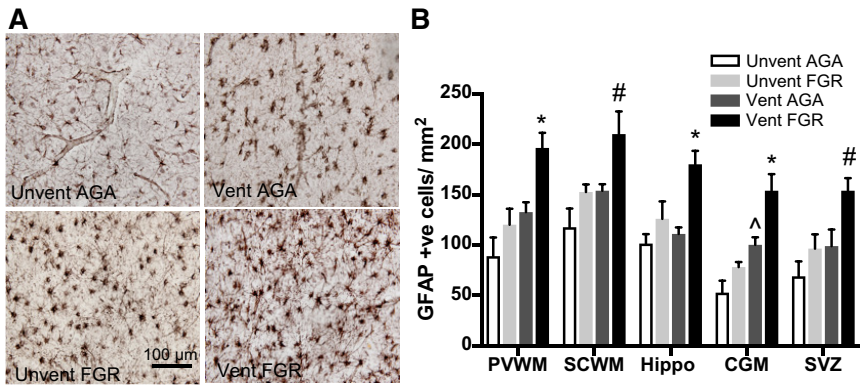


Fig. 4. *A*: representative photomicrographs of astrogliosis seen in subcortical white matter (SCWM) of unventilated (unvent) and ventilated (vent) appropriate for gestational age (AGA) and fetal growth restriction (FGR) lambs using glial fibrillary acidic protein (GFAP) immunoassay ($n = 6$). *B*: significant differences ($^*P < 0.05$) seen in periventricular white matter (PVWM), SCWM, hippocampus (Hippo), cortical gray matter (CGM), and subventricular zone (SVZ) of vent FGR lambs compared with unvent AGA, unvent FGR, and vent AGA; significant differences ($\#P < 0.05$) vent FGR compared with unvent AGA; and significant differences ($\wedge P < 0.05$) vent AGA as compared with unvent AGA lambs in respective brain regions.

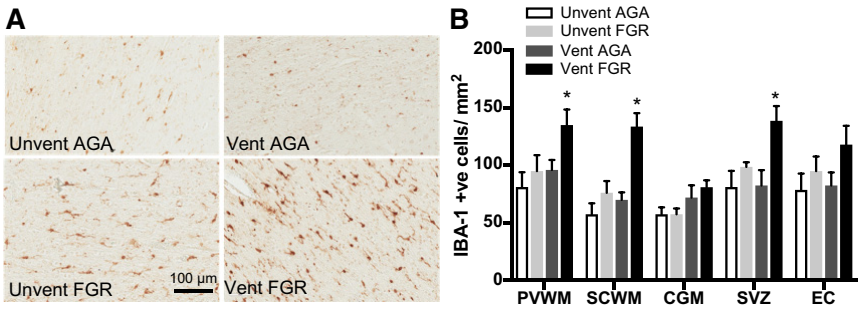


Fig. 5. *A*: representative photomicrographs of activated microglia seen in subcortical white matter (SCWM) of unventilated (unvent) and ventilated (vent) appropriate for gestational age (AGA) and fetal growth restriction (FGR) lambs ($n = 6$) using Iba-1 immunoassay. *B*: significant differences ($^*P < 0.05$) seen in Iba-1 staining in periventricular white matter (PVWM), SCWM, and subventricular zone (SVZ) of vent FGR lambs compared with unvent AGA, unvent FGR, and vent AGA lambs in respective brain regions.

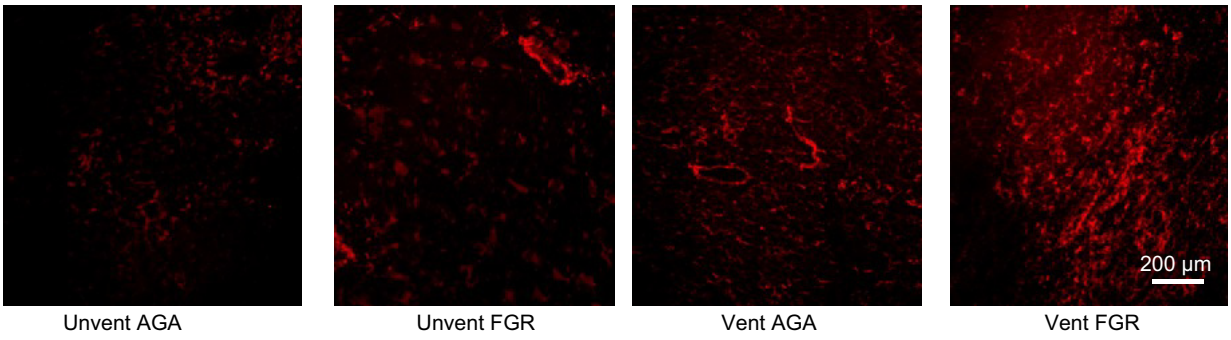
PVWM of the vent FGR lambs, to a greater extent than in vent AGA and unvent FGR animals (Fig. 7), suggesting BBB disruption associated with growth restriction.

Caspase-3-mediated cell death was examined (Fig. 8), and it was noted that caspase-3 staining was present within all groups and brain regions examined. Ventilation increased caspase-3-mediated cell death in the AGA group, with a significant elevation in cell counts within the PVWM (unvent AGA vs. vent AGA; $P < 0.05$). The most notable increase in caspase-3-mediated apoptosis was seen in the vent FGR group, in which caspase-3 cell counts increased twofold increased in PVWM, CGM, and SVZ regions of vent FGR, compared with unvent AGA and unvent FGR ($P < 0.05$ for all). Furthermore, ventilation increased cellular apoptosis in vent AGA brains compared with unvent FGR within the PVWM and CGM and SVZ ($P < 0.05$), indicative that ventilation per se exacerbates neuropathology in FGR offspring.

We examined cellular oxidative stress in the form of lipid peroxidation (Fig. 9) in white matter brain regions (PVWM and SCWM) of all groups, via immunohistochemistry for 4-HNE, one of the most bioactive and widely studied lipid peroxidation product. 4HNE-positive cells were significantly increased number ($P < 0.05$) in the PVWM and SCWM of vent FGR lambs as compared with unvent and vent AGA lambs, and vent FGR was significantly increased above unvent FGR ($P < 0.05$).

Cytokine Analysis

There was no significant change (data not shown) in any of the cytokines studied in serum or CSF in the FGR or



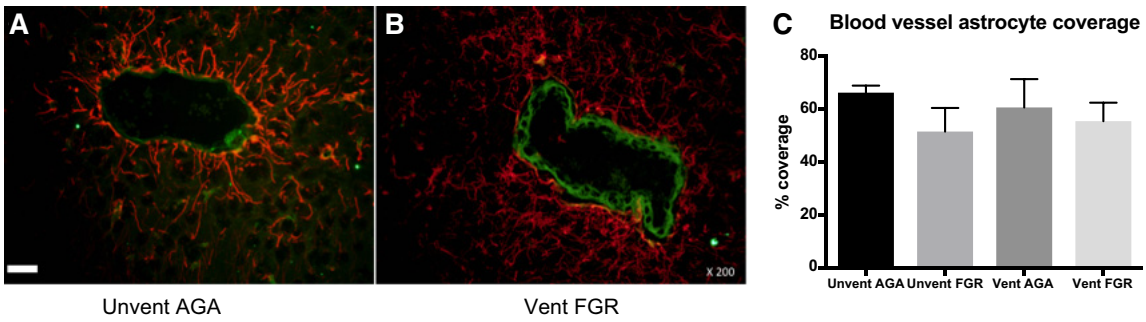


Fig. 6. *Top row*: astrocyte proliferation in lamb groups as seen on low magnification with glial fibrillary acidic protein (GFAP) fluorescent staining. *A*: representative photomicrographs of GFAP-Laminin double-label fluorescent immunohistochemistry showing astrocyte (28) relationship with blood vessel (green vessel wall) in an unventilated (unvent) appropriate for gestational age (AGA) lamb brain. *B*: note detachment of astrocyte foot processes from the vessel wall in ventilated (vent) fetal growth restriction (FGR) lamb brain. Scale bar = 100 μm. *C*: graph comparing blood vessel astrocyte coverage between groups.

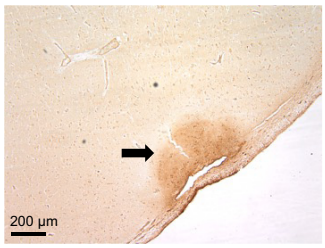


Fig. 7. Representative photomicrograph of increased blood brain barrier permeability (34) seen in the cortex of a vent fetal growth restriction (FGR) lamb brain using albumin immunoassay. Table shows number of animals (out of total in each group) with albumin extravasation in brain tissue. AGA, appropriate for gestational age lamb.

	Unvent AGA	Unvent FGR	Vent AGA	Vent FGR
Albumin extravasation	0/6	1/6	2/6	5/6

ventilated group as compared with unventilated controls or FGR lambs.

DISCUSSION

We examined short-term markers of neuropathology in response to early neonatal care and mechanical ventilation in FGR and AGA preterm lambs. Our results demonstrate that ventilation in preterm FGR lambs induces neuropathology compared with AGA lambs, with an increase in neuroinflammation, oxidative stress, altered BBB structure, and cell death. This is critical information, which provides knowledge on the injurious pathways behind the increased neurological deficits seen in preterm FGR infants and provides insight for targeted strategies to improve outcomes in this vulnerable cohort of infants.

This study is the first to mimic an early onset placental insufficiency/FGR (0.6 gestation), and subsequent preterm delivery at ~0.8 gestation, with standard neonatal care and noninjurious mechanical ventilation to examine the effects of the first 24 h of life on markers of neuropathology. Early onset FGR in the human presents in the second trimester of pregnancy and is associated with a higher risk of neurological deficits including motor, cognitive, and behavior dysfunctions (13). This may be contributed by the in utero compromise to brain development, with the period from 24 to 32 wk gestation being the period at high risk for white matter damage (7). We undertook the technique to induce FGR during this vulnerable developmental period and before the onset of myelination in the periventricular white matter of the sheep brain, when preoligodendrocytes are the predominant cell type (7). The majority of infants who are diagnosed with early onset FGR will be born preterm, with the mean gestational age for delivery of this cohort of infants between 32 and 34 wk (27). We delivered our cohort of lambs at 125–127 days gestation, which is a moderate preterm age for lung maturity and late preterm, near term age with respect to brain development (6, 33). This timing in the sheep presents a neurodevelopmental stage when cortical myelination is quite advanced but the white matter remains highly vulnerable to injury. Indeed, we observed that white matter areas within FGR lamb brains that received ventilation were highly susceptible to oxidative stress, elevated inflammatory cell activation, and apoptosis-mediated cell death. The neonatal delivery and care of FGR lambs encompassed all aspects of neonatal intensive care management in the preclinical environment, including thermoregulation, surfactant therapy, mechanical ventilation, fluid therapy, and antibiotics, enabling us to study the effects of standard early neonatal management on brain pathology. An interesting finding was an increase in liver-to-body weight ratio with ventilation in FGR and AGA groups, which we consider most likely to be due to the fluid therapy in this experiment and a relatively immature renal system (50).

During the 24-h period of neonatal care we noted that the FGR lambs demonstrated a reduced mean carotid blood flow (as a surrogate for cerebral blood flow) compared with AGA lambs. This is in keeping with our previous work to show that in the fetal and early newborn period, FGR lambs have a significantly lower CBF than appropriately grown lambs (36, 39). These results are at odds with clinical data obtained in the first 3 days of life indicative of elevated cerebral oxygenation in preterm small for gestational age infants using near infrared spectroscopy (17). In part, these differing observations probably reflect a degree of heterogeneity in the small for gestational age human infants, including duration and degree of fetal hypoxia, and also the techniques used to measure cerebral hemodynamics (carotid flow in sheep versus near infrared spectroscopy assessment of cortical vessels in humans). Reflecting this, clinical and animal studies have shown that there are brain region-specific changes in cerebral blood flow in growth-restricted fetuses, and cerebral hemodynamic change with advancing fetal compromise (23, 36). We would argue that the decrease in CBF observed in the this study is explained

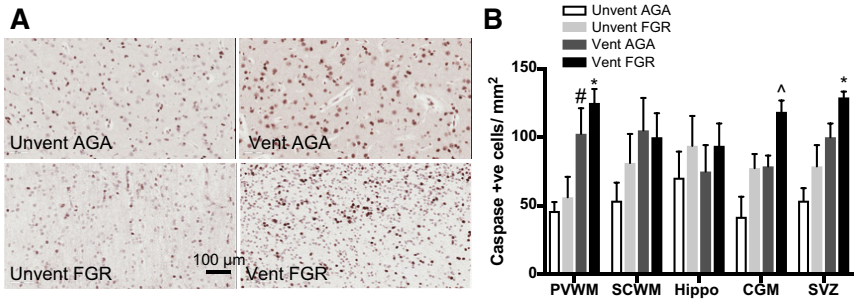


Fig. 8. *A*: representative photomicrographs of cell death using caspase-3 immunostain seen in periventricular white matter (PVWM) of unventilated (unvent) and ventilated (vent) appropriate for gestational age (AGA) and fetal growth restriction (FGR) lambs. *B*: significant differences (*$P < 0.05$) in caspase immunoreactivity in vent FGR lamb brains as compared with unvent AGA and unvent FGR; significant differences (#$P < 0.05$) between vent AGA compared with unvent AGA; and significant differences (^$P < 0.05$) between vent FGR compared with unvent AGA lambs in respective brain regions ($n = 6$ each group).

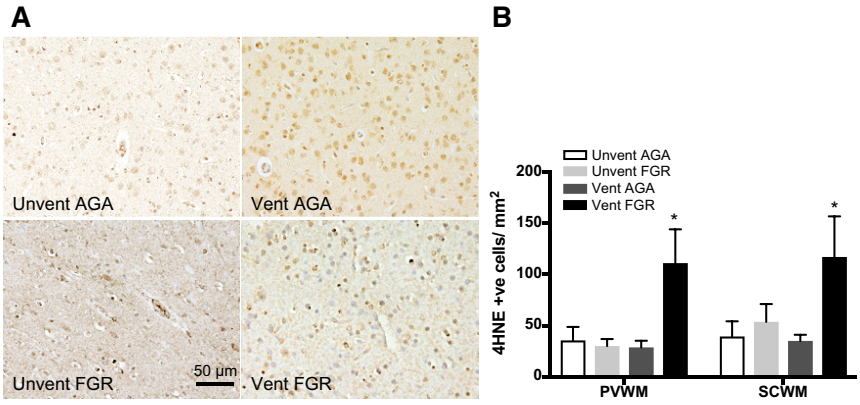


Fig. 9. *A*: representative photomicrographs of oxidative stress using 4-hydroxynonenal (4-HNE) immunostain seen in periventricular white matter (PVWM) of unventilated (unvent) and ventilated (vent) appropriate for gestational age (AGA) and fetal growth restriction (FGR) lambs. *B*: significant differences (*$P < 0.05$) in 4HNE immunoreactivity in vent FGR lamb brains as compared with unvent AGA, unvent FGR, and vent appropriate for gestational age (AGA) lambs ($n = 6$ each group). SCWM, subcortical white matter.

by structural cerebrovascular adaptations that occur in response to chronic in utero hypoxia, with a significant decrease in vascular density in white matter brain regions in FGR lambs (15). Furthermore, we observed profound neuroinflammation within FGR brains exposed to ventilation, often accompanied by disassociation of astrocyte end-feet from blood vessels. A critical role of the astrocytes is to regulate cerebral blood flow, which they do via connections with blood vessels (24). We showed an overall decrease in tissue oxygenation within the FGR brain and increased cerebral oxygen extraction. Combined, these findings are physiologically important and indicative that the developing FGR brain is very sensitive to altered cerebral hemodynamics that arise from routine neonatal care, and these can impact on cerebral metabolism and potentially contribute to subsequent brain injury seen in this high-risk population.

There is evidence to suggest that early invasive ventilation in premature infants (who are appropriately grown) is associated with increased risk of intraventricular hemorrhage (3) and white matter injury (12, 42), particularly when the ventilation is not well controlled. It is likely that ventilation-induced brain injury in preterm infants arises due to disruption of the BBB, mediated via central inflammation and oxidative stress (11). We saw evidence of reduced BBB integrity in response to ventilation, most notable in the FGR cohort of animals. This is in agreement with our previous findings of altered BBB structure and function in FGR neonates (1, 15). Structural integrity of the BBB is critical to ensure peripheral inflammatory cells and RBCs cannot access the developing brain. Similar effects of increased BBB permeability have also been noted with ventilation of well grown preterm lambs (9), but here we show that FGR increases the vulnerability for a compromised BBB, potential RBC translocation, and subsequent intraventricular hemorrhage and white matter damage.

Neuroinflammation plays a central role in the development of brain injury in FGR infants (26, 52). Until now it has not been well defined whether the neuroinflammation present in the FGR brain was primarily antenatal in origin as a result of placental insufficiency or initiated by postnatal interventions, including ventilation, or indeed a combination of both. In preterm infants, Leviton et al. (28) proposed a two-hit model of inflammation, born small and exposed to postnatal systemic inflammation. In preterm lambs, our group has shown that ventilation increases systemic and cerebral inflammatory markers (9) and that the combination of antenatal chorioamnionitis and postnatal ventilation is associated with an exacerbation of inflammatory markers and increased brain injury (10, 43). Results from this study now further demonstrate that the two-hit model of inflammation and brain injury is appropriate for FGR offspring wherein ventilation exacerbates gliosis in FGR preterm lambs. Thus the combination of chronic antenatal hypoxia and postnatal ventilation appears to have additive adverse effects on the FGR brain, which together may contribute to long-term neurological deficits.

The contribution of microglia and astrocytes is complex, as they mediate both neurorepair and damage (5, 19, 47). In the perinatal brain, it is however increasingly well described that microglial activation is a first critical step in the progression of neuroinflammation and contributes to white matter injury (19). An upregulation of activated astrocytes and microglia in response to standard ventilation in preterm FGR infants is likely to be a contributor toward increased cellular apoptosis as observed in this study. Microglia act as a neuropathology sensor in the central nervous system and can rapidly detect subtle changes in brain tissue both in the developing and adult brain (30). In the activated form, microglia cells produce a plethora of proinflammatory cytokines and chemokines, which have been implicated in the pathogenesis of a number of pathological conditions, including cerebral palsy (25). Microglia also play an indispensable role in building the normal brain architecture and are known to be involved in myelination, phagocytosis of apoptotic neurons, axonal pruning, and the development of vascular and axonal networks (44). Given the crucial role of microglia in regulating brain development, maturation, and network connectivity, modifications to microglia cells in the developing FGR brain could alter on-going developmental processes and thereby have impact on normal brain structure. This neuroinflammatory response may well be amenable to umbilical blood cell therapy as seen in a preterm brain injury model reported previously (29).

Ventilation increased oxidative stress, as measured by 4HNE within the FGR lamb brains. This is interesting, especially in the context of similar ventilation requirements between the FGR and AGA animals. Increased expression of oxidative stress markers is seen in brain injury associated with FGR (45, 51). Our group has previously shown an increase in oxidative stress markers in serum and brain tissue of FGR lambs (38). Similarly, increased oxidative stress markers have

been seen in hypoxic and ventilation-induced brain injury models of preterm birth (14, 41). An upregulation of lipid peroxidation within the immature brain may be catastrophic for white matter development (7, 38). It is possible the FGR lamb brain has a reduced antioxidant capacity (54), which is unable to respond to another stressful state (ventilation) leading to a heightened response as seen here. In this respect it will be important to follow-up on the potential for antioxidant therapies, such as melatonin, to mitigate antenatal and postnatal contributions to neuropathology in FGR offspring (38).

We used two markers of axonal injury, thioflavin and Congo red; both of which confirm the presence of abnormal amyloid deposition occurring in response to axonal damage and local accumulation of amyloid precursor protein. Previously we have shown amyloid precursor protein deposition in term unventilated FGR lamb brains (38), and in this study we examined whether ventilation might specifically induce axonal injury in FGR or AGA lambs. Our results are indicative that some, but not all, FGR brains show evidence of axonal injury within white matter regions, but that ventilation per se did not exacerbate axonal injury. This observation also supports the two-hit model of brain injury in FGR offspring, with this underlying white matter injury and axonal damage induced during the period of chronic hypoxia in utero and additional neuroinflammatory compromise possibly initiated with neonatal ventilation.

We did not see any significant or characteristic cytokine profiles in blood, CSF or brain tissue in this study. We have previously seen some increase in IL-8, a proinflammatory cytokine in brief periods of mechanical ventilation in (late onset) FGR preterm lambs (1). This suggests a brain-specific neuroinflammatory process and further highlights the case to identify and develop organ specific biomarkers for the detection and assessment of FGR-related brain injury (32).

We acknowledge the limitations of this study. By delivering these lambs at 125 days of gestation we were able to replicate the effects of ventilation on brain development equivalent to late preterm human gestation; however, lung development is slightly more immature in the sheep at this age (equivalent to 28–30 wk gestation), such that we could not maintain the lambs past 24 h. Human infants born preterm would likely be exposed to longer periods of invasive or noninvasive ventilation and other neonatal interventions (e.g., caffeine therapy, therapy for patent ductus arteriosus), which we have not accounted for, and may separately influence brain development. All animals were exposed to antenatal glucocorticoids, which also would be likely to affect our observations within the brain (37). These findings do however give us an indication of mechanisms and mediators of early brain pathology that may be potentially modifiable in FGR infants.

Perspectives and Significance

Preterm growth-restricted lambs when ventilated show an increased risk of brain injury, especially in white matter brain regions, as compared with well-grown and unventilated growth-restricted lambs. Neuroinflammation and oxidative stress are upregulated and are likely to contribute to cerebrovascular compromise and apoptosis-mediated cell death. This fits in well with the two-hit hypothesis wherein chronic in utero hypoxia and a second stress of neonatal ventilation combine to result in neuropathology associated with FGR. Targeted therapies to mitigate the effects of ventilation on the growth-restricted infant brain need to be evaluated with this information in mind.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.M., G.J., and S.L.M. conceived and designed research; A.M., M.C.-M., B.J.A., A.E.S., I.N., Y.P., A.A.d.A.R., M.C.F., G.R.P., G.J., and S.L.M. performed experiments; A.M., M.C.-M., B.J.A., and S.L.M. analyzed data; A.M., M.C.-M., B.J.A., and S.L.M. interpreted results of experiments; A.M., M.C.-M., and B.J.A. prepared figures; A.M. drafted manuscript; A.M., M.C.-M., B.J.A., A.E.S., I.N., Y.P., A.A.d.A.R., M.C.F., G.R.P., G.J., and S.L.M. edited and revised manuscript; A.M., M.C.-M., B.J.A., A.E.S., I.N., Y.P., A.A.d.A.R., M.C.F., G.R.P., G.J., and S.L.M. approved final version of manuscript.

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Chapter 3

In Chapter 1, I reviewed the neonatal morbidities associated with FGR, which indicated that FGR brain injury may be exacerbated in preterm infants. Preterm infants are frequently ventilated due to lung immaturity, but the mechanisms of how neonatal ventilation may influence FGR related brain injury have not been well studied. In Chapter 2, I investigated the mechanisms that mediate brain injury in FGR preterm lambs exposed to neonatal ventilation. I showed that neonatal ventilation exacerbates brain injury associated with FGR principally by upregulating inflammatory, and oxidative stress pathways, which in turn modifies blood-brain barrier function, and induces neurovascular deficits and apoptotic cell death.

In the following study I focused on evaluating the effects of a targeted neuroprotective therapy for brain injury in preterm FGR lambs, in this case, umbilical cord blood derived stem cells. Umbilical cord blood cells are already in clinical use, and a number of preclinical and clinical studies have been undertaken to evaluate their potential neuroprotective and neuroregenerative properties. Our group has demonstrated that umbilical cord blood derived stem cells have anti-inflammatory, anti-apoptotic, anti-oxidant, angiogenic, and neurogenic properties, which makes them a good candidate for evaluation in FGR, given some of these pathophysiological mechanisms are at play in the causation of FGR related brain injury in preterm ventilated lambs as shown in Chapter 2.

Using the lamb model of early-onset FGR and neonatal ventilation described in Chapter 2, I conducted experiments in which umbilical cord blood cells were administered intravenously to lambs at 1 hour of life to evaluate them as an early treatment option for FGR brain injury. This is linked to my overall thesis aim of developing novel strategies for the early detection and early treatment of FGR brain injury. This is the unaltered version of the paper submitted to Stem Cell Research and Therapy in September 2019.

3.1. Neurovascular effects of umbilical cord blood cell therapy in growth restricted newborn lambs

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Stem Cell Research & Therapy

Neurovascular effects of umbilical cord blood derived stem cells in growth restricted newborn lambs

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Abstract:	Background: Neonatal ventilation exacerbates brain injury in lambs with fetal growth restriction (FGR), characterized by neuroinflammation and reduced blood brain barrier integrity, which is normally maintained by the neurovascular unit. We examined whether umbilical cord blood stem cell (UCBC) treatment stabilized the neurovascular unit and reduced brain injury in preterm ventilated FGR lambs. Methods: Surgery was performed in twin-bearing pregnant ewes at 88 days' gestation to induce FGR in one fetus. At 127 days, FGR and appropriate for gestational age (AGA) lambs were delivered, carotid artery flow probes and umbilical lines inserted, lambs intubated and commenced on gentle ventilation. Allogeneic ovine UCBC (25 x 10 6 cells/kg) were administered intravenously to lambs at 1 hour of life. Lambs were ventilated for 24 hours, and then euthanized. Results: FGR (n=6) and FGR+UCBC (n=6) lambs were growth restricted compared to AGA (n=6), and AGA+UCBC (n=6) lambs (combined weight, FGR 2.3±0.4 vs. AGA 3.0±0.3 kg; p=0.0002). UCBC therapy did not alter mean arterial blood pressure or carotid blood flow but decreased cerebrovascular resistance in FGR+UCBC lambs. Circulating TNF-α cytokine levels were lower in FGR+UCBC vs. FGR lambs (p< 0.05). Brain histopathology showed decreased neuroinflammation and oxidative stress, increased endothelial cell proliferation, pericyte stability and greater integrity of the neurovascular unit in FGR+UCBC vs. FGR lambs. Conclusions: Umbilical cord blood stem cell therapy mitigates perinatal brain injury due to FGR and ventilation, and the neuroprotective benefits may be mediated by stabilization of the neurovascular unit.	
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Question	**Response**
Is this study a clinical trial?<hr>A clinical trial is defined by the World Health Organisation as 'any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes'.	No

Original Article

Neurovascular effects of umbilical cord blood derived stem cells in growth restricted newborn lambs

UCBCs for perinatal brain injury

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Keywords

Brain, cerebral palsy, intrauterine growth restriction, stem cell, ventilation

Abstract

Background: Neonatal ventilation exacerbates brain injury in lambs with fetal growth restriction (FGR), characterized by neuroinflammation and reduced blood brain barrier integrity, which is normally maintained by the neurovascular unit. We examined whether umbilical cord blood stem cell (UCBC) treatment stabilized the neurovascular unit and reduced brain injury in preterm ventilated FGR lambs.

Methods: Surgery was performed in twin-bearing pregnant ewes at 88 days' gestation to induce FGR in one fetus. At 127 days, FGR and appropriate for gestational age (AGA) lambs were delivered, carotid artery flow probes and umbilical lines inserted, lambs intubated and commenced on gentle ventilation. Allogeneic ovine UCBC (25 x 10^6 cells/kg) were administered intravenously to lambs at 1 hour of life. Lambs were ventilated for 24 hours, and then euthanized.

Results: FGR (n=6) and FGR+UCBC (n=6) lambs were growth restricted compared to AGA (n=6), and AGA+UCBC (n=6) lambs (combined weight, FGR 2.3±0.4 vs. AGA 3.0±0.3 kg; p=0.0002). UCBC therapy did not alter mean arterial blood pressure or carotid blood flow but decreased cerebrovascular resistance in FGR+UCBC lambs. Circulating TNF-α cytokine levels were lower in FGR+UCBC vs. FGR lambs ($p< 0.05$). Brain histopathology showed decreased neuroinflammation and oxidative stress, increased endothelial cell proliferation, pericyte stability and greater integrity of the neurovascular unit in FGR+UCBC vs. FGR lambs.

Conclusions: Umbilical cord blood stem cell therapy mitigates perinatal brain injury due to FGR and ventilation, and the neuroprotective benefits may be mediated by stabilization of the neurovascular unit.

Introduction

Fetal growth restriction (FGR), due to placental insufficiency, leads to progressive reduction of oxygen and nutrient supply to the developing fetus. Perinatal brain injury due to FGR is associated with impairments in brain structure and function. The neurological outcomes associated with FGR depend on the gestation at onset of FGR, severity of growth restriction, degree of fetal cardiovascular adaptation, and gestation at birth[1,2]. When FGR infants are delivered premature, they often require mechanical ventilatory support over the first few days of life. Mechanical ventilation can have detrimental effects on the immature preterm brain[3,4]. In preterm FGR offspring, neonatal ventilation leads to increased risk of brain injury[5,6], contributed by a greater susceptibility to neuroinflammation and blood brain barrier (BBB) breakdown in FGR offspring[6].

Stem cell therapies for non-hematological indications have received much attention recently, including for perinatal neuroprotection and neuroregeneration[7-9]. Preclinical studies support that umbilical cord blood cell (UCBC) therapy decreases the progression of perinatal brain injury. UCBCs are neuroprotective for the preterm brain when administered in ovine models of hypoxia and inflammation-induced preterm brain injury[10-12]. More recently, it has been shown that a single administration of UCBC therapy resulted in improvement in long-term behavioral outcomes in a rat model of neonatal hypoxic ischemic injury[13]. Umbilical cord blood collected at birth has a high cell yield and a wide variety of stem and progenitor cells, which are shown to mediate positive benefits on glial cells, neurons, and cells that maintain the BBB[7,14-16]. These and other studies show that the neuroprotective or neuroreparative benefits of UCBC for the immature brain acts through anti-apoptotic, anti-

inflammatory, pro-angiogenic, neurogenic, antioxidant, and BBB protective mechanisms[9-12].

The neurovascular unit (NVU) describes the intimate cellular relationship between neurons, glia and the neurovasculature (endothelial cells, pericytes, astrocytes), and plays a critical role in the development of brain structure and function[17]. The NVU mediates substrate supply to brain tissue, facilitates cell interactions and provides structural integrity to the BBB. Disruption to the NVU can occur in the developing brain in response to placental insufficiency and FGR[18]. In turn, altered cell interactions lead to an increased risk of BBB breakdown and may have significant adverse implications for injury-induced angiogenesis and repair[19]. Administration of UCBC in adult stroke induces changes in the neurovasculature, resulting in reduced brain neuropathology and improved neurological outcome[20,21]. It is unknown whether UCBC therapy mediates similar effects on the neurovascular unit in the neonatal brain.

Accordingly, this study evaluated the therapeutic potential of UCBC therapy to prevent or modulate mediators of brain injury in preterm ventilated FGR lambs. We have assessed the role of the neurovasculature in perinatal brain injury and repair, and examine a therapeutic target for growth-restricted babies at risk of brain injury. We hypothesized that UCBCs would mitigate the harmful effects of neonatal ventilation on the preterm FGR lamb brain by targeting and stabilizing structural components of the NVU.

Material and Methods

Ethics approval

Experiments complied with the National Health and Medical Research Council (NHMRC) of Australia guidelines for the care and use of animals for scientific purposes and were approved by Monash Medical Centre Animal Ethics Committee A. The experiments have been reported in compliance with the ARRIVE guidelines (Animal Research: Reporting in Vivo Experiments).

Surgery to induce FGR and ventilation of lambs

Procedures to induce FGR and delivery followed by ventilation in preterm lambs have been previously described[6]. In brief, surgery for single umbilical artery ligation (SUAL) was performed at ~88 days gestation to induce early onset FGR in one fetus of twin-bearing Border-Leicester Merino crossbred ewes. The other fetus acted as the control. At ~126 days gestation, the pregnant ewe was anaesthetized using thiobarbital followed by gas anesthesia and a caesarean section was undertaken. With the lamb exposed within the uterus but not yet delivered, a flow probe (size 4; ADInstruments, Castel Hill, Australia) was inserted around the carotid artery, and the lamb then delivered and the umbilical cord clamped and cut. The lambs were dried, weighed, and transferred to an infant warmer (Fisher and Paykel, Auckland, NZ) where each lamb was intubated (4.0 mm cuffed endotracheal tube), lung liquid passively drained and gentle ventilation commenced. Umbilical vein and artery catheters were immediately inserted and secured using silk sutures. A pulse oximeter probe (Masimo, Irvine, CA) was placed on the lamb's tail for measurement of transcutaneous oxyhemoglobin saturation levels (SpO_2). Near infrared spectroscopy (NIRS; Fore-Sight Tissue Oximeter, CAS Medical Systems Inc., Branford, CT) was used for continuous recording of cerebral oxygenation, via placement of probes over

the frontoparietal head region and covered with a lightproof dressing. Cerebral oxygenation was expressed as tissue oxygenation index (TOI, %) at 0.5 Hz[22]. Ventilation of the preterm FGR and AGA lambs was initiated using assist control ventilation (Babylog 8000+, Dräger, Lüberk, Germany) with an initial peak inspiratory pressure (PIP) of 30 cmH_2O and positive end-expiratory pressure of 5 cmH_2O for the first 10 minutes. The inspired oxygen fraction (FiO_2) commenced at 0.3, but was adjusted to maintain SpO_2 between 91-95% after initial resuscitation. All lambs received prophylactic surfactant via the endotracheal tube (100 $mg.kg^{1}$, Curosurf; Chiesi Pharma, Parma, Italy) at 10 minutes after birth.

Lambs were ventilated for 24 hours on volume guarantee mode with a tidal volume (V_T) set at 5-7 $mL.kg^{-1}$. Throughout ventilation, lambs were sedated by continuous infusion of Alfaxan (3 $mg.kg^{-1}.min^{-1}$; CenVet, Lynbrook, Australia) through the umbilical vein catheter. Lamb physiological well-being was assessed via umbilical arterial blood gas parameters measured at 1, 6, 12 and 24 hours post delivery. Lambs were euthanized at 24 h by intravenous pentobarbital sodium overdose (100 $mg.kg^{-1}$ I.V.; Valabarb, Rutherford, Australia). All ventilation and physiological data was digitally acquired using Powerlab (1 kHz) and Lab Chart 8 software (ADInstruments, Castle Hill, Australia).

Cell collection and preparation

Umbilical cord blood was collected at caesarean section from a separate cohort of healthy term lambs (144-145 days gestation). The umbilical cord was clamped and blood from the placental side was collected into heparinized syringes. UCBCs were then isolated from the buffy coat layer by centrifuging the blood at 3100 rpm for 12

min at room temperature, with no brake, and excess red blood cells removed using red blood cell lysis buffer. The cells were resuspended in fetal bovine serum with 10% DMSO (Merck, Darmstadt, Germany), and cryopreserved in liquid nitrogen. The cells were thawed immediately before administration and DMSO removed by washing cells with media (DMEM/F12, 10% FBS, 1% antibiotics). Cell yield and viability were assessed using trypan blue dye exclusion before administration. Cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) to enable tracking of the cells within the brain[23]. For this study, allogeneic umbilical cord blood mononuclear cells (25 million/kg) were suspended in 2-3 ml of sterile saline, and were given intravenously (via the umbilical vein) to the preterm ventilated lambs at 1 hour of life.

Brain pathology

After euthanasia, cerebrospinal fluid (CSF) was collected with an 18G needle, and 3 ml syringe and the brain removed and weighed. The left brain hemisphere was divided into four sections (frontal, middle (x2), occipital) and frozen for analysis. The right brain hemisphere was coronally cut into 5 mm slices and fixed in formalin for 48-72 hours and then embedded in paraffin (ProSci Tech, Thuringowa, Australia) for histological and immunohistochemistry analysis.

Molecular assessment and cytokine assays

Concentrations of intracellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), Interleukin (IL) 3, IL6, neuron specific enolase (NSE), decorin, interferon (IFN) γ, IL17A, IL21, IL8, IP10, monocyte induced by gamma interferon (MIG), secreted frizzled related protein (sFRP) 3, tumor necrosis factor (TNF) α, and vascular endothelial growth factor (VEGF)-A were assessed in the lamb serum and

CSF samples using a human 5-plex and ovine 10-plex quantibody array following the manufacturer's instructions (Crux Biolab, Scoresby, Australia). Ovine brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and amyloid precursor protein (APP) ELISA assays were conducted on homogenized brain white matter tissue. Lastly, endothelin-1 receptor antibody (EDNRA) ELISA (Crux Biolab, Scoresby, Australia) was conducted using manufacturer's instructions on serum samples at 1, 6 and 12 hours after birth.

Immunohistochemistry

Single label staining: Cerebral cellular apoptosis was assessed using activated caspase-3 (Cat# AF835, R&D Systems, Minneapolis, MN), astrocytes were assessed using glial fibrillary acidic protein (GFAP, Cat# G3893, Sigma-Aldrich, Castle Hill, Australia), inflammatory microglial cells were evaluated using ionized calcium binding adapter molecule 1 (Iba-1, Cat# 019-19741, Wako Pure Chemical Industries, Osaka, Japan), BBB permeability was assessed using albumin extravasation (Cat# S4265-2ML, Sigma-Aldrich, Castle Hill, Australia) and oxidative stress was assessed using 4 –hydroxynonenal (4HNE, Cat# 393207-100ul, Merck, Germany). Ki67 (Cat# RM-9106-S, Thermo Fisher Scientific, Waltham, MA) was used to study cell proliferation, and endothelial cell proliferation was evaluated via expression of glucose transporter-1, which is present on mature endothelial cells (Glut-1; Cat# AB14683, Abcam, Melbourne, Australia). Standard immunohistochemistry protocols were followed. Briefly, for any single label immunohistochemistry analysis, brain blocks at the level of caudo-putamen and the dorsal tegmental bundle containing the cortical grey matter (CGM), subcortical white matter (SCWM), periventricular white matter (PVWM), hippocampus (hippo) and subventricular zone (SVZ) were sectioned

at 10um. Serial sections were placed on SuperFrost glass slides and dewaxed in xylene followed by rehydration in serial ethanol solutions. Antigen retrieval was carried out by heating in citric acid buffer (pH 6) for 15 mins (3x5mins) and then allowing the hot buffer to cool at room temperature for a further 20 minutes. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide buffer in 50% methanol. Non-specific binding was blocked by animal serum (goat or rabbit serum in bovine serum albumin). Primary antibody was then added and sections incubated at 4^{o}C overnight. The following day, sections were incubated in corresponding secondary antibody followed by streptavidin horseradish peroxidase (HRP; Cat# GERPN1051-2ML, 1:200, Amersham Bioscience, UK). Staining was visualized using 3,3'-diaminobenzidine and coverslipping using mounting medium (DPX, Cat# 100579, Merck, Kilsyth, Australia).

Double label staining: Double-labeled fluorescent immunohistochemistry was conducted to determine the relationship of the desmin and smooth muscle actin (α-SMA), which marks the pericytes of the neurovascular unit. After dewaxing the sections, endogenous peroxidases were blocked with 0.3% hydrogen peroxide in 50% methanol, and sections were then washed with sodium borohydride (10 mg/mL) in PBS to reduce autofluorescence. Sections were subsequently treated with serum-free protein blocker (Cat# X090930-2, DAKO Australia, Campbellfield, Australia) and incubated with mouse monoclonal α-SMA (Cat# A522-200UL, 1/50; Sigma-Aldrich, USA) and rabbit polyclonal anti-desmin (Cat# D8281-1ML, 1/50; Sigma-Aldrich, USA) to identify the degree of pericyte coverage, as previously described by our group[18]. Fluorescent secondary antibodies were used to study the interaction of the two proteins in the neurovascular unit.

Quantitative analysis of brain injury

Sections were viewed at a magnification of x400 using light microscopy (Olympus BX-41, Japan) with the slides coded for blind analysis. Immunoreactive cell counts were assessed in three fields of view within regions of interest on two slides per animal, to give six fields of view per region per animal, from which an average was then calculated. Manual counts of immunopositive cells expressing GFAP (astrocytes), Iba-1 (activated or amoeboid microglia), caspase -3 (cell death), 4-HNE (oxidative stress), Ki-67 (cell proliferation) and Glut-1 (endothelial cell proliferation) were undertaken.

For cell proliferation, besides manual counting of Ki-67 positive cells in brain regions, we calculated the percentage of blood vessels with Ki-67 positive cells as compared to all blood vessels. Automated counts of % area for Glut-1 staining were also assessed. Fluorescent desmin and α-SMA colocalisation (using Mander's coefficients M1 and M2) was assessed by measuring the amount of co-localization between the red and green fluorescence within blood vessels at 400x magnification. An image processing software (Image J- Fiji 2.0.0, National Institutes of Health, MD) was used for automated analysis.

Statistics

Statistical comparisons were carried out using GraphPad Prism (GraphPad Software v7, San Diego, CA) and SPSS (v25, IBM SPSS Statistics, Armonk, NY). Data are presented as mean ± standard deviation (S.D.), or as percentage or a fraction for semi-quantitative data (blood vessel proliferation, albumin extravasation). Group data were

analyzed using one-way, two-way or three-way ANOVA, or repeated measures two-way or three-way ANOVA where appropriate and post hoc multiple comparisons test. Sources of variations for ANOVA included time factor, fetal growth (FGR vs. AGA), UCBC (FGR/ AGA+UCBC vs. FGR/ AGA) or a combination of these. Significance was accepted at $p<0.05$.

Results

Baseline characteristics

Fetal surgery was undertaken in 13 sets of twin fetal sheep; there were 2 fetal deaths, one each in FGR and AGA lamb groups. In total, 24 newborn lambs (6 in each group (AGA, FGR, AGA+UCBC, FGR+UCBC) were included in final analyses. Animal body and specific organ weights, and organ/ body weight ratios are presented in Table 1. All groups had an equal number of male and female lambs (3:3). Overall, FGR lambs (FGR and FGR+UCBC) weighed 23% less than AGA lambs at birth (AGA and AGA+UCBC) (2.3±0.4 kg vs. 3.0±0.3 kg; $p = 0.0002$). Both FGR and FGR+UCBC lambs demonstrated an increase in brain/body weight ratio compared to AGA, indicative of brain sparing. Organ weights of the liver, lung and heart were reduced in FGR lambs compared to AGA lambs; Table 1.

Physiological parameters and ventilation requirements

Blood gas parameters (Supplementary data Table 1) and ventilation parameters (Supplementary data Table 2) are shown at 1, 6, 12 and 24 hours after birth. There were no individual time point differences for any blood gas variables across the groups. Ventilation requirements were not significantly different across the four lamb

groups throughout the experiment, albeit the FGR groups were more likely to be on higher FiO2 and have lower lung compliance at 24 h than AGA groups (NS).

Hemodynamics

Figure 1 demonstrates hemodynamic measures in all groups over the 24 hours of the experiment. Mean arterial blood pressure remained stable over 24 hours in the AGA group. Overall however, there was a time-dependent decrease in MAP over the time course of the experiment ($p=0.003$). The FGR groups had lower MAP than AGA groups ($p=0.03$). UCBC treatment did not affect MAP in the AGA+UCBC or FGR+UCBC lambs. There was a significant effect of time across groups on carotid blood flow ($p=0.009$), but no differences between groups or effect of UCBCs. There was a significant effect of time on cerebrovascular resistance ($p=0.02$). Cell therapy was associated with a decrease in cerebrovascular resistance in FGR+UCBC vs. FGR lambs within 2 h after cell therapy (mean difference 69.0 mmHg/ml/kg/min (95% CI 48.9-89.1, $p<0.05$) and from 12 to 15 h after birth (13 h, mean difference 46.4 mmHg/ml/kg/min (95% CI 1.8-91.5, $p<0.05$). There was no change in the tissue oxygenation index as a result of cell therapy (Supplementary Data Figure 1). We also measured levels of the EDNRA in the serum as a marker of vascular reactivity, however there was no significant change in EDNRA levels from baseline over the duration of the experiment (data not shown).

Immunohistochemistry and molecular analysis

CFSE labeled cells were distributed diffusely in low numbers, mostly near blood vessels in the white matter of the brains of AGA+UCBC and FGR+UCBC animals. We utilized two immunohistochemical markers as measures of neuroinflammation

(Figure 2), Iba-1 to assess activated microglia and GFAP to assess astrogliosis. The number of activated microglial cells was increased in FGR brains in the PVWM, SCWM and SVZ ($p<0.05$) compared to AGA. There was no difference in the number of activated microglia in FGR+UCBC compared to AGA across all brain regions examined, but cell counts in the FGR+UCBC group were reduced compared to FGR within the PVWM, SCWM and SVZ ($p<0.05$). The number of GFAP-positive astrocytes was significantly increased in the FGR animals versus AGA across all brain regions examined ($p<0.05$), while no other group was significantly different to the AGA group. Serum TNF-α protein concentration showed a significant elevation in the FGR group above that in the FGR+UCBC group ($p=0.01$; Figure 2, panel E). There were no significant differences seen in any other cytokines measured in serum or CSF (data not shown).

We determined brain levels of oxidative stress by performing immunohistochemistry for 4-HNE in white matter brain regions (Figure 3). We observed a significant increase in cellular oxidative stress within the PVWM and SCWM of FGR brains compared to AGA ($p<0.05$). No other significant differences were observed in levels of cellular oxidative stress across groups. Caspase-3 mediated cell death was not significantly different across groups in brain regions examined (Supplementary Data Figure 2).

Neurovascular unit

Cell proliferation was examined using Ki-67 immunohistochemistry (Figure 4, A-B), and revealed a significant increase in cell proliferation in the SVZ of FGR+UCBC lamb brains. We often observed Ki-67-positive cells in association with blood vessels

(Figure 4, C-D) and, therefore, next calculated the percentage of Ki-67- positive blood vessels (Ki-67 positive/ total blood vessels in a field of view) in the PVWM and SVZ. There was a significant increase in Ki-67 positive blood vessels in both regions ($p<0.05$). We next assessed whether distribution and number of endothelial cells in blood vessels was altered by FGR or FGR+UCBC by quantifying the glucose transporter (Glut-1) present in the cerebral vasculature. Both FGR and FGR+UCBC lamb brains showed an increase in endothelial cell coverage compared to AGA brains ($p<0.05$), and the FGR+UCBC group was also significantly increased compared to all other groups (Figure 5, A-C).

Pericyte coverage of individual blood vessels was examined using desmin α-SMA double-labeled immunofluorescence (Figure 5, D). FGR lambs showed a significant decrease in co-localization as studied by Mander's coefficient. UCBC therapy normalized co-localization in FGR+UCBC lamb brains as evidenced by no difference with pericyte coverage compared to AGA (Figure 5, E). Lastly, blood brain barrier function was assessed using albumin immunohistochemistry. We observed that 5 of the 6 FGR lamb brains showed evidence of increased BBB dysfunction (albumin extravasation into the brain parenchyma surrounding blood vessels) as compared to 1 out of 6 AGA brains. Only 1 of the 6 FGR+UCBC brains demonstrated albumin extravasation into the brain parenchyma.

Discussion

FGR infants are frequently delivered prematurely and being born preterm exacerbates brain injury associated with FGR[24], but the mechanism/s underlying increased neuropathology remain largely unknown. Here we demonstrate that

neuroinflammation and oxidative stress are upregulated in the brain of preterm FGR offspring that are ventilated, and we show, for the first time, that allogeneic umbilical cord blood cell therapy has neuroprotective properties for preterm FGR offspring via modulation of these aberrant responses. This is important because ventilation-induced cerebral inflammation and oxidative stress are observed more in FGR offspring, and likely mediate additive damage to developing white and grey matter of the brain[2]. Further, our results demonstrate that the neuroprotective benefits of UCBC are mediated, at least in part, via stabilization of the neurovascular unit.

Neuroinflammation

Animal models of FGR show that neuroinflammation is upregulated in areas of white and grey matter that are vulnerable to damage[25-27]. Cerebral inflammation can disrupt neuronal and oligodendrocyte development[28] leading to life-long neurological deficits. Our results demonstrate that mechanical ventilation induces an exacerbated microglial and astrocyte response in the FGR lamb brain, relative to AGA lambs. We did not include an unventilated group in this study but we have previously shown that neuroinflammation is increased with ventilation in this preterm FGR ventilation model and other preterm lamb models[6,29,30]. This selective neuroinflammation in FGR lambs may be a contributing factor towards the additive neuropathology observed in FGR infants who are born preterm. Accordingly, therapeutic intervention soon after birth to reduce neuroinflammation is likely to be neuroprotective for the FGR brain.

The administration of umbilical cord blood stem cells to ventilated FGR lambs at 1 hour after birth significantly decreased brain inflammation when compared to untreated ventilated FGR animals. Microglial cell activation was decreased across all

white matter brain regions examined in FGR+UCBC lambs. The cerebral and systemic anti-inflammatory benefits of UCBC therapy have been reported previously in a preclinical model of preterm brain injury induced via acute hypoxia ischemia[10]. Li et al also showed a significant correlation between increasing density of activated microglia and loss of oligodendrocytes at 10 days after insult, indicative that neuroinflammation is strongly associated with oligodendrocyte development. Further, that study showed that early administration of UCBC resulted in reduced microgliosis and restoration of oligodendrocyte cell number at 10 days after treatment[11]. While our study was conducted over a shorter period of 24 hours, these results suggest that protecting the brain against neuroinflammation would have positive benefits for white matter development.

The benefits of UCBCs may be mediated by their mixed cell population. Umbilical cord blood contains a number of stem and progenitor cells, including mesenchymal stromal cells (MSCs), T-regulatory cells (Tregs), endothelial progenitor cells (EPCs), and hematopoietic stem cells (HSCs), each with differential properties that could mediate positive effects on the brain[31]. In particular, the MSCs are postulated to modulate the anti-inflammatory effects of UCBCs, but we have shown recently that the proportion of MSCs in term derived UCBC is very low, <0.01%[32], and therefore it is likely that other cell types mediate the anti-inflammatory effects observed in the brain. Interestingly, in response to acute hypoxia in term-equivalent neonatal rats, the EPCs were equally effective as mixed cell UCBCs for reducing microglial activation and peripheral inflammatory cell infiltration into the brain, and the EPCs were more effective at reducing behavioral deficits[31] than UCBC. Endogenous or therapeutically administered EPCs migrate towards regions of tissue injury, where they contribute

towards vascular repair and remodelling[33,34]. Thus, in light of our observation that UCBCs reduced neuroinflammation and improved vascular stability, the mechanistic role and therapeutic potential of EPCs should be further examined.

Markers of cerebral oxidative stress were also significantly reduced within the white matter of FGR+UCBC lambs. Oxidative stress can play a major role in brain injury associated with FGR, preterm ventilation-induced brain injury, and apoptosis and cell death[4,6,35]. Studies of UCBC therapy have also demonstrated reduced circulating oxidative stress markers in a preterm lamb hypoxic model[10] and in response to birth asphyxia[9]. Despite this, we were not able to demonstrate a significant effect of UCBC on circulating oxidative stress markers or cell death seen in the FGR+UCBC lambs brains in this study. The previous studies of Li et al 2017[10] and Aridas et al 2016[9] induced perinatal brain injury via acute and severe hypoxia-ischemia, whereas FGR fetuses in the current study are exposed to moderate hypoxia over a chronic period, secondary to placental insufficiency. Also, the timing of the examination of the brain (24 hours post cell administration) in this study might have precluded observation of changes to cell death within 24 hours of birth and ventilation.

UCBC administration and practical use

Allogeneic UCBCs were collected from healthy term sheep at caesarean delivery. We utilized allogeneic cord blood cells from term ovine pregnancies in this study for three reasons, i) we wanted to ensure that we used healthy UCBCs and the composition of stem and progenitor cells in cord blood is different and may be compromised in pregnancies complicated by preterm birth, chorioamnionitis or FGR[36]; ii) we chose to administer cells at 1 hour after birth, which was feasible with allogeneic

administration; and iii) we have shown previously that UCBCs from term ovine pregnancies has a more potent anti-oxidant capacity than UCBCs from preterm cord blood[10]. The optimal type, timing and frequency of administration of UCBC cell therapy is still to be fully elucidated[15] and in this study we chose to use unfractionated cord blood instead of a specific cell type found within UCBC. Cells were administered at 1 hour after birth because preterm infants have usually achieved relatively stable cardiorespiratory parameters by then, and it gives us an early window of opportunity for neuroprotective therapy with the best chance to prevent systemic and cerebral inflammation in this vulnerable infant population. Previous studies show that early (12 hours after insult) administration of UCBCs provides greater benefit for the preterm brain following an acute hypoxic ischemic insult when compared to later (5 days after insult) administration[11]. In a clinical feasibility study of autologous umbilical cord blood cell therapy, Cotten and colleagues found that UCBC could be collected, processed and infused in babies mostly within 7 hours of birth[7]. Consideration of feasibility of autologous transplantation of UCBC therapy is paramount for translation of such therapies into the clinic.

We examined the distribution of UCBCs within the brain after post mortem 1 day after cell administration, and we were able to observe small numbers of diffusely distributed cells. The systemic immunomodulatory effects of UCBC, instead of engraftment may thus play an important role in mediating the neuroprotective effects[31] observed in this study. Indeed. non-manipulated umbilical cord blood cells are known to engraft poorly in tissues[37]. While this is a positive outcome because it suggests a lesser risk of tumor formation, it may require multiple, repeat dosing of cells to maximize benefit[38].

Cerebral blood flow and metabolism

We did not see significant differences in carotid blood flow (CBF; as a surrogate for cerebral blood flow) across the lamb groups during study, although it is interesting that CBF remained highest in the AGA group and lowest in the FGR group, while CBF in the FGR+UCBC animals was midway between the two. Previous studies in rodent models of stroke report different findings on the effects of UCBCs, either having no effect, or causing transient improvement in cerebral blood flow [39,40]. In any case, we did not find that CBF was adversely affected in FGR lambs after birth, at least not during the 24 hours of our study. The effect of UCBC on stabilizing cerebrovascular resistance is fascinating, as we did not observe significant independent effects of UCBC on MAP or CBF, both of which contribute to cerebrovascular resistance. It is likely that the stabilization is due to a combination of improvement in CBF as a result of stabilization of the neurovascular unit (discussed below) or improved cerebrovascular metabolism. This is the first study to report any benefits of UCBC therapy on cerebrovascular resistance.

Neurovascular unit

The importance of the neurovascular unit and its component cells have been increasingly recognized in the regulation of health and disease[41]. The neurovascular unit is critical in maintaining the integrity of the BBB. One of the major constituents of the BBB is the endothelium lining the microvessels, whose unique features largely account for the integrity of the barrier. Glut-1 was used in this study to mark the vascular endothelium, with this marker highly expressed by brain endothelium, and it is also utilized to assess glucose transport into the brain[42]. Maintaining the BBB

requires significant energy, which is obtained by the uptake of glucose by endothelial cells and, as such, the endothelial cells regulate the integrity of the BBB and transport of glucose into the brain [43]. In turn this process mediates energy-dependent survival of other glial and neuronal cells within the CNS[44]. GLUT-1 depletion is also closely related to the pathogenesis of cerebral oedema[45]. We observed a significant increase in endothelial cell proliferation in the white matter regions of the brain and subventricular zone, which we hypothesize might be a response aimed at stabilization of the energy supply to the brain. The increase in endothelial cell coverage seen with cell therapy could indicate that the UCBCs are supporting BBB integrity, which is confirmed by the results of albumin extravasation, thereby improving vascular stability. It is well described that the role of endothelial cells and astrocytes in the regulation of cerebral blood flow is crucial, and impairment in their number and function around the blood vessels leads to changes in cerebral blood flow [46]. Whether the neurovascular unit plays a similar role in the regulation of CBF in the developing brain still needs to be confirmed[47].

Together, the pericytes and astrocytes play critical regulatory roles in the NVU, including regulating capillary hemodynamic responses, angiogenesis, neuroinflammation and contributing to the integrity of the BBB[48]. Interestingly, cell therapy did not alter the astrogliosis observed in ventilated FGR lambs. We did, however, observe an increased co-localization/ association of the smooth muscle proteins of the vascular basal lamina with the pericytes in the NVU. This association would assist in the maintaining stability of the NVU, and regulation of cerebral blood flow and metabolism. Improved astrocyte or pericyte attachment with blood vessels is also likely to stabilize the BBB and decrease permeability to circulating proteins, as

evidenced by reduced albumin extravasation around white matter blood vessels in FGR+UCBC brains.

Overall effects

The overall effects of cell therapy on the neurovascular unit of FGR lambs are summarized in Figure 6. We demonstrate that early administration of UCBC to the FGR lamb reduces injurious processes following ventilation onset. UCBC administration provides cerebrovascular stability via a decrease in inflammation, mediated by A) systemic decrease in pro-inflammatory factors coupled with B) dampening of the cellular inflammatory activation of microglia. There may be some beneficial effects also on oxidative stress, and stabilization of the BBB of the vulnerable FGR lamb group by C) increasing endothelial cell proliferation and D) increased pericyte co-localization and stability.

Limitations

We acknowledge that there are limitations to this study, most notably that the lambs were studied for only 24 hours after birth, which is a relatively short period in the time course of the developing neuropathology, and were not subjected to other interventions that preterm infants may experience in neonatal care, including caffeine or inotrope administration. This period of 24 hours was not sufficient to observe the full development of brain injury, although it well described that cerebral inflammation and oxidative stress are pathogenic in the immature brain[6,49]. We utilized ovine UCBCs because we wished to examine the effects of allogeneic cell administration, however the use of ovine cells precludes characterization of subsets of stem and progenitor cells of interest[10]. Finally, while we were particularly interested in the

effects of UCBC on the brain, FGR infants are at risk for other neonatal morbidities, particularly related to cardiovascular and respiratory functions[2], and the effects of UCBC on these systems should also be examined.

Potential and Impact

The results of this study are most encouraging with respect to potential clinical translation, and this is the first study to demonstrate that UCBCs are neuroprotective for the brain of preterm growth restricted offspring. It is well described that early onset and severe (less than third centile for weight) FGR infants born preterm are at greatest risk for long term neurodevelopmental deficits[24]. There are no neuroprotective or neuroreparative treatments that are currently offered to severe early onset FGR infants after preterm birth, but neonatal cord blood stem cell therapy is already being trialed in preterm and term brain injury in neonates showing feasibility and safety[7,50]. We now propose that the results of this study lay the foundation for a novel therapeutic option using cord blood stem cells as an early intervention therapy for FGR infants.

Conclusions

We have previously shown that brain inflammation and oxidative stress are significantly upregulated in the FGR brain relative to the appropriately grown brain, at 24 h after birth following mechanical ventilation. Umbilical cord blood cell therapy in the early neonatal period reduces neuroinflammation and oxidative stress in FGR lambs in response to neonatal ventilation. Our results demonstrate that neuroprotective benefits of UCBCs are mediated, at least in part, by stabilization of the neurovascular unit with the FGR brain. These results are a foundation step

towards a novel neuroprotective therapy with cord blood stem cells that could be applied in the neonatal period to infants diagnosed with perinatal brain injuries like severe early onset fetal growth restriction.

Supplementary information

A supplementary file containing 2 tables, and 2 figures is available online.

Declarations

Ethics approval

Experiments complied with the National Health and Medical Research Council (NHMRC) of Australia guidelines for the care and use of animals for scientific purposes and were approved by Monash Medical Centre Animal Ethics Committee A.

Funding

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Author contributions

All experiments were conducted in the animal house and laboratory facilities of the Hudson Institute of Medical Research, Melbourne. AM, GRP, GJ, and SLM

contributed to the concept and design of the study; AM, MCM, BJA, AES, IN, YP, CAM, MCF, GRP, GJ and SLM contributed to acquisition, analysis and interpretation of data. AM drafted the initial manuscript and all authors edited the manuscript critically for important intellectual content, and approved the final version for submission. The authors further confirm that they are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Disclosures/ Conflicts of Interest

The authors do not have any disclosures or conflicts of interest to declare.

Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author.

Consent for publication

Not applicable

Abbreviations

AGA	appropriate for gestational age
BBB	blood brain barrier
CBF	cerebral blood flow
CGM	cortical grey matter
EPC	endothelial progenitor cell

FGR	fetal growth restriction
GFAP	glial fibrillary acidic protein
HNE	hydoxynonenal
HSC	haematopoietic stem cell
MAP	mean arterial pressure
MSC	mesenchymal stromal cell
NVU	neurovascular unit
PVWM	periventricular white matter
SCWM	subcortical white matter
SVZ	sub ventricular zone
UCBC	umbilical cord blood cell

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Tables

Table 1. Body, organ weights and organ/ body weight ratios for the lamb groups.

	AGA (n=6)	FGR (n=6)	AGA+UCBC (n=6)	FGR+UCBC (n=6)
Body weight (kg)	3.0±0.4	2.5±0.4*	3.1±0.3	2.0±0.2*
Brain weight (g)	43.9±2.2	43.9±4.7	46.4±3.7	42.4±3.6
Brain/body weight (g/kg)	14.8±1.7	17.9±2.5*	15.2±1.8	20.8±2.6*
Liver weight (g)	127.2±26.1	94.4±30.2*	121.4±16.7	89.0±15.3*
Liver/ body weight (g/kg)	42.1±3.6	37.4±7.6	39.5±4.5	41.1±21.6
Lung weight (g)	93.5±11.2	80.0±37.3	95.7±9.3	71.8±4.2*
Lung/ body weight (g/kg)	31.6±5.5	31.4±10.2	31.4±4.6	33.3±17.4
Heart weight (g)	26.7±4.3	20.4±6.8*	25.2±2.5	16.4±1.8*
Heart/ body weight (g/kg)	8.9±1.5	8.1±1.9	8.2±1.3	7.5±3.9

Data expressed as mean±SD. 2-way ANOVA analysis was applied for each parameter. *Denotes significant differences between corresponding FGR vs. AGA lamb groups.

Figure legends

Figure 1. A: Mean arterial blood pressure (MAP) across lamb groups over the course of the experiment. B: Carotid blood flow (CBF) as a surrogate for cerebral blood flow across lamb groups. C: Cerebrovascular resistance for lamb groups. #Significant differences between FGR vs. FGR+UCBC group at highlighted time points, $p<0.05$. Dotted lines denote timing of UCBC administration; 3-way RM ANOVA was applied across all parameters.

Figure 2. A: Representative photomicrographs of Iba-1-positive inflammatory cells in SCWM across groups. B: Quantitative analysis of Iba-1 cell counts across brain regions; 2-way ANOVA applied. C: Representative photomicrographs of GFAP-positive astrocytes in SCWM across groups. D: Quantitative analysis of GFAP cells across brain regions; 2-way ANOVA analysis applied. D: Serum levels of pro-inflammatory cytokine, TNF-α across groups over the course of the experiment; 2-way RM ANOVA applied. No significant rise in pro-inflammatory cytokines in FGR as compared to AGA lamb groups, but significant decrease in TNF-α levels seen in FGR+UCBC vs. FGR. * denotes FGR significantly different to AGA. # denotes FGR+UCBC significantly different to FGR; significant differences accepted at $p<0.05$. All scale bars = 50 μm.

Figure 3. A: Representative photomicrographs of 4-HNE -positive cells demonstrating oxidative stress in SCWM across groups. B: Quantitative analysis of 4-HNE cell counts across white matter brain regions; 2-way ANOVA applied. * denotes FGR significantly different to AGA, significant differences accepted at $p<0.05$. All scale bars = 50 μm.

Figure 4. A: Representative photomicrographs of Ki-67-positive cell proliferation in SVZ across groups. B: Quantitative analysis of Ki-67 cell counts across brain regions; 2-way ANOVA applied. C: Representative photomicrographs of Ki-67-positive blood vessels in PVWM in FGR+UCBC animals. D: Quantitative analysis of Ki-67-positive blood vessels across brain regions. # denotes significant difference between FGR+UCBC vs. all other groups. ^ denotes significant difference between FGR+UCBC vs. AGA+UCBC group. * denotes significant difference between FGR+UCBC vs. all other groups; significant differences accepted at $p<0.05$. All scale bars = 50 μm.

Figure 5. A: Representative photomicrographs of Glut-1-positive endothelial cell proliferation in PVWM across groups. Scale bar=50 μm. B: Quantitative analysis of Glut-1 cell counts across brain regions; 2-way ANOVA applied. C: Quantitative analysis of Glut-1 positive % area in PVWM across groups. D: Representative photomicrographs of Desmin-SMA double label fluorescent staining showing poor (A, FGR) and good (B, FGR+UCBC) co-localization within the neurovascular unit. Scale bar = 100 μm. * denotes significant difference between FGR vs. AGA groups. # denotes significant difference between FGR+UCBC vs. all other groups. E: Quantitative analysis of co-localization coefficients (M1 and M2), showing significant reduction (*) in co-localization coefficients in FGR lambs, which is normalized with UCBC therapy; significant differences accepted at $p<0.05$.

Figure 6. Schematic showing the possible mechanisms of action of UCBC therapy on the neurovascular unit in FGR lambs. Image courtesy: Dr Jean Tan.

Figure 1

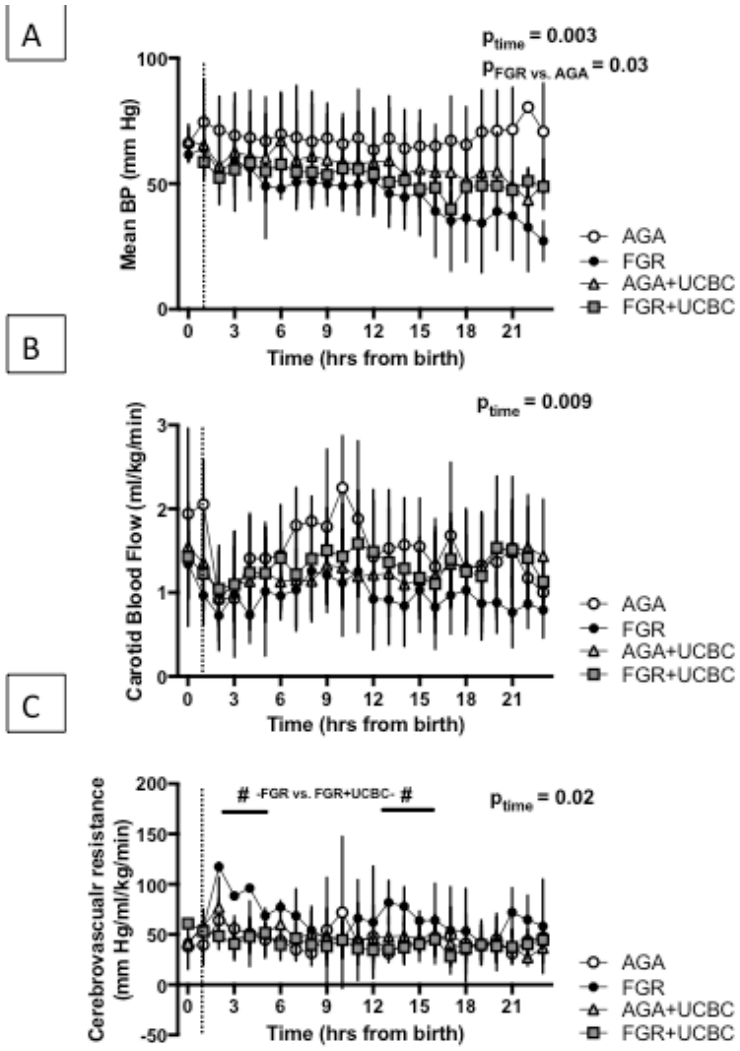

A
p_{time} = 0.003
$p_{FGR\ vs.\ AGA}$ = 0.03
Mean BP (mm Hg)
100
50
0
0 3 6 9 12 15 18 21
Time (hrs from birth)
AGA
FGR
AGA+UCBC
FGR+UCBC
B
p_{time} = 0.009
Carotid Blood Flow (ml/kg/min)
3
2
1
0
0 3 6 9 12 15 18 21
Time (hrs from birth)
AGA
FGR
AGA+UCBC
FGR+UCBC
C
-FGR vs. FGR+UCBC-
p_{time} = 0.02
Cerebrovascualr resistance (mm Hg/ml/kg/min)
200
150
100
50
0
-50
0 3 6 9 12 15 18 21
Time (hrs from birth)
AGA
FGR
AGA+UCBC
FGR+UCBC

Figure 2

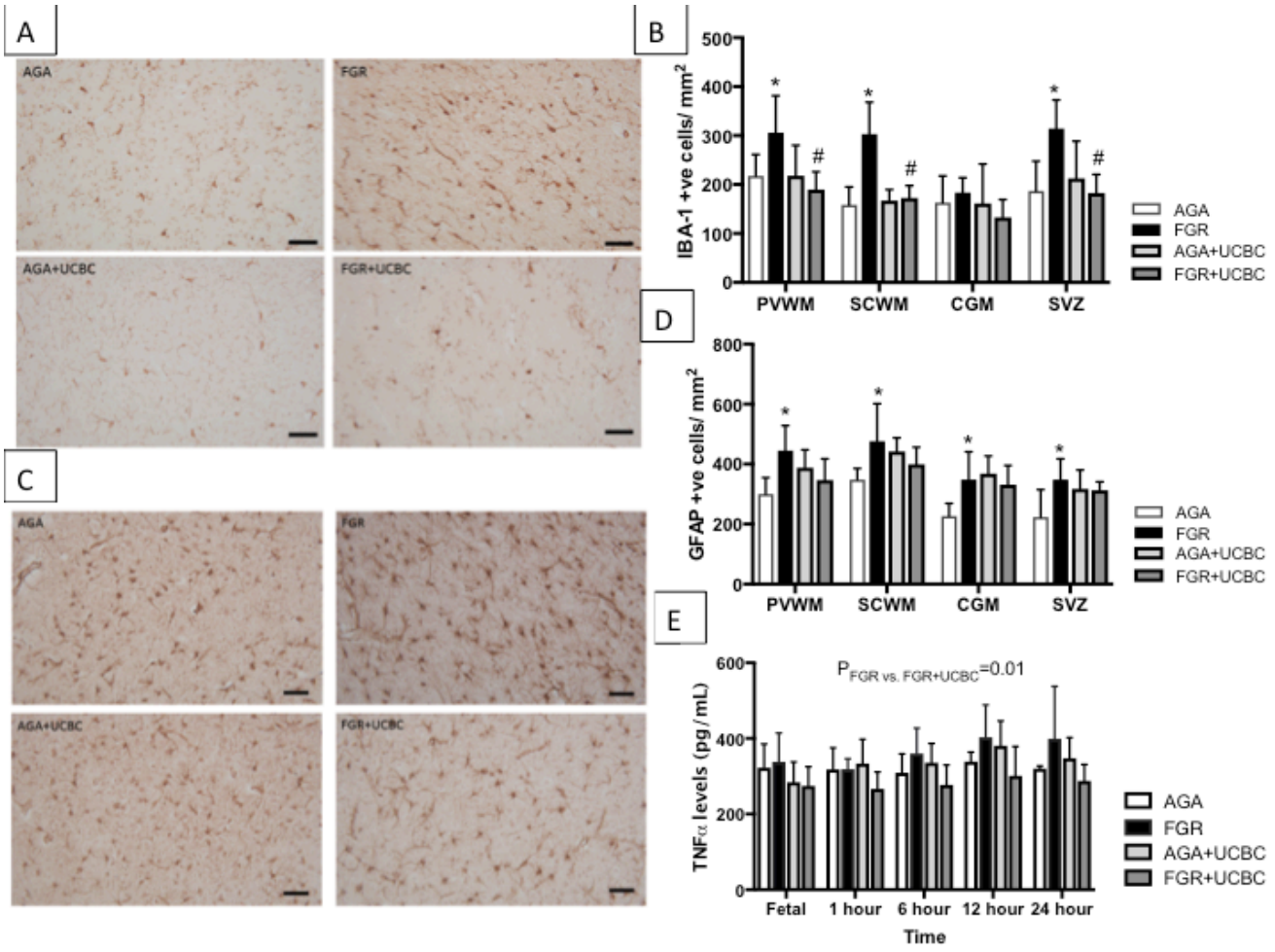

A
AGA
FGR
AGA+UCBC
FGR+UCBC
B
IBA-1 +ve cells/ mm²
500
400
300
200
100
0
PVWM
SCWM
CGM
SVZ
AGA
FGR
AGA+UCBC
FGR+UCBC
C
AGA
FGR
AGA+UCBC
FGR+UCBC
D
GFAP +ve cells/ mm²
800
600
400
200
0
PVWM
SCWM
CGM
SVZ
AGA
FGR
AGA+UCBC
FGR+UCBC
E
P FGR vs. FGR+UCBC=0.01
TNFα levels (pg/mL)
600
400
200
0
Fetal
1 hour
6 hour
12 hour
24 hour
Time
AGA
FGR
AGA+UCBC
FGR+UCBC

Figure 3

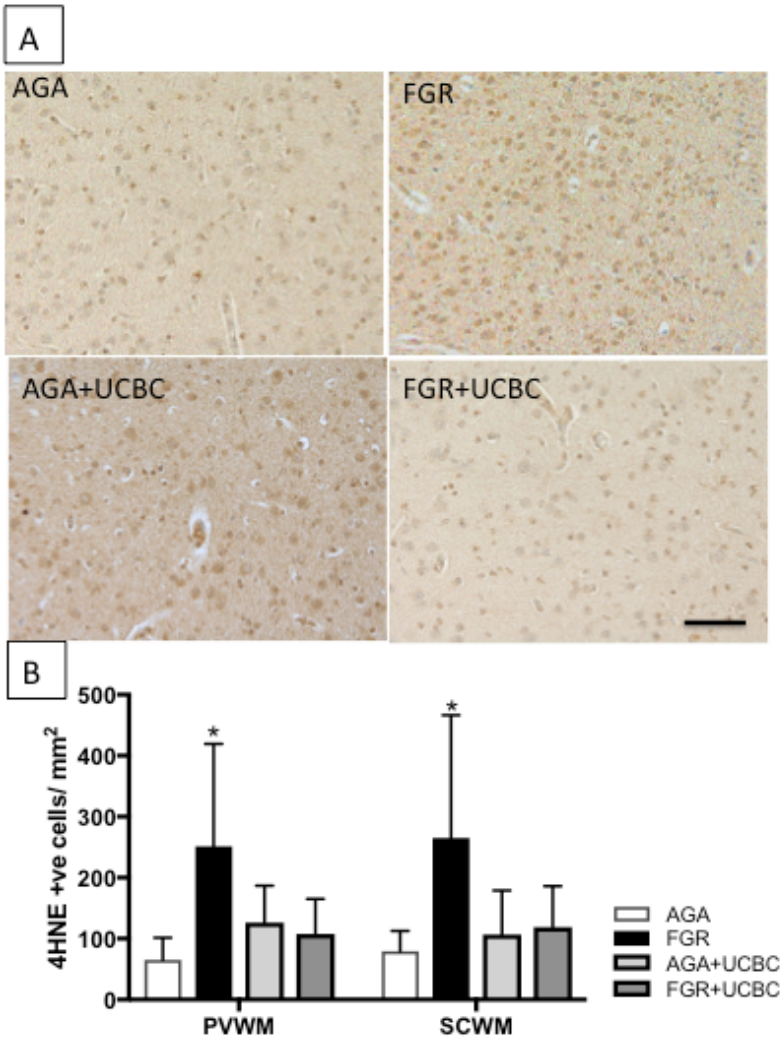

A
AGA
FGR
AGA+UCBC
FGR+UCBC
B
4HNE +ve cells/ mm²
500
400
300
200
100
0
*
*
PVWM
SCWM
AGA
FGR
AGA+UCBC
FGR+UCBC

Figure 4

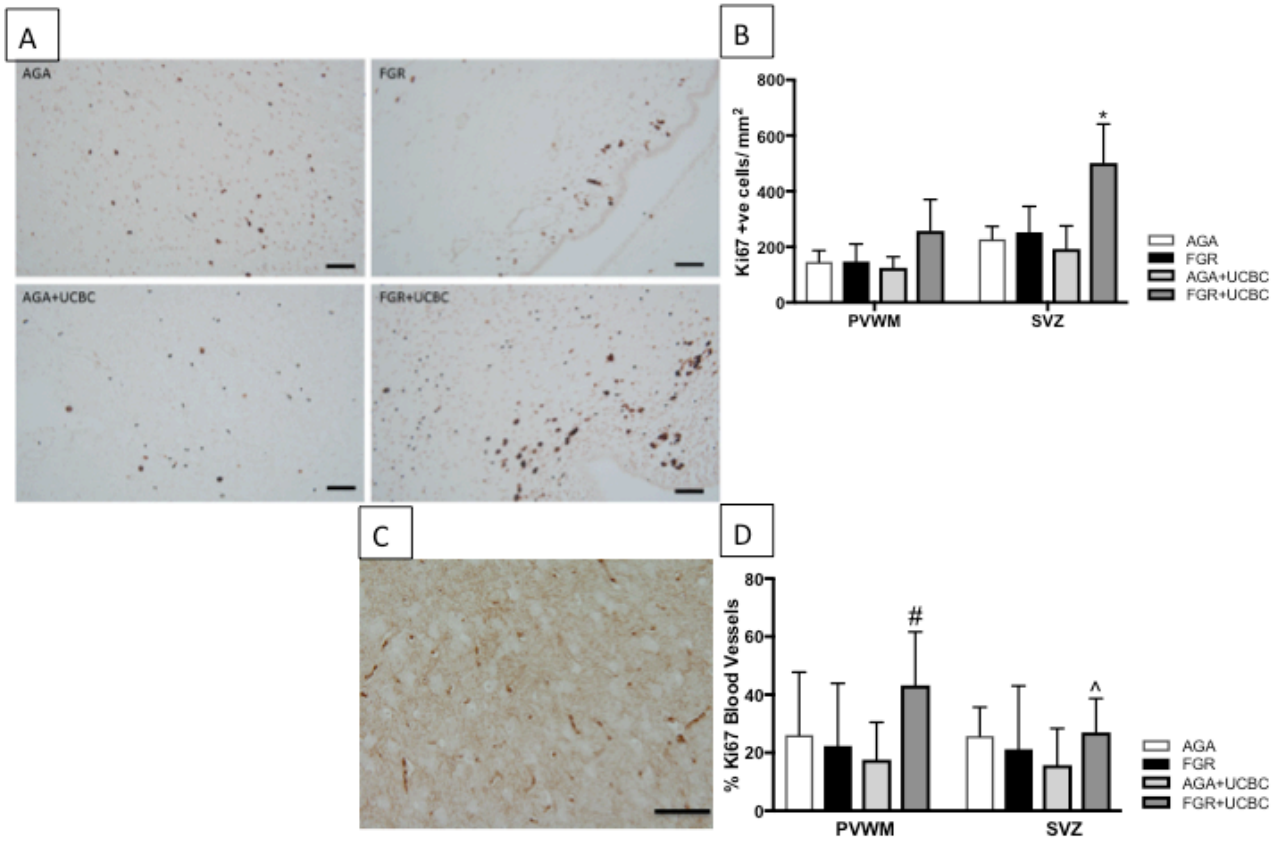

A
AGA
FGR
AGA+UCBC
FGR+UCBC
B
Ki67 +ve cells/ mm²
800
600
400
200
0
*
PVWM
SVZ
AGA
FGR
AGA+UCBC
FGR+UCBC
C
D
% Ki67 Blood Vessels
80
60
40
20
0
#
^
PVWM
SVZ
AGA
FGR
AGA+UCBC
FGR+UCBC

Figure 5

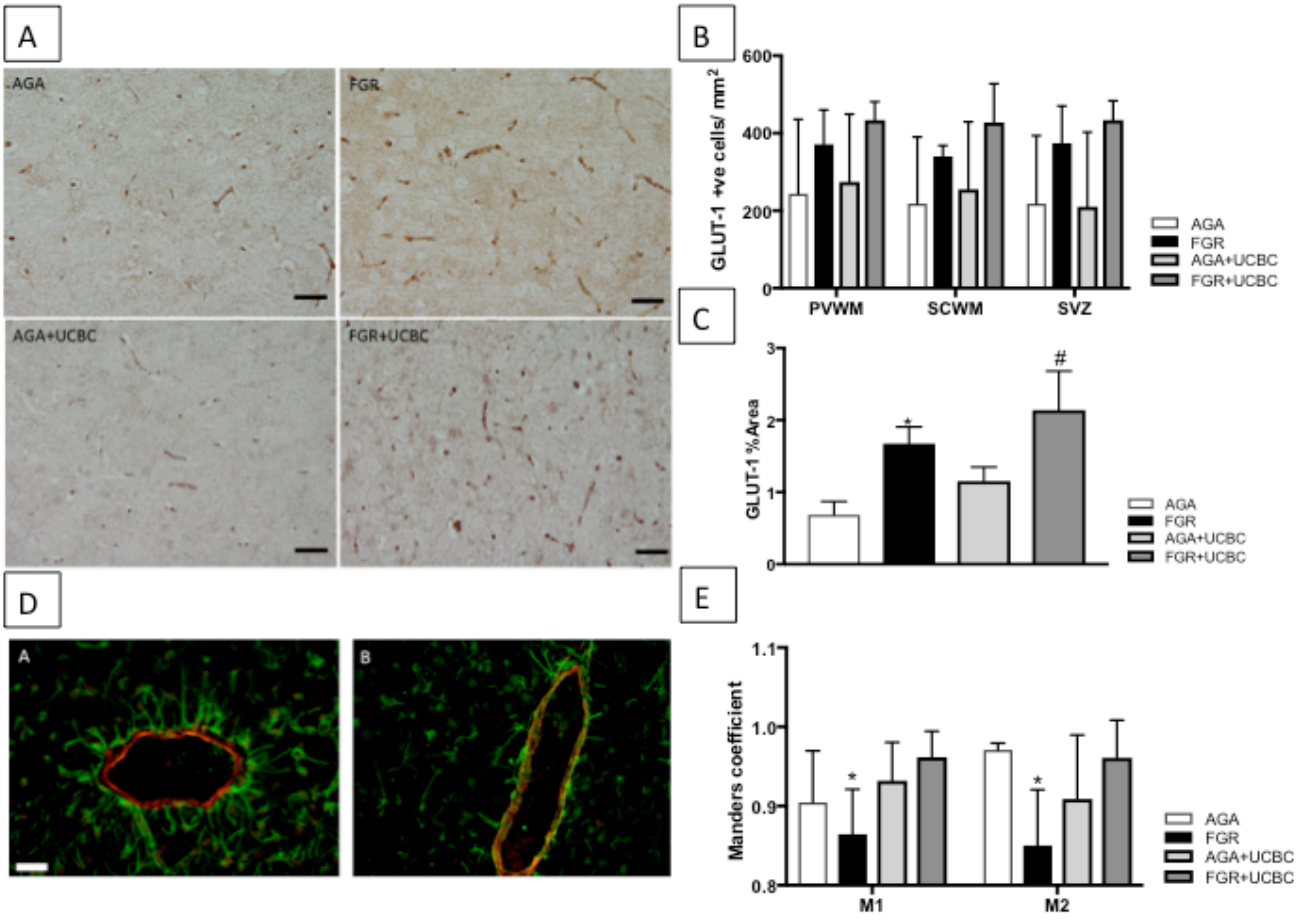
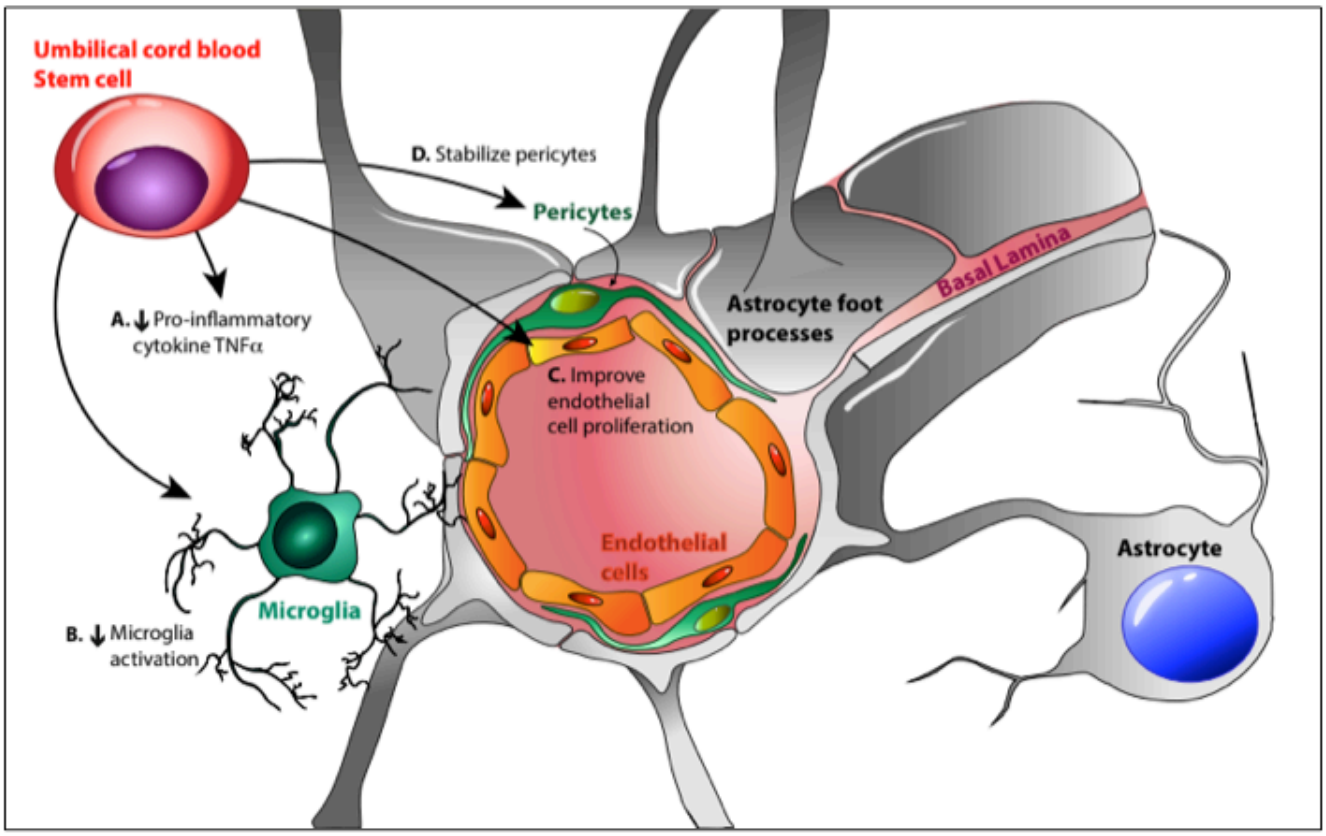

A
AGA
FGR
AGA+UCBC
FGR+UCBC
B
GLUT-1 +ve cells/ mm2
600
400
200
0
PVWM
SCWM
SVZ
AGA
FGR
AGA+UCBC
FGR+UCBC
C
GLUT-1 %Area
3
2
1
0
*
#
AGA
FGR
AGA+UCBC
FGR+UCBC
D
A
B
E
Manders coefficient
1.1
1.0
0.9
0.8
*
*
M1
M2
AGA
FGR
AGA+UCBC
FGR+UCBC

Figure 6


Umbilical cord blood
Stem cell
D. Stabilize pericytes
Pericytes
Basal Lamina
Astrocyte foot
processes
A. ↓ Pro-inflammatory
cytokine TNFα
C. Improve
endothelial
cell proliferation
Endothelial
cells
Astrocyte
Microglia
B. ↓ Microglia
activation

SUPPLEMENTARY FILE

Supplementary Table 1. Blood gas parameters during the course of the experiments across the lamb groups

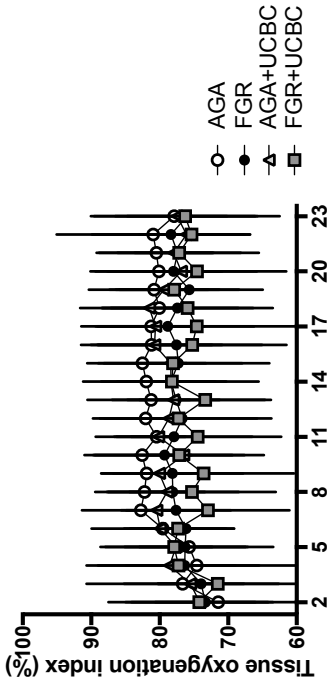
		1 hour	6 hours	12 hours	24 hours
pH	**AGA**	7.30±0.03	7.45±0.05	7.35±0.1	7.36±0.19
	FGR	7.27±0.08	7.47±0.08	7.37±0.1	7.33±0.19
	AGA+UCBC	7.14±0.10	7.39±0.02	7.37±0.05	7.34±0.06
	FGR+UCBC	7.25±0.15	7.33±0.10	7.29±0.12	7.21±0.19
pO_2 (mmHg)	**AGA**	38.0±11.8	47.3±11.9	34.6±7.1	39.0±7.9
	FGR	35.3±7.2	34.9±4.1	38.2±9.4	41.8±13.5
	AGA+UCBC	37.0±5.3	45.4±12.3	43.7±9.2	40.0±5.7
	FGR+UCBC	30.4±5.8	41.3±13.0	42.2±10.7	35.3±9.1
pCO_2 (mmHg)	**AGA**	51.3±8.9	39.8±5.5	58.7±15.8	45.6±15.2
	FGR	52.2±6.6	35.1±8.6	49.9±7.8	37.6±14.6
	AGA+UCBC	58.1±15.0	44.8±2.7	52.0±7.2	54.4±10.6
	FGR+UCBC	56.1±15.3	53.7±15.0	59.5±17.3	62.3±17.0
Lactate (mmol/L)	**AGA**	3.5±0.6	2.1±0.7	1.6±0.4	1.4±7.1
	FGR	4.2±1.3	3.0±0.5	1.8±0.3	1.8±3.4
	AGA+UCBC	4.6±1.8	1.7±0.2	1.4±0.3	1.3±0.4
	FGR+UCBC	4.0±1.9	1.9±0.4	1.6±0.5	2.6±3.1
HCO_3^- (mmol/L)	**AGA**	27.9±4.0	27.5±1.4	31.2±2.8	24.3±10.4
	FGR	23.6±5.1	25.5±3.1	27.4±3.3	15.9±12.4
	AGA+UCBC	20.0±4.5	26.8±1.9	28.6±1.6	27.5±4.2
	FGR+UCBC	24.8±3.7	26.9±5.1	28.1±5.5	26.3±6.3

Data expressed as mean±SD. No significant differences seen between any lamb groups.

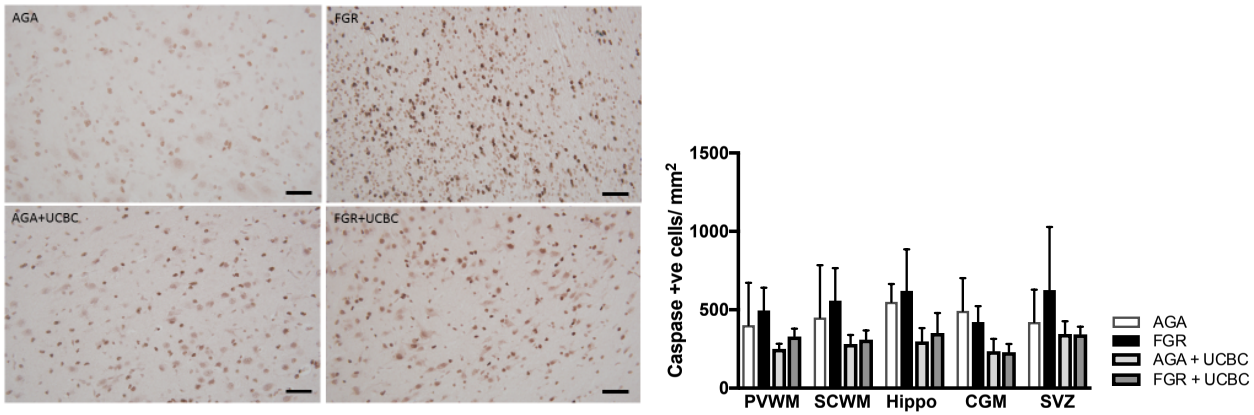
Supplementary Table 2. Ventilation parameters during the course of the experiments across the lamb groups

		1 hour	6 hours	12 hours	24 hours
Compliance (ml/g/cm H_2O)	**AGA**	0.016±0.020	0.016±0.020	0.018±0.008	0.020±0.013
	FGR	0.011±0.003	0.016±0.000	0.011±0.001	0.008±0.001
	AGA + UCBC	0.013±0.001	0.020±0.001	0.016±0.001	0.013±0.003
	FGR + UCBC	0.014±0.002	0.016±0.003	0.016±0.002	0.010±0.003
Peak pressure (cm H_2O)	**AGA**	16.8±1.6	21.0±1.6	21.0±2.6	25.0±3.4
	FGR	18.6±6.4	20.0±3.6	19.6±3.4	26.0±6.4
	AGA + UCBC	21.6±1.7	15.8±1.3	15.6±0.8	21.7±1.9
	FGR + UCBC	18.8±1.5	19.2±1.4	20.5±2.0	24.7±1.7
Tidal volume (ml/kg)	**AGA**	5.2±0.3	4.9±0.3	5.0±0.3	5.6±0.5
	FGR	5.2±0.4	5.0±0.1	4.6±0.3	4.8±1.0
	AGA + UCBC	4.9±0.0	4.9±0.0	4.9±0.1	4.9±0.1
	FGR + UCBC	5.0±0.0	5.0±0.0	4.9±0.1	4.9±0.1
FiO_2	**AGA**	40.0±11.2	22.6±1.0	26.0±5.0	31.0±10.0
	FGR	36.5±10.5	22.0±1.2	27.6±4.0	52.8±15.0
	AGA + UCBC	43.1±6.9	25.8±4.8	21.0±0.0	24.0±1.2
	FGR + UCBC	35.4±6.8	29.5±2.8	30.5±3.7	48.4±13.4

Data expressed as mean±SD. No significant differences seen between any lamb groups.



Supplementary Figure 1. Tissue oxygenation index as measured (mean±SD) in the lamb groups across the duration of the experiment.



Supplementary Figure 2. Left panel: Representative photomicrographs of cell death seen as apoptotic cells (Caspase-3) in SCWM in lamb groups. Scale bar=50um. Right panel: Quantitative analysis (mean±SD) of Caspase-3 cells across brain regions. 2-way ANOVA analysis applied. No significant differences seen with cell therapy.

Chapter 4: Literature Review B

The Chapter 1 review brought together clinical data to show that FGR infants born preterm have an increased risk for neurological deficits. However, this raises novel questions around how brain injury can be optimally detected (and treated) in preterm FGR infants. Experimental animal data from Chapters 2 and 3 finds that neuropathology is significantly increased in FGR lambs at preterm birth, and exacerbated with neonatal ventilation, and provides us with information regarding the nature of the brain injury and the white matter brain regions that are most at risk. It is however not easy to detect this FGR related brain injury in the clinic. Early detection of FGR related brain injury is critical for the assessment of brain injury for the clinician, the affected infant and the family. The next two chapters of this thesis focus on the "early detection" of FGR brain injury in the neonatal period in the clinical and preclinical situation. These are relevant to the overall aim of my thesis, which was to develop and evaluate novel strategies for the early detection and early treatment of FGR related brain injury.

In my clinical experience, I have encountered numerous occasions when brain injury is not evident on conventional brain imaging in the fetal and newborn period in FGR, but these infants go on to develop neurodevelopmental deficits in infancy and childhood. In this chapter, I reviewed the tools that are currently available to detect and assess brain injury in the growth restricted fetus and neonate. A scoping review and narrative synthesis of relevant data is presented. The findings of this literature review chapter directly pertain to the research I conducted on using advanced MRI brain analysis techniques for the detection of FGR related white matter brain injury, which is reported in Chapter 5. This is the unaltered version of the review paper published in Pediatric Research.

4.1. Detection and assessment of brain injury in the growth restricted fetus and neonate

*Atul Malhotra[1,2,3], Michael Ditchfield[2,4], Michael C Fahey[2,3], Margie Castillo-Melendez[3,5], Beth J Allison[3,5], Graeme R Polglase[3,5], Euan M Wallace[3,5], Ryan Hodges[3,5], Graham Jenkin[3,5], Suzanne L Miller[3,5] (2017) Aug; 82(2):184-193. doi: 10.1038/pr.2017.37

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Review

Detection and assessment of brain injury in the growth-restricted fetus and neonate

Atul Malhotra[1], Michael Ditchfield[2], Michael C. Fahey[3], Margie Castillo-Melendez[4], Beth J. Allison[4], Graeme R. Polglase[4], Euan M. Wallace[5], Ryan Hodges[5], Graham Jenkin[4] and Suzanne L. Miller[4]

Fetal growth restriction (FGR) is a common complication of pregnancy and, in severe cases, is associated with elevated rates of perinatal mortality, neonatal morbidity, and poor neurodevelopmental outcomes. The leading cause of FGR is placental insufficiency, with the placenta failing to adequately meet the increasing oxygen and nutritional needs of the growing fetus with advancing gestation. The resultant chronic fetal hypoxia induces a decrease in fetal growth, and a redistribution of blood flow preferentially to the brain. However, this adaptation does not ensure normal brain development. Early detection of brain injury in FGR, allowing for the prediction of short- and long-term neurodevelopmental consequences, remains a significant challenge. Furthermore, in FGR infants the detection and diagnosis of neuropathology is complicated by preterm birth, the etiological heterogeneity of FGR, timing of onset of growth restriction, its severity, and coexisting complications. In this review, we examine existing and emerging diagnostic tools from human and preclinical studies for the detection and assessment of brain injury in FGR fetuses and neonates. Increased detection rates, and early detection of brain injury associated with FGR, will offer opportunities for developing and assessing interventions to improve long-term outcomes.

Fetal growth restriction (FGR) or intrauterine growth restriction (IUGR) affects more than 10% of pregnancies worldwide, with substantial implications for short-term and long-term well-being of the infant. FGR is strongly associated with stillbirth, preterm birth, and, in newborn survivors, increased risk of developing neonatal complications (1). FGR is also a causal factor in the development of adverse neurodevelopmental sequelae in childhood (2,3).

FGR defines a fetus that has failed to reach its genetically determined birth weight. Unfortunately, there is a lack of a consensus definition for fetal growth restriction. Pragmatically, FGR is defined by the criteria of estimated fetal weight, or birth weight, being less than the 10th centile for age and sex. However, many studies do not discriminate between infants whose birth weight is less than the 10th centile for age but who are small and otherwise healthy, termed small for gestational age (SGA), compared with the pathologically small babies who did not grow fully (true FGR). Further, some fetuses will be growth-restricted, but have a birth weight $>$10th centile. Such fetuses remain at an increased risk of stillbirth or perinatal morbidity.

The causes of FGR are diverse, including fetal, maternal, or placental factors (4). Poor placental function is the most important contributor clinically (5–7), resulting in chronic fetal hypoxia and hypoglycemia in an otherwise normal fetus (8–10). In turn, chronic fetal hypoxemia and nutrient insufficiency directly decrease fetal growth rate, and hypoxia induces a redistribution of cardiac output (11–14). This redistribution of fetal cardiac output tends to protect brain and heart growth relative to other organs, termed brain-sparing or central redistribution, but this does not ensure normal brain growth (4,9,15).

The specific neuropathology of FGR is complex and distinct from that in both infants born preterm without FGR and in term infants exposed to a severe acute hypoxic event (2,16). Human FGR imaging studies and postmortem examination, together with animal experimental studies of placental insufficiency and FGR, describe reduced total brain volume, with loss of both gray and white matter substructure. At the cellular level, gray matter areas are shown to have reduced cell number (17) with sparse and disorganized cortical structure (18). The white matter of the FGR brain is described as immature, with delayed oligodendrocyte maturation (19), more unmyelinated axons, and thinner myelin coverage (20), with evidence of astrogliosis and inflammation (21). More recently, it has also been shown that the structural connectivity of the FGR brain is significantly altered, particularly along motor and cortico-striatal-thalamic tracts. Importantly, these measures of reduced tractography correlate with poor neurodevelopmental outcomes in young children who were born FGR (22,23).

FGR is associated with an increased risk for neurodevelopmental impairment, with the degree of impairment

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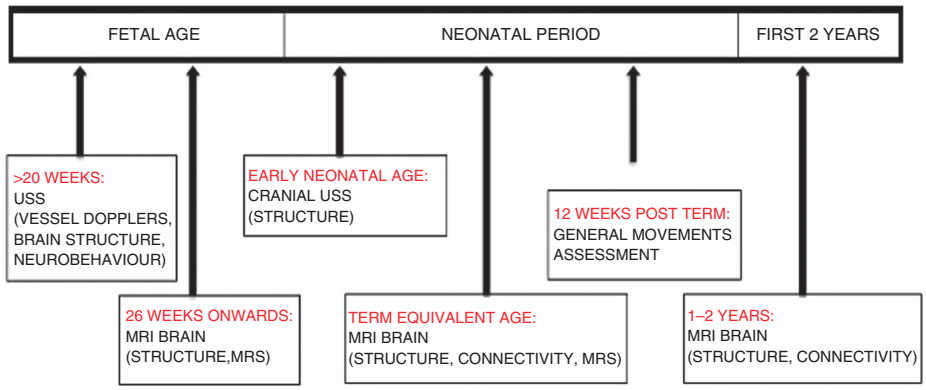

Figure 1. Timeline for the detection and assessment tools available for FGR-related brain injury.

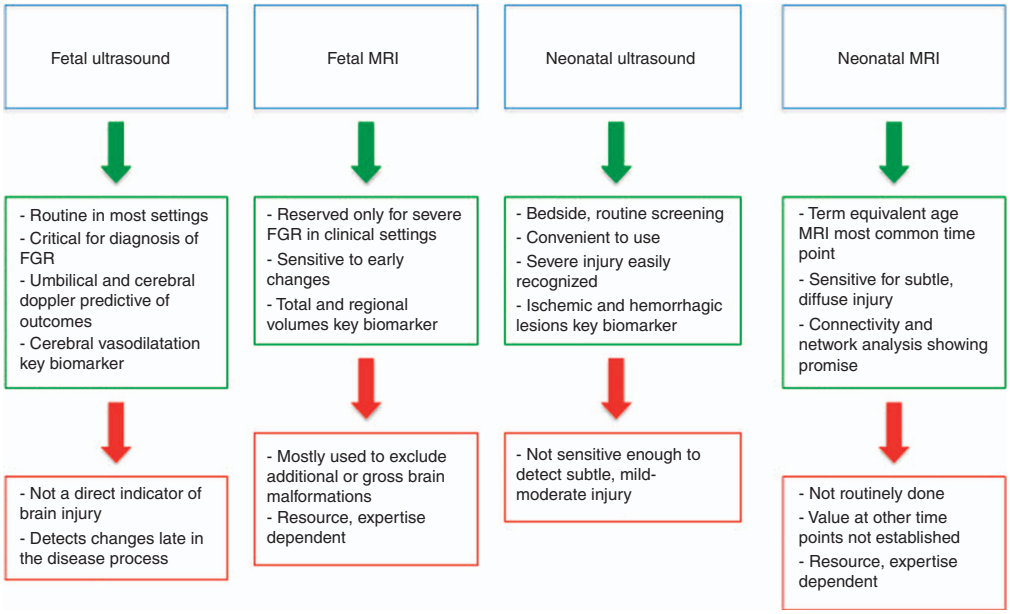

Figure 2. Current status of fetal and neonatal imaging for FGR-related brain injury.

related to (i) the severity of growth restriction, (ii) the onset of FGR (early or late), and (iii) gestation at birth (preterm or term) (2). FGR children born preterm or with evidence of brain-sparing are considered to be at greatest risk for deficits in brain development (24). The neurodevelopmental outcomes of children born after early-onset FGR are worse than outcomes for late-onset FGR. This likely reflects both a greater degree of placental dysfunction and hypoxia adversely affecting brain development, and the impact of preterm birth (25). In addition, preterm FGR infants demonstrate an elevated risk for neonatal complications such as pulmonary hypertension, metabolic disturbances, and necrotizing enterocolitis, which in turn may induce acute hypoxia/ischemia leading to increased brain injury (26). Late-onset FGR infants are also at risk for altered outcomes, particularly infants with brain-sparing, who show abnormal neurobehavior in the neonatal period and at 2 years of age (27–29). Impairments in school-age children who have FGR encompass gross and fine motor deficits, cognition and learning problems, and behavioral dysfunctions (30,31), and neurological dysfunctions continue into older childhood and adolescence (32). Furthermore, FGR is associated with high risk for diagnosis of cerebral palsy. The rate of cerebral palsy for early-onset FGR is up to 12% for infants delivered at <32 weeks of gestation (25,33,34).

The complex and heterogeneous adverse outcomes observed in FGR children demonstrate the need for accurate neurological assessments that can be applied either antenatally or postnatally, and for the provision of a diagnostic link between the injury observed and long-term consequences. The objective of this review is to bring together the available evidence for the detection and assessment of brain injury linked to FGR, in both the fetus and neonate. We acknowledge that, with no strict definition for FGR, this is imperfect; however, here we have only included published work in which the population was described as "FGR" or "IUGR".

ASSESSMENT OF FGR-RELATED BRAIN INJURY IN THE FETUS

Fetal Ultrasound

Ultrasound-based fetal surveillance is an established component of modern perinatal care of high-risk pregnancy, including the monitoring of FGR (**Figures 1** and **2**) (35–37). A major aspect of assessment of fetal well-being and, indirectly, neuropathology in FGR relies on Doppler assessment of fetal and uteroplacental circulations. As placental pathology is considered the principal cause of true FGR (7,10), recent definitions of FGR include fetal umbilical artery Doppler flow velocimetry assessment (36,38). However, it is suggested that a definition of true FGR should not rely on parameters of fetal umbilical artery Doppler alone, as this parameter identifies only severe, early-onset placental insufficiency (39). Instead, FGR should be diagnosed by the presence of poor fetal growth combined with any Doppler observation associated with suboptimal perinatal outcome in umbilical or uterine artery (UA), or cerebral arteries. A recent study in a cohort of FGR fetuses confirmed that evaluation of Doppler parameters, rather than gestational age at birth, allowed better risk stratification of FGR preterm fetuses for neonatal neuropathologies (40).

Fetal ultrasound—umbilical artery Doppler. Growth-restricted fetuses with absent or reversed end-diastolic flow in the UA have increased rates of fetal and neonatal mortality, and a higher incidence of long-term permanent neurologic damage (41). The importance of following UA Doppler status is demonstrated particularly in early-onset FGR, where end-diastolic velocity is reversed in the UA or aorta, whereas cerebral vascular impedance changes are apparent in both early- and late-onset FGR (38). Absent diastolic flow in the UA is a sign of placental resistance and vascular stress, with increasing placental resistance leading to reversal of flow in an already compromised fetal placental unit. Fetal Doppler indices, such as absent or reversed end-diastolic flow in the UA and absent or reversed "a" wave in the ductus venosus (DV), are considered good predictors of neonatal intraventricular hemorrhage (IVH) and death in growth-restricted infants (42,43). Infants who demonstrate an altered UA have poorer motor outcomes at 2 years and at school age when compared with their appropriately grown preterm or term counterparts (44,45). It is pertinent to note that the detection of placental and fetal circulatory abnormalities via ultrasound does not provide a direct assessment of brain injury but, along with fetal head circumference, severity of growth restriction, and gestational age at delivery, they are very useful determinants of the degree of placental dysfunction, which is, in turn, associated with neurodevelopmental outcomes (46). The overall sensitivity and specificity of reverse end-diastolic flow in UA or DV and adverse perinatal outcomes is 60–80%, with a positive predictive value (PPV) of ~50% and negative predictive value (NPV) of 80% (47,48).

Fetal ultrasound—cerebral doppler. Assessment of the fetal cerebral circulation is particularly useful to observe hemodynamic changes associated with chronic hypoxia and the severity of FGR. The gold standard for fetal brain hemodynamic evaluation is middle cerebral artery (MCA) flow (3) and pulsatility index (49). Reduced pulsatility index in the MCA demonstrates cerebral vasodilatation (and brain-sparing), and a number of studies show that MCA vasodilatation predicts neurodevelopmental deficits after birth (28,50–52). All available data to date indicate that vasodilation of the MCA reflects an advanced and severe stage of growth restriction and brain injury, with high risk for abnormal neurodevelopment (53,54). The anterior and posterior cerebral arteries might also provide cerebral hemodynamic insight, characteristic of the onset and the degree of brain-sparing (50). Scherjon and colleagues showed that fetal brain-sparing with elevated umbilical/cerebral ratio was associated with normal neurodevelopmental outcome at 3 years of age but, at 5 years, infants with brain-sparing had an IQ score 9 points lower than expected (30,55). Fetal blood flow redistribution in favor of the fetal brain can also be detected and quantified by the Doppler cerebral/umbilical ratio (C/U ratio = cerebral resistance index (CRI)/umbilical resistance index) (56) and the fractional moving blood volume estimation (57). Fetal deterioration in chronic and severe hypoxia is characterized by the disappearance of physiological cerebral vascular variability, followed by an increase in cerebral vascular resistance (56). However, studies on growth-restricted and hypoxic human fetuses have shown that perinatal brain lesions can develop even before the loss of cerebrovascular variability (27). The cerebroplacental ratio (ratio of Doppler indices of MCA and UA) is an important predictor of adverse perinatal and later outcomes. The sensitivity and specificity of an abnormal cerebroplacental ratio for an adverse perinatal outcome lies between 60% and 80% (58).

Fetal ultrasound—others doppler studies. Aortic isthmus Doppler has been proposed as a novel method to interrogate oxygenation of the cerebral circulation in the presence of brain-sparing. When downstream placental vascular resistance is high and cerebral vascular resistance is low, blood leaving the right ventricle may take the path of least resistance and flow retrograde through the aortic isthmus. This results in poorly oxygenated blood from the right ventricle, destined for the placenta, instead being shunted through the aortic isthmus to the cerebral circulation (59). In the setting of early-onset severe FGR, retrograde flow increases the likelihood of IVH and periventricular leukomalacia (60). Not surprisingly, such

retrograde flow has been linked with adverse neurodevelopmental outcomes at the age of 2–5 years (61).

UA Dopplers have also been studied, but their role in the assessment of brain injury or outcomes in FGR is not clear (62). Overall, fetal Dopplers greatly assist in the assessment and prediction of FGR, particularly the more severe cases, and, although these Doppler indices do not detect brain injury *per se*, they provide an essential first screening to identify fetuses at greatest risk of brain injury. Less severe FGR fetuses are more difficult to detect via Doppler assessments and, therefore, FGR and associated neuropathology may be missed.

Fetal ultrasound—direct assessment of brain structure. In addition to assessment of the fetoplacental circulation, ultrasound also offers opportunities to assess fetal brain structure. Prenatal 3D ultrasound can detect smaller brain volume in FGR fetuses (63), and can detect coexistent neuropathologies including intracranial hemorrhage and hydrocephalus, which may not be due to FGR but contribute to adverse outcomes (64). FGR fetuses show differences in the volume of many intracranial structures compared with appropriate for gestational age fetuses, with the largest difference observed in the frontal region. Nomograms exist for the ultrasonographic dimensions of the fetal corpus callosum, allowing for prenatal diagnosis of abnormal callosal development (65,66). Cerebellar size, measured by ultrasound, is correlated with the severity of FGR, and therefore the trans-cerebellar diameter may also have prognostic significance (67). Although there are some studies that have correlated fetal corpus callosum and trans-cerebellar diameter changes with neurobehavior and neurodevelopment (68–70), these remain relatively rudimentary detecting only the most overt structural changes.

Fetal ultrasound—assessment of fetal behavior. Fetal biophysical profile, which assesses fetal tone, breathing, and body movements, has been traditionally used in the surveillance of high-risk pregnancies, and its accuracy in prediction of perinatal and neonatal outcomes continues to be debated (7). Kurjak *et al.* (71)proposed a scoring system for the assessment of fetal neurological status by 4D sonography named "*Kurjak Antenatal Neurodevelopmental Test* (KANET)". This test assesses fetal behavior in a qualitative and quantitative manner (72); however, it remains unvalidated in large studies by independent operators and requires skill development and expertise that currently limits its widespread use.

In summary, a number of fetal ultrasound tools are available to directly and indirectly detect, assess, and prognosticate on neurological outcomes of FGR fetuses. Comprehensive evaluation of heterogenous FGR fetuses in large studies will aid elucidation of the most reliable and feasible neuroimaging assessments capable of predicting neurodevelopmental outcomes.

Fetal MRI

Fetal brain MRI has revolutionized early detection of intrauterine CNS injury in high-risk fetal and pregnancy conditions. Fetal MRI can be technically challenging, with acquisition of diagnostic quality fetal brain MRI affected by the trans-abdominal intrauterine environment and fetal movement. Fetal MRI brain is ideally done in a center with good radiological expertise, in late gestation when the fetal head is fixed in the maternal pelvis. In FGR, fetal brain MRI is currently used predominantly as a clinical tool to exclude gross brain malformations, and as a research tool for the evaluation of FGR-related brain injury; hence, predictive values for adverse outcomes are not yet available.

Fetal MRI—brain structure. Fetal MRI provides a sensitive and detailed assessment of the developing brain in high-risk conditions, including for growth-restricted fetuses, with the capacity to correlate fetal brain structural anomalies with neurodevelopmental outcomes (73). MRI of the fetus and the fetal brain has been used to confirm circulatory redistribution —brain-sparing—in FGR fetuses via assessment of fetal organ volumetry (13) or superior vena caval and umbilical vein perfusion (74). Fetal brain MRI has also been used in late-onset FGR fetuses to demonstrate an abnormal pattern of cortical development (75). Brain function, especially childhood development, is tightly linked to the development of the cortex in late gestation, and MRI with post-processing image analyses can provide insight into cortical development (76). This is reflected in the use of conventional MRI and diffusion tensor imaging (DTI) of appropriately grown and FGR fetuses, which has elucidated the relationship between changes in intracortical layering and cortical folding, where FGR is associated with altered cortical development (76). Fetal MRI of the corpus callosum has also confirmed that this structure is significantly smaller in FGR fetuses, and this is correlated with adverse neonatal neurobehavioral outcomes (68).

Fetal MRI—brain metabolism. Magnetic resonance spectroscopy (MRS) is a standard tool used in the early neonatal period to examine brain biochemistry wherein changes in MRS can predict neurological outcomes in perinatal brain injury, especially birth asphyxia (77). More recently, MRS has been successfully undertaken on the fetal brain, and to date demonstrates similar findings to those observed in neonatal populations with respect to altered brain metabolite concentrations within the compromised brain. MRS is particularly useful when subtle changes are present on conventional fetal MRI sequences (78). Brain-sparing in FGR fetuses is associated with altered brain metabolism evidenced by a reduction in the peak ratios of the metabolites N-acetylaspartate: Choline (NAA:Cho) and N-acetylaspartate: Creatine (NAA:Cr) (79). NAA is a very useful marker of neuronal cell integrity, and therefore reduction in these ratios principally reflects a loss of neurons within the FGR brain. Furthermore, frontal lobe NAA:Cho ratio in FGR and appropriately grown fetuses shows a strong association with corpus callosum development (80). The brain metabolite myo-inositol, considered a good marker of glial astrocyte cells, has also been examined in FGR and appropriately grown fetuses via

MRS, but levels in key brain regions are not shown to be significantly different between cohorts (81).

In summary, a number of promising MRI and MRS tools for use in the fetus are currently being investigated. It is apparent that they can provide excellent direct assessments of structure and biochemistry of the developing brain, and to date results suggest that fetal MRI and MRS outcomes show strong predictive value for long-term neurodevelopmental outcomes. Fetal MRI is, however, not readily available across obstetric and birth centers, and requires specialist expertise, adequate training of radiology personnel involved, and resources to obtain reliable, clinically useful, and relevant information. Moving forward, it will be critical for individual centers to evaluate the pros and cons of obtaining fetal brain MRI assessments, with consideration for the expertise and resources available.

DETECTION OF BRAIN INJURY IN THE FGR NEONATE

In high-resource clinical settings, approximately half of the FGR fetuses are detected antenatally (82) (**Figures 1** and **2**). The findings presented above demonstrate that fetal assessment of the FGR brain could effectively be incorporated into routine clinical care to detect neuropathology associated with FGR, particularly in the most severe cases. However, in the remaining (antenatally undiagnosed) FGR neonates, it is critical that effective screening and detection strategies are in place for the assessment of neuropathology after birth. In the first instance, an important consideration is therefore the neonatal identification of the growth-restricted infant who would be appropriate for a newborn imaging examination. This allows for the possibility of neuroprotective strategies to treat FGR-related brain injury to commence shortly after birth, and certainly provides clinicians and parents with knowledge regarding diagnosis and follow-up requirements across the spectrum of potential outcomes.

Neonatal Ultrasound

Cranial ultrasound enables bedside, easily available serial cerebral assessment, and is commonly used as the primary brain-imaging modality in high-risk neonates, especially those born preterm. It is well described that significant brain abnormalities evident on neonatal cranial ultrasound are associated with adverse neurodevelopment (83). Neonatal cranial ultrasound is considered the gold-standard screening method for neonatal brain injury, for the detection of major or significant abnormalities of the brain, most notably severe IVH or cystic periventricular leukomalacia in the preterm infant. It is, however, not generally considered sensitive enough to detect and assess subtle or diffuse brain pathologies in the neonatal period (84), and most term-born growth-restricted infants are unlikely to have a brain lesion easily identifiable by neonatal cranial ultrasound. Overall, the sensitivity of neonatal ultrasound to detect any brain injury predictive of adverse motor outcomes at 2–3 years ranges between 20% and 60%, with specificity of 80–95% especially with severe injury, PPV of 20–60%, and NPV of 85–100% (85).

The interaction of prematurity and FGR on neonatal hemorrhagic and ischemic brain damage, as detected by cranial ultrasound, has been described and debated for over 20 years (86,87). There is inconsistent evidence on whether placental insufficiency and FGR is directly linked to IVH and other neonatal cranial ultrasound abnormalities (88–91). Some studies have shown FGR to be associated with an increased prevalence of IVH and white matter damage detectable on ultrasound brain scans in preterm neonates (92–94). In contrast, other studies have reported a reduced rate of IVH in FGR infants (95,96) or have shown no change in the incidence of neonatal cranial ultrasound abnormalities in FGR infants compared with appropriately grown preterm infants (91,97,98). More recently, two studies have found an increase in cranial ultrasound abnormalities in preterm FGR infants compared with matched controls (40,99). This spectrum of outcomes may exist because of different definitions of FGR, inclusion criteria, and quality of ultrasound technology used.

Neonatal MRI

MRI of the brain in the neonatal period is a gold standard for non-invasive structural assessment of the brain with excellent sensitivity and prognostic utility. Neonatal brain MRI is considered supplementary to routine and sequential cranial ultrasound, and is most commonly used in term and preterm infants with suspected brain injury or in infants considered high risk (for example, infants born extremely preterm). Advances in MRI technology and post-processing have greatly progressed our understanding of, and ability to detect, neonatal neuropathology, resulting in a broad shift from simply using neonatal MRI for the detection of severe cystic brain lesions toward the assessment of subtle and/or diffuse injury, or injury that is region-specific (84). MRI provides for a range of assessments of the neonatal brain that can be correlated with outcome measures of childhood motor and cognitive function, behavior, and learning (100). Specifically for motor outcomes, neonatal MRI demonstrates 80–100% sensitivity and specificity, PPV between 30 and 90%, and NPV of 90–100% (85).

Neonatal MRI—brain structure. More than a decade ago, Tolsa and colleagues used echo-planar MRI to demonstrate that FGR neonates imaged within 2 weeks after preterm birth demonstrate region-specific alterations in brain development, with decreased total brain volume and cortical (gray matter) volume compared with appropriately grown infants. A second MRI at term-equivalent age confirmed that the reduction in total intracranial and gray matter volume in FGR infants, with reduced cortical volume at term, correlated with worse behavioral outcomes (27). MRI examination of preterm-born FGR and appropriately grown infants at term-equivalent age found no differences in the incidence of gross brain lesions, or in the degree of morphological brain maturation between the

two groups; however, there was a delay in myelination within the FGR cohort (101). Preterm FGR neonates have also been shown to have discordant gyrification and cortical folding observed on MRI soon after birth, which can predict term-equivalent-age cerebral volumes and neurobehavioral development (18). There are a number of studies that have shown structural differences (including in the gray matter) in the brain of FGR infant as compared with the appropriately grown infant at a later age (70,102).

The hippocampus is a highly vulnerable brain structure that is altered in response to chronic fetal hypoxia and therefore frequently reported as abnormally developed in human and animal experimental FGR (2). The hippocampal structure of preterm-born FGR infants has been examined using 3D MRI at term-equivalent age, demonstrating reduced hippocampal gray matter volume. Hippocampal volume reduction was associated with functional behavioral differences at term-equivalent age, but not at 24 months of corrected age (103).

Neonatal MRI—brain metabolism. Although MRS is regularly used in the early assessment and diagnosis for acute neonatal encephalopathy associated with birth asphyxia (hypoxic ischemic encephalopathy), MRS has not been routinely used for assessment of the neonatal FGR brain. This is likely contributed by the difficulty in imaging FGR neonates very soon after birth, at a time when brain biochemical metabolites may be still be influenced by the chronic disturbances resultant from placental insufficiency. A recent study in neonatal FGR rabbits showed reduced NAA in the cerebral cortex and hippocampus, likely because of a loss of neuronal cells, and higher levels of glycine in the striatum. These metabolic changes were correlated with decreased brain volume (104). Similarly, regional differences in brain neurochemical profiles have been observed in FGR rats (105).

Neonatal MRI—brain organization and networks. A relatively new imaging tool allows the examination of brain organization using diffusion MRI, which we now appreciate, has the potential to describe complex brain connection networks, and to correlate these with neurodevelopmental outcomes. Specifically, MRI-based connectomics is an emerging approach to extract information from MRI data that exhaustively maps inter-regional connectivity within the brain to build a graph model of the neural circuitry forming the brain network (106,107).

DTI assessment of fractional anisotropy (FA), which provides microstructural information on the density and organization of white matter tracts, provides an excellent assessment modality for white matter development within the brain. FGR is associated with a complex pattern of brain reorganization (as demonstrated by FA) in specific regions of the brain, as determined from voxel-based analysis in the neonatal period (107,108). A recent study in FGR neonates showed hyper-connected but poorly organized brain networks that were most notable within the frontal, cingulate, and lingual cortices (109). A number of further brain connectivity studies have been performed at a later age (typically around 1 year of age or beyond) showing altered brain network organizations in infants born growth-restricted (22,23,102,110–112). Importantly, two recent studies demonstrate that brain connectivity is predictive of subsequent functional delays in preterm and/or FGR infants (23,113). The future study of neuropathology in FGR infants should incorporate examination of brain connectivity, which is emerging as a predictive assessment of complex brain reorganization that occurs in FGR infants in response to placental insufficiency.

In summary, advanced neonatal MRI of brain structure and microstructure, incorporating the use of DTI, is increasingly being used for the detection and assessment of FGR-related brain injury in infants after birth. MRI provides high resolution and therefore the capability for detecting subtle, but clinically important, brain-imaging information. It is becoming apparent that neonatal brain examination of FGR infants must incorporate detection of white matter injury and altered brain connectivity, and link with robust follow-up data. Altogether, these will provide evaluation on how brain microstructural changes correlate with long-term neurodevelopmental outcomes in FGR infants.

OTHER TOOLS AND TECHNIQUES TO DETECT FGR-RELATED BRAIN INJURY

A range of indirect assessment tools that have been used to identify the presence or severity of brain injury have been the subject of trials in growth-restricted fetuses and newborns. Visual evoked responses using magnetoencephalography provide a simple and non-invasive assessment of brain function, and are delayed in FGR fetuses (114). A number of other tools available in the neonatal period may predict long-term neurodevelopmental outcomes in FGR infants. In the first instance, this may be as simple as the measure of smaller head circumference in FGR infants, which is a good predictor for poor neurodevelopmental outcome (115). Although head circumference is an important predictor of neurodevelopmental outcome independent of gestational age, overall growth delay in the fetal period as impacted by the severity of FGR probably has the greatest impact in early-onset FGR (46). Overall, postnatal growth restriction, especially poor head growth, can have an additive impact on adverse neurodevelopment (116). Higher postnatal venous hematocrit and lower cerebral blood flow velocity have also been suggested as prognostic markers for adverse neurodevelopment in FGR neonates (117). Nuclear magnetic resonance spectroscopy-based analysis of umbilical vein blood in FGR infants has shown interesting patterns of metabolite change. Increased lipid levels were present in umbilical vein samples from both early and late FGR infants, whereas glucose was decreased and acetone increased in early FGR infants. FGR cases also showed increased glutamine and creatine levels, whereas the amounts of choline, valine, leucine, phenylalanine, and tyrosine were decreased in cord blood samples (118). S100B is a glial astrocyte protein that is

released from brain astrocytes in response to injury, and elevated cord blood levels of S100B are associated with subsequent diagnosis of cerebral palsy (119).

Last, qualitative assessment of general movements (GMs) (120) is a powerful diagnostic method to evaluate brain dysfunction in "at-risk" preterm and term infants (121). Many infants with growth restriction have transient abnormal GMs in the early newborn period, indicating the importance of obtaining serial observations. As is the case with preterm infants, the quality of fidgety movements (when examined at 12 weeks post term age) is predictive for neuromotor outcome in term and preterm FGR infants (122,123). Irrespective of imaging and other diagnostic modalities used, FGR infants should be closely followed up after birth to ascertain the impact of FGR on long-term neurodevelopmental outcomes, incorporating motor, cognition, and behavioral assessments, in this vulnerable population.

CONCLUSIONS

Detection and assessment of neuropathology in the fetus or neonate is a major challenge for modern perinatal medicine, allowing for timely delivery of the fetus, prediction of long-term consequences, and neuroprotective interventions. FGR is a common complication of pregnancy, and FGR infants have a greatly elevated risk for fetal and neonatal brain injury, such that strategies for the detection and treatment of FGR neuropathology are of great interest. We suggest that optimizing outcomes for FGR infants requires a collaborative approach, incorporating improved detection of true FGR infants during pregnancy via Doppler assessment of the degree of growth restriction. These infants in whom FGR is confirmed antenatally should provide a reference group for further validation studies incorporating biomarkers of brain injury, general movement assessment after birth, and direct assessment of the brain via cranial ultrasound and MRI in the neonatal period. For all FGR newborns, it is clear that early assessment of brain abnormalities should be a principal aim and, where possible, advanced MRI should be incorporated to provide clinicians and parents with accurate diagnostic information. There are currently no interventions or treatments that are available to improve brain development in FGR infants, and this should be a research focus to reduce the burden of neurodevelopmental impairments associated with FGR.

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Chapter 5

In Chapter 4, I reviewed the imaging and other tools currently available to detect and assess FGR related brain injury in the FGR fetus and neonate. This review focused on the advantages and disadvantages of each available imaging tool. It also shed light on the sensitivities and specificities of each available tool for the detection of FGR brain injury.

The review also highlighted the need to develop novel and advanced imaging techniques to detect FGR associated brain injury, as conventional brain imaging techniques may not be sensitive enough to detect subtle brain injury in FGR neonates. In this final chapter of the published papers of my thesis, I evaluated the role of advanced MRI analysis techniques in the early detection of FGR related brain injury in FGR lambs. Advanced MRI analysis, describing sophisticated processing of diffusion MRI data, is increasingly being used in a number of brain injury models and conditions in children and adults. The rationale and aims of this chapter relate directly to the overall aim of my thesis, which was the development of novel strategies for the early detection and early treatment of FGR brain injury. If we can devise better ways to detect and assess FGR brain injury early in the neonatal period, this will not only lead to better risk stratification of affected neonates, but also open avenues to objectively assess and monitor the impact of novel therapies and interventions for FGR brain injury.

Using our preclinical lamb model of FGR, we conducted MRI brain studies in newborn lambs born preterm. A number of post-image processing and analysis techniques were applied to generate results for the advanced imaging analysis of the brain structure. This was followed by histological analysis of relevant brain regions to correlate structural brain changes observed on advanced MRI analysis with histological evidence of white matter injury. This is the unaltered version of the paper published in Neuroimage: Clinical in August 2019.

5.1. Advanced MRI analysis to detect white matter brain injury in growth restricted newborn lambs

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Advanced MRI analysis to detect white matter brain injury in growth restricted newborn lambs

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ABSTRACT

Background: Fetal growth restriction (FGR) is a serious pregnancy complication associated with increased risk of adverse neurodevelopment and neuromorbidity. Current imaging techniques, including conventional magnetic resonance imaging (MRI), are not sensitive enough to detect subtle structural abnormalities in the FGR brain. We examined whether advanced MRI analysis techniques have the capacity to detect brain injury (particularly white matter injury) caused by chronic hypoxia-induced fetal growth restriction in newborn preterm lambs.

Methods: Surgery was undertaken in twin bearing pregnant ewes at 88–90 days gestation (term = 150 days) to induce FGR in one fetus. At 127 days gestation (~32 weeks human brain development), FGR and control (appropriate for gestational age, AGA) lambs were delivered by caesarean section, intubated and ventilated. Conventional and advanced brain imaging was conducted within the first two hours of life using a 3T MRI scanner. T1-weighted (T1w) and T2-weighted (T2w) structural imaging, magnetic resonance spectroscopy (MRS), and diffusion MRI (dMRI) data were acquired. Diffusion tensor imaging (DTI) modelling and analysis of dMRI data included the following regions of interest (ROIs): subcortical white matter, periventricular white matter, cerebellum, hippocampus, corpus callosum and thalamus. Fixel-based analysis of 3-tissue constrained spherical deconvolution (CSD) of the dMRI data was performed and compared between FGR and AGA lambs. Lambs were euthanised immediately after the scans and brain histology performed in the regions of interest to correlate with imaging.

Results: FGR and AGA lamb (body weight, mean (SD): 2.2(0.5) vs. 3.3(0.3) kg, $p = .002$) MRI brain scans were analysed. There were no statistically significant differences observed between the groups in conventional T1w, T2w or MRS brain data. Mean, axial and radial diffusivity, and fractional anisotropy indices obtained from DTI modelling also did not show any statistically significant differences between groups in the ROIs. Fixel-based analysis of 3-tissue CSD, however, did reveal a decrease in fibre cross-section (FC, $p < .05$) but not in fibre density (FD) or combined fibre density and cross-section (FDC) in FGR vs. AGA lamb brains. The specific tracts that showed a decrease in FC were in the regions of the periventricular white matter, hippocampus and cerebellar white matter, and were supported by histological evidence of white matter hypomyelination and disorganisation in corresponding FGR lamb brain regions.

Conclusions: The neuropathology associated with FGR in neonatal preterm lambs is subtle and imaging detection

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may require advanced MRI and tract-based analysis techniques. Fixel-based analysis of 3-tissue CSD demonstrates that the preterm neonatal FGR brain shows evidence of macrostructural (cross-sectional) deficits in white matter subsequent to altered antenatal development. These findings can inform analysis of similar brain pathology in neonatal infants.

1. Introduction

Fetal growth restriction (FGR) is a serious pregnancy complication that can lead to significant perinatal compromise, neonatal morbidity and risk of long term adverse outcomes (Malhotra et al., 2019). Placental insufficiency is the principal cause of FGR, leading to progressive restriction of oxygen and nutrient delivery to the developing fetus which, in turn, is linked with impairments in brain structure and function (Miller et al., 2016). White matter injury is the most common neuropathology responsible for the adverse neurological and neurodevelopmental outcomes of FGR infants (Tolcos et al., 2011; Saunavaara et al., 2017; Tolcos et al., 2017), but neuropathology is generally multicellular, diffuse and often subtle (Miller et al., 2016). Adverse neurological outcomes depend on the age of onset of FGR, severity of growth restriction, degree of fetal cardiovascular adaptation (traditionally known as “brain sparing”) and gestation at birth (Malhotra et al., 2019).

The early and accurate detection of brain injury in the growth restricted fetus and newborn remains a significant challenge (Malhotra et al., 2017). Current diagnostic imaging tools are essentially limited to antenatal and more commonly postnatal cranial ultrasound and, in selected and severe cases, magnetic resonance imaging (MRI). However, clinical implementation of these techniques is subject to high variability, and generally poor sensitivity and specificity for detecting FGR related brain injury (Malhotra et al., 2017). MRI is the most promising of the imaging tools available: it is capable of detecting subtle changes in brain structure and volume in FGR fetuses and infants (Bruno et al., 2017; Polat et al., 2017). There has also been interest in using advanced MRI modalities such as diffusion MRI (dMRI), complemented by specific processing and analysis tools that include diffusion tensor imaging (DTI) and fixel-based analysis (FBA) of constrained spherical deconvolution (CSD) methods, to better delineate and understand the micro-, macro-structural and functional impairments of the FGR fetal and neonatal brain (Eixarch et al., 2012; Arthurs et al., 2017; Pannek et al., 2018). Further, Kannan et al. have performed high fidelity 2D and 3D simulations for predicting and quantifying local and global injuries for organs that include the brain and the lung. These simulations noninvasively “numerically penetrate” the tissues and help reconstruct the optical properties (the presence of water, oxygenated and de-oxygenated blood in tissue), which can predict the extent and severity of organ haemorrhage/injury (Kannan and Przekwas, 2011, 2012). However, most of these advanced MRI analysis techniques have not been studied or applied extensively in the newborn brain, and are still in the early development or research stage for their use to detect FGR brain injury (Malhotra et al., 2017). Early and accurate detection of structural and functional brain impairment is not only essential to risk stratify and monitor the affected FGR infant, but also to monitor the response to targeted experimental therapies for FGR, which are increasingly being examined (Nawathe and David, 2018).

In this study, we induced early-onset FGR in fetal sheep (Malhotra et al., 2018) to evaluate the use of advanced MRI brain analysis techniques to detect FGR-related brain injury in the early neonatal period. We then collected the brain for histological analysis to substantiate the presence, distribution and cellular nature of neonatal white matter brain injury in preterm FGR versus appropriately grown newborn lambs.

2. Methods

2.1. Ethics approval

Experiments complied with the National Health and Medical Research Council (NHMRC) of Australia guidelines for the care and use of animals for scientific purposes and were approved by Monash Medical Centre Animal Ethics Committee A.

2.2. Experiment design

Procedures to induce early-onset FGR at mid-gestation and then delivery followed by ventilation at preterm age have been described previously (Alves de Alencar Rocha et al., 2017; Malhotra et al., 2018). In brief, sterile surgery was performed after inducing anaesthesia in twin-bearing Border-Leicester Merino crossbred ewes at 88–90 days gestation (term is 150 days) to induce early onset FGR by single umbilical artery ligation (SUAL) in one of the fetal lambs. The other lamb in the twin pairs was used as the control (appropriately grown for gestational age, AGA).

At 125–127 days gestation, following administration of antenatal steroids in the two days preceding birth, FGR and AGA lambs were delivered and umbilical cord clamped and cut. The lambs were dried, weighed, and transferred to an infant warmer (Fisher and Paykel, Auckland, NZ) where each lamb was intubated (4.0 mm cuffed endotracheal tube), lung liquid passively drained and gentle ventilation commenced. Umbilical venous and artery catheters (internal diameters 5 Fr) were inserted and secured using silk sutures. A pulse oximeter probe (Masimo, Irvine, CA, USA) was placed on the lamb's tail for measurement of transcutaneous oxyhaemoglobin saturation levels (SpO_2). Ventilation of the preterm FGR and AGA lambs was initiated using assist control ventilation (Babylog 8000+, Dräger, Lüberk, Germany) with an initial peak inspiratory pressure of 30 cm H_2O and positive end-expiratory pressure of 5 cm H_2O for the first 10 min and then volume targeted ventilation with tidal volumes of 5–7 $ml.kg^{-1}$ for the remainder of the experiment. The inspired oxygen fraction (FiO_2) was commenced at 0.3 and then adjusted to maintain lamb SpO_2 between 85 and 95% after initial resuscitation. All lambs received prophylactic surfactant (100 $mg.kg^{1}$, Curosurf; Chiesi Pharma, Parma, Italy) via the endotracheal tube 10 min after ventilation onset.

As soon as the lamb was stabilised (within 1 h after birth), it was transferred to the MRI scanner (Siemens Skyra 3.0 T, Erlangen, Germany). Lambs were scanned in a supine position and ventilation was maintained using a BabyPac MR compatible ventilator (Pneupac, Smiths Medical, Kent, UK). Throughout ventilation, lambs were sedated by continuous infusion of Alfaxan (Alfaxalone, Jurox, Rutherford, NSW, 3 $mg.kg^{-1}.min^{-1}$) through an umbilical venous catheter. The MRI acquisition protocol comprised structural imaging sequences (T1w, T2w), dMRI, and single-voxel proton magnetic resonance spectroscopy (MRS). The total acquisition time was between 60 and 75 min.

At the completion of the experiment, lambs were euthanised by intravenous pentobarbital sodium overdose (100 $mg.kg^{-1}$ I.V.; Valabarb, Jurox, Rutherford, NSW, Australia).

2.3. MR Image acquisition

MRI data were acquired using a 15-channel transmit/receive knee coil on the Siemens 3T Skyra (Siemens, Erlangen, Germany) running software version vd13C. Diffusion MRI data were obtained with a single

shot echo planar imaging protocol, using the following parameters: coronal slices, repetition time/echo time (TR/TE) = 10,500/99 ms, 1.2 × 1.2 × 1.2 mm^3 isotropic voxels, 128 × 128 acquisition matrix, GRAPPA = 2 and number of excitations (NEX) = 1. Multi-shell data were acquired with the following shells: 30 directions at b = 500 and 750 s/mm^2, 64 directions at b = 1000, 1250 and 1500 s/mm^2, and five volumes without diffusion weighting (b = 0). Additional pairs of b = 0 images were acquired with reverse phase encoding to correct for image distortions. A 3D T2-weighted image was acquired using a fast spin echo sequence with the following parameters: 0.5 × 0.5 × 0.5 mm^3 isotropic voxels, 384 × 384 acquisition matrix, TR/TE = 1000/130 ms and NEX = 2, Echo Train Length (ETL) = 64. A T1-weighted image was generated using a spin echo sequence with inversion recovery. The parameters were as follow: TR/TE = 1440/3.92 ms, inversion time = 900 ms, NEX = 1, 0.8 × 0.8 × 0.8 mm^3 isotropic voxels and acquisition matrix = 256 × 256. A 3D SW image was acquired using a 3D FLASH sequence with the following parameters: TR/TE = 28/20 ms, NEX = 2, flip angle = 15°, 0.6 × 0.6 × 0.6 mm^3 isotropic voxels, and acquisition matrix = 288 × 216.

2.4. MRS

MRS data were acquired using two water suppressed PRESS Spin Echo sequences with TR = 2000, TE = 35 ms and 270, NEX = 128 and 192, respectively. The scans were also repeated with no water suppression. A single 1.4 cm × 1.5 cm × 2 cm voxel in the deep grey matter of the brain (globus pallidus, Fig. 1) was used to calculate absolute metabolite values and metabolite ratios by a using peak area under the MRS curve. Metabolites studied include lactate (Lac), choline (Chol), creatine (Cr), and *n*-acetylaspartate (NAA) and ratios assessed were Lac/choline, Lac/NAA, NAA/choline and choline/creatine.

2.5. Diffusion MRI pre-processing

dMRI data were corrected for head motion, eddy currents, and susceptibility distortions using TOPUP and EDDY tools, as implemented in FSL (FMRIB Software Library) (Jenkinson et al., 2012; Andersson and Sotiropoulos, 2016). FSL-BET (Brain Extraction Tool) was used to extract the brain and create a brain mask. The data were denoised using MP-PCA denoising (Veraart et al., 2016) implemented in MRtrix. DTI metrics were calculated from a single shell (b-value = 1500 s/mm^2) using FSL and included fractional anisotropy (FA), axial diffusivity (AD), radial diffusivity (RD) and mean diffusivity (MD).

2.6. Region of Interest voxel-based analysis

A MATLAB (Version R2017b, The MathWorks, Natick, MA, USA) script was used to automate the generation of spherical regions of interest (ROIs) with identical 3D volumes from a manually determined point on high-resolution T2-weighted brain images. ROIs evaluated

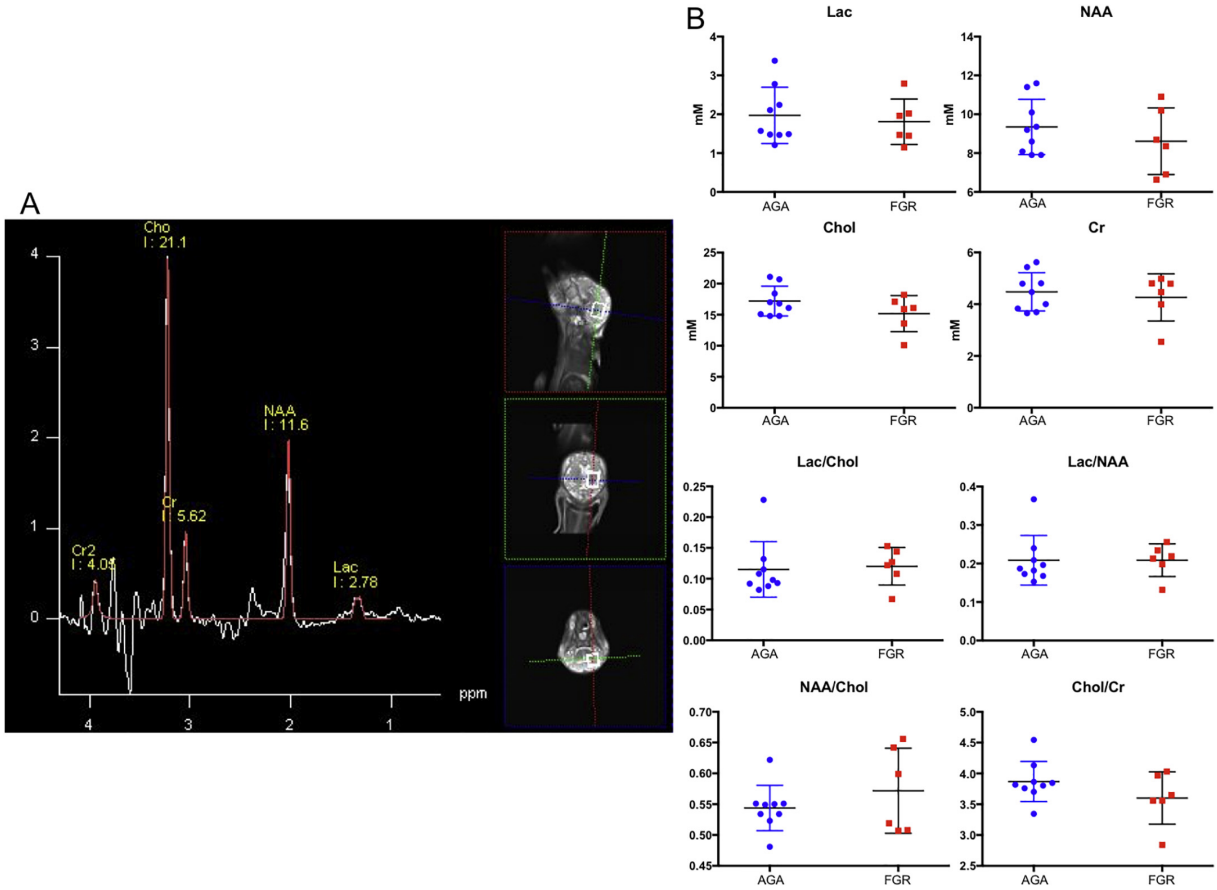


Fig. 1. A: Representative screenshot of spectrogram showing the deep brain voxel used to study brain metabolites. B: Graphs showing absolute values and ratios of metabolites in AGA (blue) and FGR (red) lamb brains studied by MRS. Lac - lactate, NAA - *n*-acetylaspartate, Chol - choline, Cr - creatine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

included the thalamus, hippocampus, cerebellum, corpus callosum, periventricular white matter (PVWM) and subcortical white matter (Fig. 2). Each ROI was manually inspected to ensure that they covered the same region in all images. T2-weighted and distortion corrected b = 0 images were registered using FSL-FLIRT and the resulting transforms were used to register the ROIs to the dMRI space. The mean and standard deviation of each of the DTI metrics was calculated within all ROIs using MATLAB.

2.7. 3-Tissue modelling and fixel-based analysis

3-tissue response functions for every animal were estimated from the data themselves using an unsupervised method (Dhollander et al., 2016; Dhollander et al., 2019) available in MRtrix3Tissue (https://3Tissue.github.io/), an MRtrix3 fork. The estimated response functions per tissue type were then averaged across all lambs to obtain a unique group response function for each tissue type (white matter-like, grey matter-like and cerebrospinal fluid-like). The dMRI data were up-sampled to an isotropic voxel size of 1 mm and 3-tissue CSD was performed using the multi-shell multi-tissue spherical deconvolution algorithm (Jeurissen et al., 2014), resulting in a white matter Fibre Orientation Distribution (FOD). Global intensity normalisation was performed using multi-tissue informed log-domain intensity normalisation across lambs to correct for global intensity differences between scans. A study-specific population template (Fig. 3) was generated from 5 AGA and 5 FGR lambs by non-linear registration of the white matter FOD images (Raffelt et al., 2011). Equal numbers of AGA and FGR lambs were used to avoid biasing the template to one particular group (Wright et al., 2017). White matter FOD images of all AGA and FGR lamb brain scans were subsequently registered to this population template (Raffelt et al., 2011; Raffelt et al., 2012). A white matter template analysis fixel mask was then computed from the FOD template (Fig. 3).

Fixel-specific fibre density (FD), fibre cross-section (FC), and fibre density and cross-section (FDC) were computed and statistically analysed using connectivity-based fixel enhancement with 5000 permutations, fully corrected for family-wise error (Raffelt et al., 2015; Raffelt et al., 2017). Group comparison was performed between AGA and FGR lambs. The fixel-based analysis was performed using MRtrix3 software (Tournier et al., 2019). It was hypothesised that there might be differences between AGA and FGR lamb brains even without macroscopic

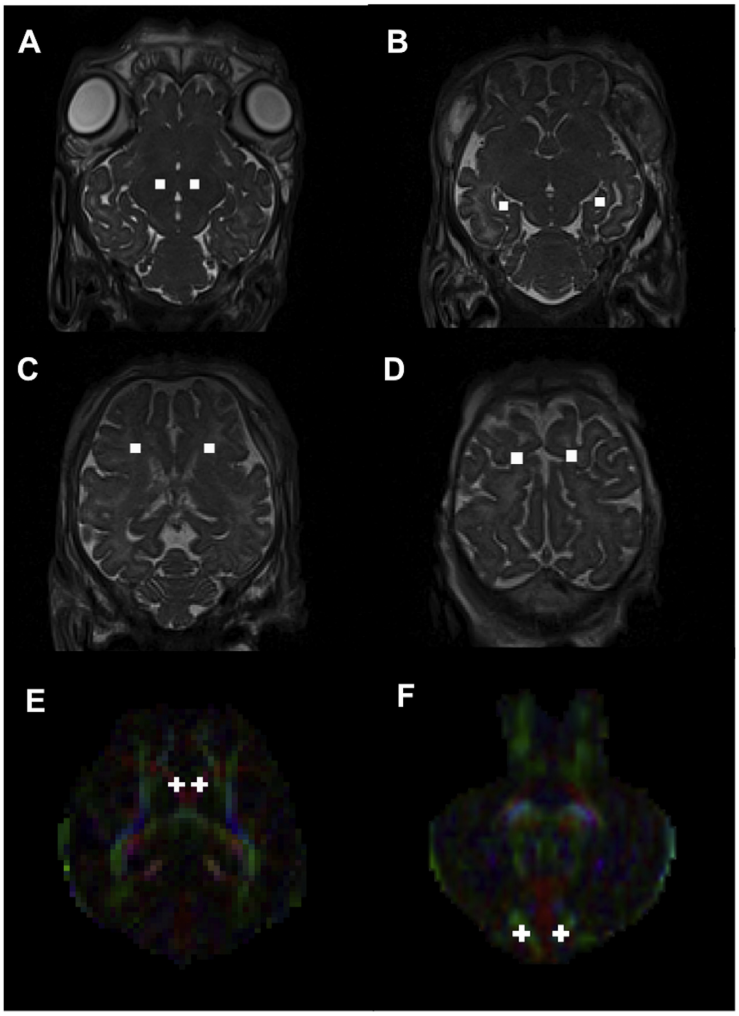


Fig. 2. Examples of regions of interest (ROI) from an exemplar lamb brain. A) Thalamus, B) Hippocampus, C) Periventricular white matter and, D) Subcortical white matter regions were first manually identified on T2 images, and then warped to the diffusion space. E) Corpus callosum and, F) cerebellum were accurately visualised and selected on principal diffusion direction maps.

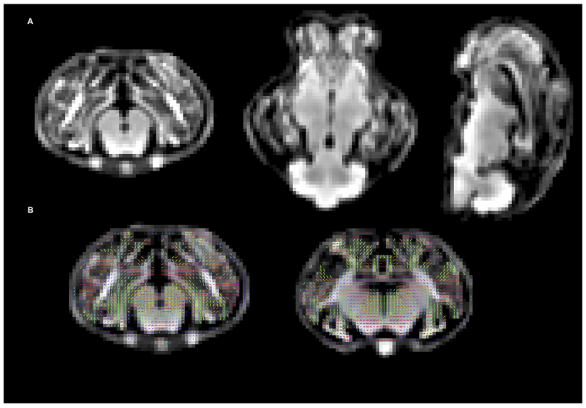


Fig. 3. A: White matter FOD templates. Left to right: Coronal, axial and sagittal views. B: White matter template analysis fixel mask, computed from the FOD templates by applying a threshold of 0.08. The white matter fixels are colour-encoded according to orientation: red for left/right; green for anterior/posterior; blue for superior/inferior. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

evidence of brain abnormality on conventional MRI, and that FGR lambs would show white matter injury compared to AGA lambs, which would be supported by reduction in FC, FD and FDC in the FGR lambs.

2.8. Brain pathology

After euthanasia, the brain was removed and weighed. The left brain hemisphere was divided into four sections and frozen for analysis. The right brain hemisphere was cut coronally into 5 mm slices and fixed in formalin for 48–72 h and then embedded in paraffin (ProSci Tech, Thuringowa, Australia) for histological analysis.

2.9. Histological staining

Luxol fast blue (LFB) was used to stain lipoproteins present in the myelin sheath of the white matter using a standard protocol in order to detect demyelination under light microscopy (Alves de Alencar Rocha et al., 2017). Hydrated brain paraffin sections were placed in luxol fast blue solution overnight. Excess stain was rinsed off, sections placed in dilute lithium carbonate, differentiated in 70% ethanol, further stained in dilute fuschin and picric acid, and mounted in resinous media. Brain sections were analysed for semi-quantitative assessment of myelin injury and demyelination by a blinded examiner, and scored for presence and quality of luxol fast blue staining, myelin breaks and vacuolation in white matter. Brain sections were examined at the level of the subventricular zone and the paraventricular thalamus (sections 0760 and 1120 respectively, Michigan University Sheep Brain Atlas). Brain sections were thoroughly examined for any signs of demyelination with particular ROIs based on imaging data, including PVWM, corpus callosum, hippocampus and cerebellar white matter.

2.10. Statistics

Birth weight, MRS, and ROI-based dMRI metrics are presented as mean and standard deviation (S.D.). Statistical comparisons (*t*-tests between AGA and FGR groups) were conducted using GraphPad Prism (v7, GraphPad Software, San Diego, CA). Significance was accepted when $p < .05$. As mentioned above, fixel-based statistical analysis of grouped data was carried out using the MRtrix3 software (Tournier et al., 2019), also accepting significance when $p < .05$.

Table 1
Demographic characteristics of included lambs.

	AGA	FGR
Lambs – live born	12/12	9/12
Lambs - MRI analysis	9/12	6/9
Sex, male: female	4:5	3:3
Birth weight (kg), mean (SD)	3.3 (0.3)	2.2 (0.5)*
Birth order, first: second	6:3	2:4

* Denotes significant difference ($p = .002$, unpaired *t*-test).

3. Results

Ten pregnant ewes bearing twin pairs of fetal lambs were surgically instrumented under anaesthesia at 88–90 days lamb gestation to induce FGR in one of the lambs. There were two stillbirths in the FGR cohort, and one early neonatal death in the AGA cohort. Nine AGA and eight FGR lambs completed the MRI brain studies (Table 1). However, two FGR lamb brain scans were excluded due to severe motion artefact and/or failure in acquisition of one or two b-values for dMRI data.

At birth, the body weight of the FGR lambs was significantly reduced compared to the AGA lambs, 2.2 ± 0.5 kg vs. 3.3 ± 0.3 kg, respectively (t-test, $p = .002$). Lambs were scanned in the 3T MRI scanner in the order they were born (AGA: 6 first born, FGR: 2 first born). Lamb scanning was completed within a median of 3 (2.5–4) hours after birth.

Assessment of structural brain scans (T1w, susceptibility weighted imaging) of all lambs was conducted by a paediatric radiologist (MD) blinded to the groups. There were no structural brain lesions identified in any scans. MRS metabolite absolute values and metabolite ratios are shown in Fig. 1. There were no statistically significant differences found in any of the metabolites or ratios between the lamb groups. ROI-based analysis of dMRI data generated mean FA, AD, MD and RD values for each region of interest, and these were plotted for both lamb groups (Fig. 4). There were no statistically significant differences seen for any DTI metric in any of the ROIs studied.

Fig. 5A and B illustrate the results of the group comparison between AGA and FGR group using the fixel-based analysis of dMRI data for coronal and sagittal views, respectively. The results are overlaid on the fixel template and highlight the fibres with statistically significant ($p < .05$) differences. Fig. 6 visualises these results using streamlines, specifically for the fibre cross-section metric. FC was significantly reduced in FGR lambs compared to AGA within the PVWM, hippocampus, and cerebellar white matter regions of lamb brains. The changes were observed in bilateral hemispheres of the lamb brains (Fig. 6). There were no statistically significant differences observed between the groups for the other fixel measures, FD and FDC.

Luxol fast blue was used to assess abnormalities in myelin organisation and evidence of demyelination. Luxol fast blue staining showed substantial hypomyelination in the white matter of FGR lambs compared with AGA lambs, most notable in the corpus callosum, the subcortical and PVWM, the subcallosal bundle, external capsule, the cingulum bundle and white matter tracts of the hippocampus and cerebellum. Myelin disruptions were evident as the presence of patchiness in the staining of myelin tracts, and demyelination as an overall reduction in the density of luxol fast blue staining in the FGR group compared to the AGA group. Within cerebellar white matter, FGR lamb brains showed myelin breaks and a decrease in luxol fast blue staining, thinning of the white matter tracts in the cerebellar lobes, and the presence of vacuoles, throughout the white matter (Fig. 7). In the PVWM and corpus callosum, irregularities and loss of luxol fast blue staining were seen in FGR lamb brains compared with AGA brains. The white matter abnormalities observed in these brain regions were seen as areas completely devoid of staining (Fig. 7). In the hippocampus, myelin irregularities were detected in the fornix, alveus, angular bundle and fimbrial regions of some FGR lamb brains. The relative numbers of

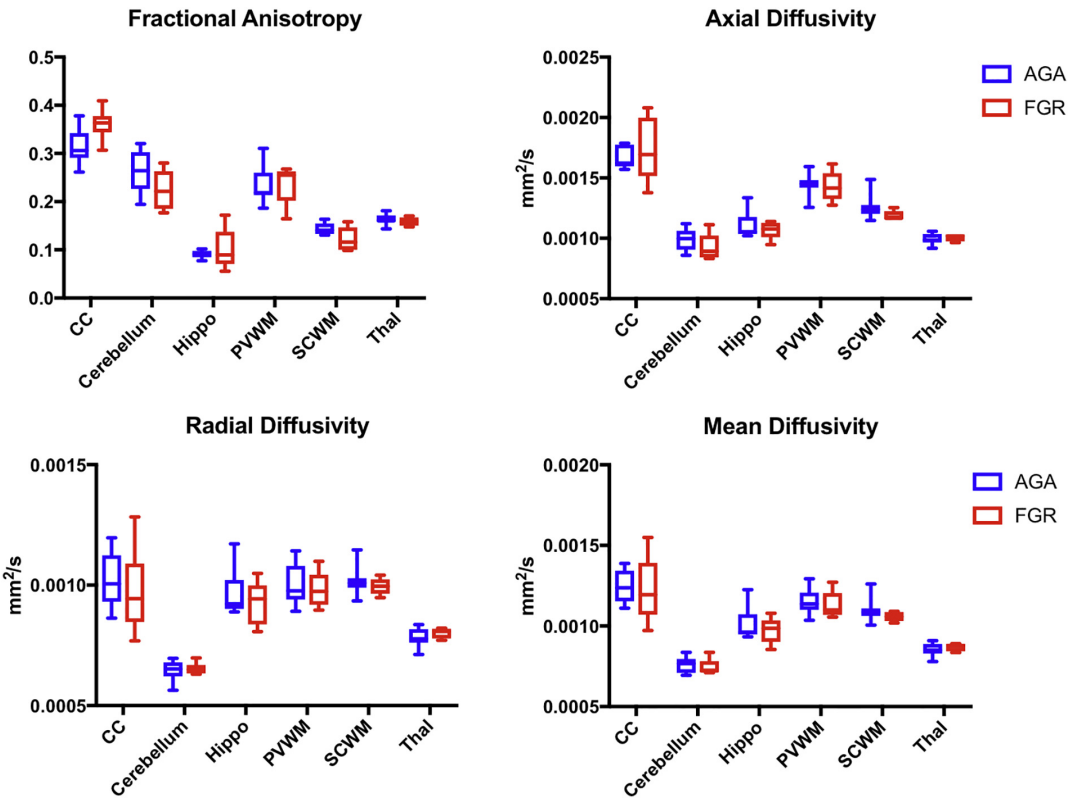


Fig. 4. DTI characteristics of ROIs calculated from ROI-based analysis. Thal - thalamus, Hippo - hippocampus, PVWM - periventricular white matter, SCWM - subcortical white matter, CC - corpus callosum.

brain regions of FGR and AGA lambs that showed evidence of abnormalities in the white matter are presented in the table (Fig. 7).

4. Discussion

In the current study we used advanced MRI brain imaging techniques for the identification of FGR related brain injury in newborn lambs and, for the first time, the presence of FGR related brain injury observed

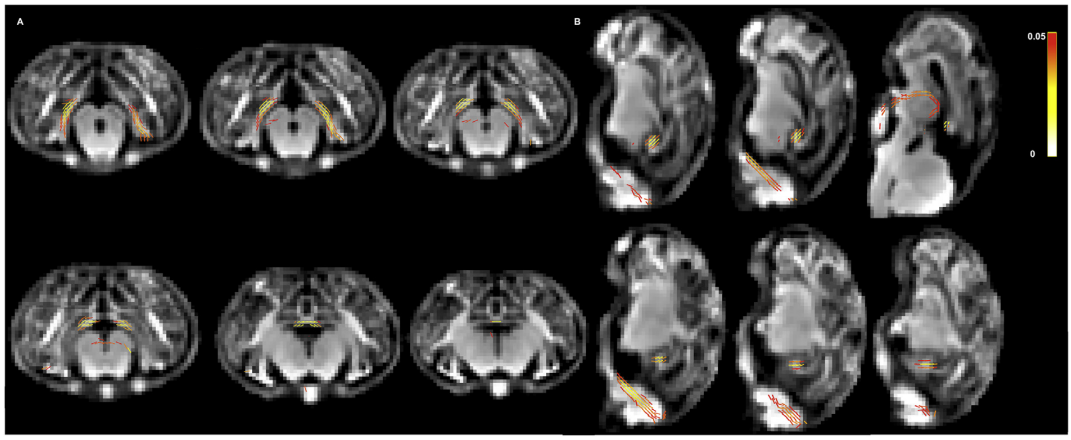


Fig. 5. A: Fixel-based analysis of 3-tissue constrained spherical deconvolution of dMRI data (coronal view). Specific tracts in hippocampus, thalamus, periventricular white matter and cerebellum showed a decrease in fibre content (FC) in FGR vs. AGA lamb brains (seen as coloured tracts in family-wise error corrected fixel mask). B: Fixel-based analysis of 3-tissue constrained spherical deconvolution of dMRI data (Sagittal view). Specific tracts in hippocampus, periventricular white matter, thalamus, and cerebellum show a significant decrease in fibre content (FC) in FGR vs. AGA lamb brains (seen as coloured tracts in family-wise error corrected fixel mask).

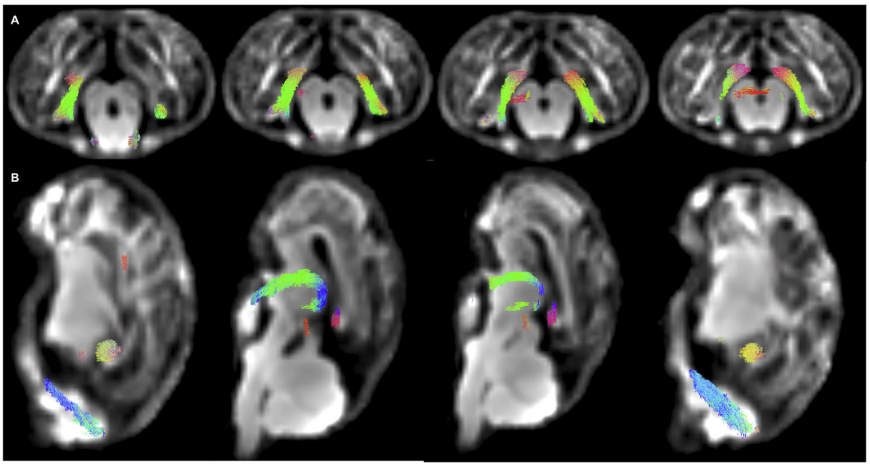
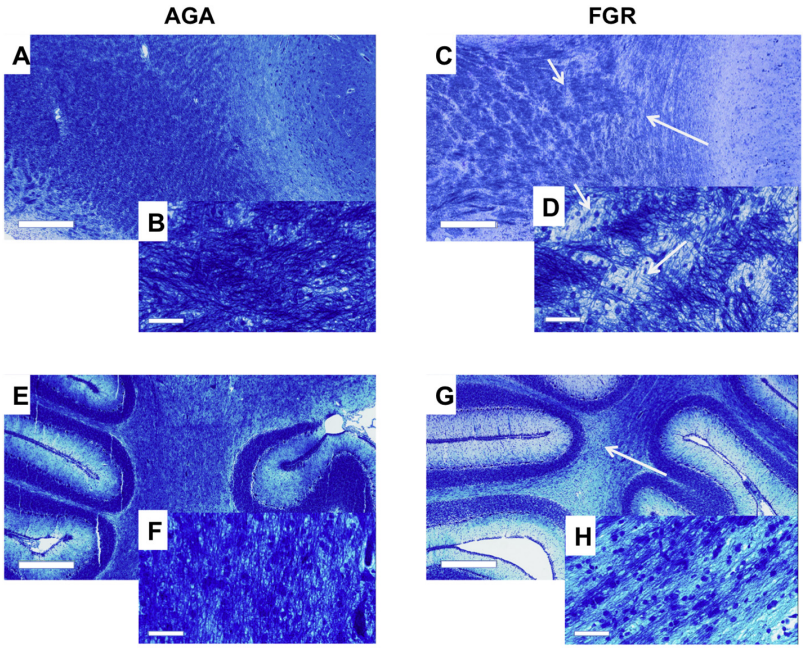


Fig. 6. A: Coronal views of significant fixel based analysis results in log FC as streamlines. Colours represent the direction of the tracts. Red for left/right; green for anterior/posterior; blue for superior/inferior. B: Sagittal view of significant results in FC as streamlines. Colours represent the direction of the tracts. Red for left/right; green for anterior/posterior; blue for superior/inferior. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

on imaging was confirmed on histological staining. No differences in brain structure and metabolite levels were observed on conventional MRI sequences and MRS between FGR and AGA lambs. However, advanced fixel-based analysis revealed macrostructural changes in the form of reduced FC in the PVWM, hippocampus, and cerebellar tracts of the FGR lamb brain, which was confirmed on histological staining of tracts in those regions.

The early detection and assessment of perinatal brain injury is critical in conditions that put the fetal or neonatal brain at risk, such as in FGR (Malhotra et al., 2017). The neuropathology associated with



	AGA	FGR
Periventricular white matter	0/9	5/6
Hippocampus	2/9	3/5
Corpus callosum	0/7	6/6
Cerebellar white matter	2/7	5/6

Fig. 7. Representative photomicrographs (low and high magnification) of luxol fast blue (LFB) staining of regions of interest in AGA and FGR lamb brains. A-B: Periventricular white matter in AGA lambs showing uniform LFB staining. C-D: Periventricular white matter in FGR lambs showing patchiness in LFB staining (arrows). E-F: Cerebellar white matter in AGA lambs. G-H: Cerebellar white matter in FGR lambs showing breaks in the myelin, thinning of myelin tract and areas devoid of LFB stained myelin (arrow) (H). Scale bars: large: 500 μm, small: 50 μm.

placental insufficiency and FGR is often subtle, and therefore not always apparent on conventional imaging, particularly in the neonatal period (Malhotra et al., 2015; Malhotra et al., 2017). Studies of the newborn FGR brain using MRI and MRS studies have shown variable results. Some studies have shown decreased total volume and/or specific regional volume changes when compared to AGA infants (Tolsa et al., 2004; Dubois et al., 2008), while others show no significant differences (Ramenghi et al., 2011). In some studies, the differences in brain structure become more apparent on MRI scans later in infancy (Padilla et al., 2011; Padilla et al., 2014). Brain metabolite concentrations were altered in a preclinical study in newborn FGR rabbits, with lower levels of aspartate and *n*-acetylaspartate in cortical and hippocampal regions, and increased glycine in the striatum (Simoes et al., 2015). MRS undertaken during pregnancy shows that growth restricted fetuses with brain sparing have lower NAA:Cr and NAA:Cho ratios compared to AGA fetuses, indicative of reduced neuronal number and/or function (Story et al., 2011). In contrast, a study on small for gestational age infants studied at two time points early in the neonatal period found no differences in cerebral metabolism (Roelants-van Rijn et al., 2004). Similarly in our lamb study, we did not observe differences using a single voxel estimation of deep grey matter metabolism in FGR and AGA lamb brains. This result suggests that, at least in deep grey matter encompassing basal ganglia, there is no loss of neuronal number or function within the FGR brain. It is of course possible other brain regions might have a different metabolite profile. It is also possible that the chronic but stable nature of antenatal compromise induced via placental insufficiency in this lamb model precluded any changes in brain metabolite concentrations as seen on MRS.

There is very limited literature on DTI based analysis in the FGR newborn brain. A study in FGR (small for gestational age infants) and AGA neonates showed lower FA and higher diffusivity (AD, MD, RD) in FGR infants as compared to AGA infants in a number of brain regions out of a total of 122 brain regions studied (Wang et al., 2017). Similarly, a study in FGR rabbits also showed altered brain networks and decreased FA in the hippocampus (Illa et al., 2018). We did not observe any changes in DTI measures in the six brain regions studied within the FGR lamb brain. DTI metrics are voxel-specific, not fibre-specific and hence, it is possible that the voxels included white matter regions with crossing fibres that have differing DTI characteristics, hence masking the effects of a disorganised white matter structure (Jeurissen et al., 2013; Wright et al., 2017). It is also possible that specific brain regions may have differing characteristics within a diseased brain or disease model. We used a ROI specific model for the DTI analyses while the FBA analyses studied the whole white matter region in the template. This may also account for the lack of DTI changes despite significant pathology seen on histological staining.

FBA was used to study fibre specific changes in the brain. This technique enables detection of the morphological alterations in axonal tracts that may or may not contribute to differences in voxel-based morphometry (Raffelt et al., 2017). FBA allows the assessment of tissue microstructure (FD), local macrostructure (FC), and the combined effect of both microstructure and macrostructure (FDC). Disease-associated axonal loss typically leads to reduced FD (Raffelt et al., 2015), while a reduced FC generally represents axonal loss associated with the extra-axonal space filled with extracellular matrix and cells, related to inflammation or gliosis (Raffelt et al., 2012). Reduced FC may also result from long term atrophy of the fibre bundle (as seen in Alzheimer's or motor neuron disease) but in most cases neurodegenerative diseases lead to reduction in FDC, the combined measure (Raffelt et al., 2017). Pannek et al. recently used fixel-based analysis to study the micro- and macrostructural differences in brain scans of preterm- and term-born infants at term equivalent age (Pannek et al., 2018), and found that this analysis technique was useful for detection of both microstructural and macrostructural abnormalities in infants born preterm. In the current preclinical study, we observed differences in fixel-based analysis results in specific white matter regions of the FGR lamb brains compared to animals that were appropriately grown. In particular, differences in FC were noted between FGR and AGA lambs in the PVWM, hippocampus and cerebellar tracts of the lamb brains. A decrease in FC may signify altered tissue macrostructure, possibly as a consequence of decreased myelination (Gajamange et al., 2018). This was confirmed on histological staining for the first time, which is important, as the changes we see on FBA may signify subtle structural changes previously not appreciated on MRI. The changes in white matter tracts of the FGR brain on FBA, coupled with evidence of hypomyelination and disorganisation on neuropathology, confirm the vulnerability of white matter development in an environment of chronic antenatal hypoxia associated with FGR. That we could detect macrostructural white matter deficits immediately after (preterm) birth confirms that brain injury occurs antenatally. We have previously shown axonal changes and axonal injury in brain white matter regions in both fetal and neonatal studies of FGR sheep (Miller et al., 2014; Alves de Alencar Rocha et al., 2017; Malhotra et al., 2018), which may be associated with the FBA changes seen here. In turn, neonatal white matter deficits and altered brain connectivity may underlie neurodevelopmental disorders that become evident in childhood (Miller et al., 2016).

The animal model lends us the distinct advantage of being able to study brain histological changes that have been identified as areas of interest using brain imaging. We have reported previously that white matter hypomyelination and disorganisation are principal neuropathologies present in FGR lambs (Miller et al., 2014; Alves de Alencar Rocha et al., 2017), but the current study extends the preclinical utility of this work into a clinical understanding of how early detection of FGR brain injury may be improved, and its histological basis. Further work on how other neuropathologies in high risk perinatal conditions may correlate with FBA should to be undertaken to ascertain whether this technique will also be useful to detect other subtle brain changes (Rana et al., 2019). It is also interesting that while we could observe demyelination and disorganisation on histological assessment, this did not result in significant alteration in FD or FDC, or indeed other DTI metrics, which also highlights the need for further studies to specifically correlate imaging with histological analysis. It is not known what degree or nature of white matter injury results in functional deficits, and therefore further preclinical lamb studies should combine brain imaging with functional and histological assessments to obtain a global picture of the relationships between these outcomes. Therefore, future FBA studies should include correlating FBA changes with neurodevelopment in childhood, and whether antenatal or postnatal therapies modify macrostructural and microstructural white matter development. An advantage of undertaking this study in lambs is that we could deliver all animals preterm, at an age equivalent to moderate to late-preterm human brain development ($\geq$32 weeks human gestation) (Alves de Alencar Rocha et al., 2017). In doing so, we were able to detect macrostructural deficits within the FGR brain. We do however note that delivering all lambs preterm is also a study limitation, because we cannot then compare with a term-born group to further characterise potential differences between preterm and term birth, as shown by Pannek recently in a human cohort (Pannek et al., 2018).

5. Conclusions

Advanced MRI brain analysis techniques offer novel modalities to image and study perinatal brain injury. In this preclinical study of placental insufficiency and subsequent FGR, we found significant differences in fibre bundle cross-section, indicative of reduced tissue macrostructure within white matter regions. Histological analysis of the same white matter regions showed that these FC changes could be attributed to hypomyelination and disorganisation of axonal tracts. The introduction and use of fixel-based analysis to study perinatal brain structure and function may be an important addition for the comprehensive assessment of brain injury in high-risk populations, including growth restricted infants.

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Disclosures

The authors do not have any disclosures or conflicts to declare.

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Chapter 6

6.1. General Discussion

6.1.1. Overview

Fetal growth restriction leading to low birth weight is a common pregnancy complication. In Australia, around 3-5% of all infants or close to 20,000 infants are born low birth weight or FGR each year (AIHW 2016). As a neonatal clinician looking after a vulnerable population, I am constantly striving to improve the outcomes of babies born prematurely and those with low birth weight. Most infants with severe FGR are also born prematurely, leading to a further increased risk of neonatal morbidity and mortality. Although the impact of FGR on neonatal and childhood outcomes in premature infants is relatively well known, the mechanisms behind their worse neurological outcomes compared to well grown infants of equivalent gestational age are not well understood. In particular, in undertaking this thesis, I wanted to investigate the impact neonatal invasive ventilation has on brain structure and function in premature FGR infants. If we understand the mechanisms behind the brain injury better, we are more likely to be able to design targeted therapies to mitigate the effects of neonatal interventions on FGR brain development – I wanted to evaluate the use of umbilical cord blood derived stem cells for FGR brain injury. Also, frequently, premature FGR infants are not identified with significant brain injury on conventional imaging in the neonatal period, even though they go on to develop significant neurodevelopmental disorders in childhood – I wanted to test advanced MRI brain analysis for this purpose. Thus the overall aim of the studies undertaken for my thesis was to develop novel strategies for the early detection and early treatment of FGR-related brain injury in premature infants.

6.1.2. Lamb model of FGR

Animal studies, or so-called ‘pre-clinical models’ are frequently used to mimic or *model* human conditions. There are a number of FGR animal models and each has its own advantages and disadvantages (Swanson & David, 2015). Placental insufficiency, chronic fetal hypoxia and hypoglycaemia are the pathophysiological mechanisms most commonly employed to cause reduced

fetal growth in these animal models (Basilious, Yager, & Fehlings, 2015). I have chosen to use an animal model of placental insufficiency and subsequent FGR to address my research questions. Our lab group has been using the ovine single umbilical artery ligation (SUAL) model for inducing placental insufficiency and FGR in fetal sheep for a number of years (Alves de Alencar Rocha et al., 2017; Miller et al., 2014; Supramaniam et al., 2006). SUAL employs a surgical technique that leads to partial infarction of the ovine placental cotyledons, thereby inducing fetal growth restriction. In previous studies, we induced FGR at 105 days lamb gestation (of ~148 days full gestation) but, for the studies in this PhD, placental insufficiency via SUAL was induced at 88-90 days of gestation. In terms of human fetal development, these gestational ages roughly equate to 0.7 and 0.6 gestation, respectively, and therefore provide us with early- and late-onset preclinical equivalents of placental insufficiency and FGR in human pregnancy (Alves de Alencar Rocha et al., 2017). We are confident that this fetal sheep model is clinically relevant, given that it causes progressive placental insufficiency and an increasing degree of chronic hypoxia and hypoglycaemia; in turn, this induces asymmetric restriction of fetal growth with brain sparing.

We conducted a direct comparison of our early and late onset placental insufficiency models to examine the effects on fetal physiological response and brain development (Alves de Alencar Rocha et al., 2017), demonstrating a progressive degree of fetal hypoxia and hypoglycaemia in the 88-day FGR induction model. In contrast, when placental insufficiency was induced at 105 days, there was a striking acute fall in fetal oxygenation and glucose delivery to the fetus after SUAL surgery, almost equivalent to a mild acute hypoxic insult to the fetus. Thus, we are confident that inducing placental insufficiency at 88 days is a clinically useful technique to mimic early-onset fetal growth restriction in human pregnancy. This mid-gestation (88-day) lamb model has since been used for further studies in our group, including a study on the placental and fetal effects of antenatal sildenafil treatment in FGR pregnancy (Inocencio et al., 2019).

In humans, early onset severe FGR infants comprise a smaller proportion of all FGR infants compared to late onset, but these are the infants at highest risk of preterm birth and adverse neurological development (Baschat, 2014). Accordingly, these are the high-risk infants for which treatments are most highly sought after. In the studies described in this thesis, the use of an accurate and standardised large animal model of early-onset fetal growth restriction has allowed us to characterise some of the challenging neuropathological processes in growth restricted fetuses and neonates. We chose to deliver the lambs prematurely at 126-128 days gestation, corresponding to a moderate preterm age of approximately 32 – 34 weeks gestation in humans, reflecting a period of high risk for preterm delivery with early-onset FGR (Groom et al., 2019). A great advantage of undertaking this study in lambs is that we were then able to implement a neonatal intensive care procedure - exactly as would be undertaken in human FGR infants born preterm and using the same equipment – to examine the inter-play between antenatal and postnatal development of FGR brain injury. Finally, we were also then able to use novel neonatal magnetic resonance imaging (MRI) on these lambs to conduct early postnatal MRI studies and trial early umbilical cord blood stem cell therapy for treatment of brain injury.

6.1.3. Neuropathology in FGR

Fetal growth restriction leads to a number of neonatal morbidities, which can have consequences in childhood and adulthood (Malhotra et al., 2019). Understanding the pathophysiology behind the neurological consequences of placental insufficiency and FGR was the focus of my literature review. It is now understood that the impact of FGR on brain structure, growth and function depends on the age of onset of FGR, its severity including the extent of cardiovascular adaptation *in utero*, and gestation at birth (Malhotra et al., 2019; Miller, Huppi, & Mallard, 2016). FGR is associated with structural deficits in the developing brain, which in turn affect long-term function (Miller et al., 2016). Fetal MRI studies in human pregnancies have shown reduced brain volume,

altered cortical folding and grey and white matter pathology in growth restricted infants with placental dysfunction (Arthurs et al., 2017; Huppi, 2011; Tolsa et al., 2004). Placental insufficiency and subsequent chronic fetal hypoxia and hypoglycaemia are the principal causes for altered brain development and neuropathology in FGR. As placental insufficiency tends to peak during an important period of white matter development in the fetus, disruption to white matter (WM) development and maturation is an important consideration (Tolcos et al., 2017). Postnatally, neuropathology associated with FGR is confirmed on imaging as primarily affecting the white matter, hippocampus, and basal ganglia, but also with cortical and cerebellar involvement (Bruno et al., 2017; Lodygensky et al., 2008; Ramenghi et al., 2011).

A specific aim of my project was to characterise antenatal and postnatal contributions towards the progression of neuropathology in FGR infants, and particularly the effects of the interaction of neonatal ventilation and FGR in preterm infants (lambs). In my study, we showed that neuropathology associated with neonatal ventilation-induced brain injury in FGR was principally mediated by neuroinflammation, oxidative stress, and increased permeability of the blood brain barrier (BBB), possibly due to destabilisation of the neurovascular unit (NVU) (Malhotra et al., 2018). There is only one other publication (also from our group), which describes the neuropathology related to neonatal ventilation in FGR newborn lambs (Allison et al., 2017). In the study by Allison et al, lamb brains were examined after 2 hours of neonatal ventilation and showed abnormalities in the neurovascular unit, altered density of vascular tight junction markers and increased blood-brain barrier permeability in ventilated FGR lambs. Brain development is compromised in the antenatal period in the FGR lamb, and invasive ventilation and neonatal care likely exacerbates this neuropathology. Similar adverse additive effects of neonatal ventilation are also seen in brains of animals that have been affected by other antenatal conditions such as chorioamnionitis (Barton et al., 2014). The role of the NVU and altered cellular interactions in FGR

brain injury has only recently been recognised (Allison et al., 2017; Castillo-Melendez et al., 2015). These studies have characterised that blood vessel development is altered, the BBB lacks stability and maintenance of vascular permeability is compromised in white matter regions of the FGR brain. New knowledge to show that the neonatal period may contribute to the pathophysiology that is evident in FGR preterm newborns is critical if we are to develop strategies and treatments to reduce the long-term neurological deficits associated with FGR. Further, here we showed that FGR infants represent a specific, and particularly vulnerable cohort, of preterm babies who are distinct from appropriately grown preterm babies, and whose postnatal care likely needs to be tailored to this population.

6.1.4. Mechanisms of action of UCBC for FGR related brain injury

Umbilical cord blood stem cells (UCBCs) are already in different phases of clinical trial for perinatal brain injury (Cotten et al., 2014; Yang et al., 2018) and in children with established cerebral palsy (McDonald et al., 2018; Min et al., 2013; Romanov et al., 2015). In my studies, I wanted to investigate whether UCBCs might also be an effective neuroprotective option for perinatal brain injury associated with FGR. Preclinical studies in fetal and neonatal lambs allows assessment of the mechanisms of action of UCBC in specific animal models of perinatal brain injury, and this is the first report of cell administration to preterm FGR newborns, undertaken with clinical translation in mind. In the study described in Chapter 3, we chose to use allogeneic UCBCs, as this is likely to be the most clinically relevant scenario, given that FGR preterm infants can be expected to have a low yield of UCBCs from autologous cord blood (often due to short and small cords as well as low blood volume being preterm) (Mazzoccoli et al., 2016). We chose to administer the UCBCs at 1 hour of life, as we believe that early administration of allogeneic cells is clinically feasible and likely to be most protective for ventilation-induced brain injury in this vulnerable population of preterm infants. Our group has published a number of studies evaluating

the role and mechanisms of action of UCBCs in a variety of preclinical models mimicking term and preterm human brain injury (Aridas et al., 2016; Li et al., 2016; Paton et al., 2019). UCBCs in these fetal and neonatal brain injury models have been given at a variety of time points relative to the onset of insult, from a few hours to a few days after the initial insult. A common theme in mechanisms of action of UCBCs in these studies has been the anti-inflammatory properties of UCBC leading to mitigation of brain injury, especially in the white matter region in preterm models (Li et al., 2016; Paton et al., 2019). Although a mitigation of some of the inflammatory response was seen in our experimental model of FGR and neonatal ventilation, it did not appear to be to the same extent as that seen in other studies undertaken by us to date (Li et al., 2016; Paton et al., 2019). UCBCs decreased microglial activation and pro-inflammatory cytokines, but did not have significant effect on astrocytosis or cell death in ventilated FGR+UCBC lamb brains. This could be related to fundamental differences between the FGR brain injury vs. the acute hypoxia related brain injury models: the hypoxia ischemia or inflammation models induce a profound neuroinflammatory load within the term or preterm brain whereas chronic hypoxia and FGR tends to activate inflammatory cells, but does not produce widespread proliferation of astrocytes and microglia (Alves de Alencar Rocha et al., 2017; Li et al., 2016).

Secondly, in the current study the lambs were euthanized and brains studied within 24 hours of UCBC therapy, as opposed to 7-10 days after administration in the other studies. It is possible that we may have seen more distinct benefits of UCBC with longer duration of the experiment. Similarly, we did not see a significant effect of UCBC therapy on cell death markers in the brain, which was a salient finding in our study on term acute hypoxia ischemia (Aridas et al., 2016). We showed in Chapter 2 that caspase-3 (a marker for cell death) is upregulated with ventilation, however it is possible that 24 hours does not allow sufficient time for cells to moderate the caspase pathway, or alternatively, that cells did not impact cell death in this neuropathological process.

We have proposed that UCBC therapy may impact specific components of the neurovascular unit in FGR (Chapter 3). In brief, UCBCs have some effect on mitigating inflammatory response of cells of the NVU, stabilising the pericytes and strengthening the BBB, which are all impacted by ventilation and FGR. The neurovascular unit has not been well studied with regards to its role in perinatal brain injury and how stem cell therapies influence its structure and function needs to be explored further. Adult studies have shown that stabilising the neurovascular unit may be beneficial in improving short-term outcomes of stroke patients (Huang et al., 2017; Laskowitz et al., 2018). Given that poor BBB function is also related to white and grey matter damage of the developing brain, stabilisation of the BBB should help mitigate the burden of brain injury seen in vulnerable infant populations (Moretti et al., 2015). Future work from our laboratory, and others, will hopefully tease out the intricate relationship of NVU and brain development in perinatal health and disease. It is important to note that there are also clinical treatments that preterm infants, including preterm FGR infants, are likely to be exposed to, with the most common of these being antenatal steroids and magnesium sulphate. Antenatal steroids are administered to mature the fetal lungs prior to preterm birth, while magnesium sulphate is administered as a neuroprotective therapy. Both antenatal steroids and magnesium sulphate have effects on the immature brain. In the current study all lambs were exposed to antenatal steroids (via maternal betamethasone administration) but were not treated with magnesium sulphate. It remains unknown whether UCBCs have different or complimentary actions on brain structure to steroid and/or magnesium sulphate treatment. The specific effects of these clinical interventions, as well as novel experimental interventions like erythropoietin for preterm and term brain injury, and their potential interactions, requires further study with regard to efficacy and timing of treatments.

6.1.5. Challenges in detection of FGR related brain injury

Detection of FGR related brain injury remains a challenge, particularly early detection. The neuropathology present in FGR is generally quite subtle and at a cellular level, making diagnosis by neurological examination or conventional imaging in the neonatal period difficult. While a significant cardiovascular adaptation in growth restricted fetuses in utero is associated with a worse neurodevelopmental outcome in childhood (Vossbeck et al., 2001), the degree, nature, severity and impact of specific antenatal ultrasound abnormalities on neonatal morbidity is not clear (Malhotra et al., 2017). Similarly, a number of studies using neonatal cranial ultrasound and MRI for detection of FGR related brain injury have produced conflicting results on the impact of FGR on neurological deficits and injury seen on imaging (Cruz-Martinez et al., 2015; Malhotra et al., 2017; Malhotra et al., 2015). Currently, neonatal cranial ultrasound is routinely used to detect major abnormalities, such as intraventricular haemorrhage and periventricular leukomalacia in FGR infants, while neonatal MRI is reserved for the extremely preterm infant or for those who show significant cranial ultrasound changes. We wanted to evaluate the role of advanced MRI analysis techniques, including voxel- and fixel-based analysis, to study FGR related brain injury and to correlate our findings with histological staining (Chapter 5).

6.1.6. Role of advanced MRI analysis techniques in the detection of FGR related brain injury

Advanced MRI analysis includes, but is not limited to, diffusion tensor imaging based modelling of dMRI data, sophisticated volumetrics, tractography (including fixel-based analysis) and functional MR. Many of these techniques are not in widespread clinical use due to challenges in their implementation and/or interpretation. Despite this, there is a significant growing interest in using advanced MRI analysis techniques to study brain injury in the perinatal period. Advanced MRI analysis techniques have also been explored in a limited number of studies for FGR related brain injury (Malhotra et al., 2017). Voxel-based analysis (global and regional DTI parameters) revealed

fractional anisotropy differences in a number of cerebral grey and white matter structures within the FGR rabbit brain as compared to controls (Eixarch et al., 2012). Regional FA changes correlated with worse outcomes in neurobehavioral tests in the FGR rabbits. Similar voxel-based and connectivity-based differences in the brain have also been shown in a long-term follow-up study in rabbits by the same group (Illa et al., 2013). The fixel-based analysis that we employed in our study has not been previously employed in a FGR brain injury animal or clinical study. Fixel-based analysis is a relatively new diffusion MRI analysis technique that delineates fibre-specific differences in injured brains as compared to the traditional region specific differences (Raffelt et al., 2012), thereby providing a greater sensitivity to detect subtle regional differences. Pannek et al recently employed fixel-based analysis to characterise brain development in ex-preterm infants at term equivalent age as compared to term controls (Pannek et al., 2018). Their study showed micro- and macro-structural differences detectable in fixel-based brain imaging of the preterm cohort, not previously identified by DTI approaches.

In our study, we saw tract-based differences in white matter tracts associated with the hippocampus, periventricular white matter and cerebellum of the FGR lambs on fixel-based analysis, which was consistent with altered density and organisation of white matter tracts that were observable on histological findings. No differences were identified between AGA and FGR lamb brains on conventional MRI, MRS or DTI (voxel-based) analysis. This is significant, as we now have evidence from both human studies (Pannek et al., 2018) and this preclinical study, that advanced imaging analysis techniques like fixel-based analysis may help to detect subtle brain injury in the neonatal period which may previously have been missed. Further, the correlation with histologyprovides insights into the structural changes in the white matter for the first time in FGR brain injury.

6.1.7. Limitations of the experiments

Fetal growth restriction in clinical practice presents a progressive state of compromise that likely incorporates many factors and small insults rather a single, standardised insult as occurs with our animal model using single umbilical artery ligation to induce FGR in this study. For example, clinical FGR can often be associated with birth defects, and both FGR and birth defects independently and together are strong predictors of cerebral palsy, and this combination can be found in a significant number of cases of cerebral palsy (McIntyre et al., 2013). The use of this standardised animal model does however allow for a well-controlled experimental design and consideration of the ethics involving replacement, reduction and refinement. A limitation noted in this study is that the animal experiments in chapter 2 and 3 were conducted for 24 hours, which may not be a long enough period to allow optimal assessment of either the impact of ventilation or the neuroprotective benefits of UCBC in FGR. Prior to the commencement of my PhD, we discussed the trade-off between using a longer ventilation and therapy model that could be undertaken in the fetus, or a shorter neonatal ventilation and therapy model that could be undertaken postnatally. It was agreed that for effective translation of my results to the clinic, I would be best placed to mimic the neonatal setting (with full NICU set-up) to examine the effects of UCBCs. The mechanisms associated with effects of ventilation and UCBC on FGR brain injury are likely to have similar mechanisms irrespective of the duration of the ventilation, and the fundamental science, particularly the assessment of neuropathology, increases our understanding of what may be occurring clinically with routine management of FGR human infants.

6.1.8. Future – Questions that remain unanswered

The thesis asked important and novel questions around revealing the pathophysiology, early detection and early treatment of neuropathology in preterm FGR offspring. I studied the pathophysiology, which may lead to increased brain injury associated with preterm birth and

neonatal ventilation in FGR, and showed for the first time that brain injury is present at birth but exacerbated by neonatal ventilation. We observed notable changes within the neurovascular unit of the brain in FGR offspring, mediated predominantly by neuroinflammation and oxidative stress, and this information provides the basis for further studies to assess the critical role of the neurovascular unit in normal and compromised brain development. My data from Chapter 3 presents new evidence that targeted therapy with umbilical cord blood derived stem cells may be a therapeutic option for FGR brain injury, but it is essential to confirm this positive finding over a longer experimental period before this can be applied to clinical testing. Some of the questions that the preclinical work presented in this thesis brings to the fore include:

- What are the long-term effects of the altered neurovascular unit on neurodevelopment?
- What are the long-term developmental correlates of the white matter deficits observed with advanced MRI analysis techniques?
- Which type of stem cells or components of umbilical cord blood are most feasible and efficacious in preventing or treating perinatal brain injury?
- What is the most appropriate dose, time and frequency of umbilical cord blood stem cell therapy in perinatal brain injury?

I will be addressing some of these questions in my post-doctoral work.

6.1.9. Implications for clinical practice and conclusions

I used a lamb model of early onset FGR to investigate novel strategies to detect and treat FGR related brain injury in the neonate. The combination of FGR and neonatal ventilation led to worse neuropathology in preterm lambs as compared to control and unventilated lambs, most likely mediated by a neuroinflammatory response in the white matter regions of the brain, associated with instability of the blood brain barrier and the neurovascular unit. This is an important observation,

fitting well with the 'two-hit hypothesis' that antenatal FGR plus a second stress of neonatal ventilation to exacerbate neuropathology in FGR infants. The clinical implications of these results indicate that FGR infants, particularly those born preterm, are a particularly vulnerable and high-risk group of preterm infants that require special and possibly individualised attention, and are a cohort of infants that would likely benefit from early diagnosis of brain injury and early neurotherapeutic intervention. Umbilical cord blood stem cell therapy mitigated critical aspects of the neuropathology associated with FGR and ventilation, acting to stabilise the neurovascular unit and thereby potentially decreasing the burden of FGR related brain injury. Whilst umbilical cord blood derived stem cell therapy requires further evaluation in preclinical work conducted over a longer period, cord blood stem cells show excellent promise as a future therapy for FGR brain injury. Advanced MRI analysis using fixel-based analysis is an emerging imaging tool that could be utilised for early detection of subtle and regional FGR related brain injury in newborn lambs, and which we showed was consistent with histological evidence of white matter abnormalities. Advanced MRI analysis tested here in a FGR model is being implemented in a number of clinical settings. Indeed, I am about to extend these preclinical findings to high-risk preterm human infants and use the advanced imaging techniques utilised in Chapter 5 to embark on a post-hoc analysis of MRI brain scans that have already been undertaken. This aspect of my thesis work is ready for clinical practice and the experience and lessons learnt from experiments in this thesis will be crucial for my clinical team as we develop more expertise in these techniques. To conclude, I am confident my PhD studies have led to increased understanding, knowledge, detection and potential treatments for FGR related brain injury.

Chapter 7

7.1. References

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